International Academic Institute for Science and Technology

International Academic Journal of Innovative Research
Vol. 3, No. 12, 2016, pp. 53-64.

ISSN 2454-390X

International Academic Journal of Innovative Research

www.iaiest.com

Innate Immune Responses In The Silkworm, *Bombyx mori* (L).

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Abstract

The silkworm, *Bombyx mori* (L) is an important economic insect for silk production. However, many kinds of pathogens cause serious economic loss every year during sericulture. B. mori lack an acquired immune system but have a sophisticated innate immune system against foreign microorganisms. The innate immunity system of B. mori is composed of humoral and cellular immune reactions, including production of antimicrobial peptides (AMPs), phenoloxidase, phagocytosis, nodule formation, encapsulation and melanization. Three immunity pathways had been reported in the B. mori to control the gene expression and defending invading microorganisms: Toll pathway, Imd pathway and JAK-STAT pathway. It has been shown that gene families associated with recognition, modulation and effectors are conserved from vertebrates to invertebrates. Recent accomplishment in molecular immunology of B. mori has been reviewed in this paper. The development of zinc-finger nuclease technique and silkworm genome information is expected to accelerate silkworm immunity studies.

Key words: *Bombyx mori*, innate immunity, signal pathway, pattern recognition receptors.

Introduction

The silkworm *Bombyx mori* has been domesticated for sericulture in the past 5,000 years. From the beginning of the nineteenth century, the silkworm has been used for basic science studies, such as genetics, physiology, and pathology, because of its large body size, its importance in sericulture, easy rearing, and a large number of described mutants (Willis, *et al*., 1995). Insects possess an efficient and potent innate immune system to discriminate and eliminate invading pathogens and parasites, but lack acquired immunity or immunological memory similar to that present in vertebrates (Lemaitre and Hoffmann, 2007). Drosophila melanogaster, the type model of insects, has been particularly extensively

studied in terms of immunity (Pal and Wu, 2009). However, the recognition and elimination systems of insects may varied, thus the data from D. melanogaster may not always be applicable to other insects. The silkworm, *Bombyx mori*, domesticated more than 5, 000 years ago, is an important economic insect for silk production, and is also a good model lepidopteran (Goldsmith *et al*., 2005). In many rural areas of China, India, Brazil and so on, sericulture is one of the main sources of income for farmers. Every year, severe economic losses happen in the sericulture industry, mainly due to the variety pathogens (Pandiarajan *et al*., 2011) he innate immune system of B. mori is also divided into two major reaction types: humoral and cellular immune responses. The first-line defense against microbes consists of structural barriers that include the epidermis, the midgut peritrophic membrane and tracheal respiratory organs and then in the hemocoele. Once in the hemocoele, the infectious microorganisms are fought by humoral and cellular responses (Tanaka *et al*., 2008). However, silkworm immunity studies, particularly at the molecular level, are not as advanced as those on the common vinegar fly and mosquitoes. Although some progress related to innate immunity of *Bombyx mori* has been published recently using silkworm genome information and modern technology, very little is known about the mechanism of innate immunity (Tian *et al*., 2010). So the comprehensive understanding the mechanism of the B. mori immunity will give us more information on the control of pathogens, such as virus, bacteria and fungi. Understanding the interaction between host and pathogen will help us to know the insect pathological and defense mechanism, also to exploit new biological insecticides for agriculture pest. The purpose of this review is to summarize the current knowledge on B. mori innate immunity, focusing on the results of recent studies, which are intended to gather as much as possible the information about the immunology of B. mori.

Humoral and cellular responses to infection in silkworm

There are at least five types of hemocytes in the hemolymph of silkworm larvae, which can be distinguished morphologically: prohemocytes, plasmatocytes, granular cells, spherule cells and oenocytoides. The celluar responses include phagocytosis, nodule formation and encapsulation by host cells. In invertebrates, in addition to lots of enzyme cascades, a variety of agglutinin-lectins and reactive oxygen producing and phagocytic systems cooperate with immune reactions to kill invading pathogens (Bogdan *et al*., 2000). Invaders are detected by the defense systems, then engulfed by phagocytes, such as macrophage-like, neutrophillike and dendritic cell, and then foreign invaders are internalized as phagosomes and finally killed (Greenberg and Grinstein, 2002). The humoral responses include the activation of cascades of constitutive proteins present in the hemolymph, such as those in the prophenoloxidase cascade, leading to melanization (deposition of melanin pigments onto pathogens and the wounded sites) and the coagulation cascade, and the activation of intracellular signaling pathways that produce defense proteins such as anti-microbial peptides in the immune responsive tissues and cells. The antimicrobial peptide (AMPs) genes are induced by microbial challenge in the fat body (equivalent of the mammalian liver), followed by the secretion of these peptides into the haemolymph, which is the hallmark of the humoral reactions (Hoffmann and Reichhart, 2002). These antimicrobial peptides from several families reach high concentrations in the hemolymph and efficiently kill invading microorganisms (Ferrandon *et al*., 2007), in the midgut of insect, the reactive intermediates of oxygen or nitrogen play a important role in killing the invaders (Bogdan *et al*., 2000; Nappi and Vass, 2001). In holometabolous

insects, such as B. mori, AMP gene transcription is inducible. Absent from uninfected insects, AMPs are rapidly produced upon infection by the fat body or by epithelia such as the gut or the trachea. The humoral and cellular responses are not easy to divide, as many humoral factors affect hemocyte function and hemocytes are an important source of many humoral molecules. There also is considerable overlap between humoral and cellular defenses in processes like the recognition of foreign intruders.

Signal pathway in the host defense of silkworm

There are two immunity pathways in the body to control the gene expression, defending invading microorganisms. The Toll pathway is activated primarily in response to fungal and some Gram-positive bacterial infections, whereas the Imd pathway is activated predominantly in response to Gram-positive and other Gram-negative bacterial infections. It is well known that Toll activation during the immune responses is strictly dependent on the product of the Spaetzle gene. This Spaetzle protein is a cystine-knot molecule with structural similarities to mammalian neurotrophins, and requires proteolytic cleavage for full biological activity (Mizuguchi *et al*., 1998). This cleavage is induced by a proteolytic cascade activated as an early result of infection. Toll receptors, which are characterized by the extracellular leucine rich repeat (LRR) arrays and an intracellular Toll–interleukin-1 receptor (TIR) domain, play important roles in innate immunity in invertebrates and adaptive immunity in vertebrate animals (Taylor *et al*., 2008). There have three partners interacted with the intracytoplasmic TIR domain of Toll, each of them has a death domain. Two of these are adaptor proteins: the Drosophila homologue of MyD88 (Horng and Medzhitov, 2001; Sun *et al.*, 2004; Tauszig-Delamasure *et al*., 2002), which in addition to the death domain has a TIR domain similar to that of Toll with which it associates, and tube. Tube has no obvious mammalian homologue. The third death domain protein in this receptor-adaptor complex is Pelle, which has a serine–threonine kinase domain and is homologous to mammalian IRAKs (interleukin-1 receptorassociated kinases) (Janssens and Beyaert, 2003). The TIR [Toll–inter- leukin-1(IL-1)] receptor domain of Toll binds to the TIR domain of MyD88, however, binding occurs only when the receptor is active (Horng and Medzhitov, 2001; Sun et al., 2004; Tauszig-Delamasure et al., 2002). Subsequently, through interactions of death domains, assembly of the signaling complex containing MyD88, Tube and Pelle occurs (Sun et al., 2004).The increased local concentration of Pelle might lead to transphosphorylation and stimulation of the Pelle kinase activity (Shen and Manley, 2002). The activated Pelle, in an as yet undefined manner, acts on the cytoplasmic Dorsal-Cactus and Dif-Cactus complexes. Dif and Dorsal are NF-κB homologues and are normally retained in the cytoplasm by the IkB-related inhibitor Cactus (Brennan and Anderson, 2004; Hoffmann, 2003; Hultmark, 2003). After signal-induced degradation of Cactus, Dif and Dorsal translocate to the nucleus and activate the expression of antimicrobial peptide genes. The Imd pathway controls the expression of many antimicrobial peptide genes in response to Gram-negative bacterial infection (Brennan and Anderson, 2004; Hoffmann, 2003; Hultmark, 2003; Lemaitre, 2004). Activation of this pathway triggers a cascade of kinases (DmTAK, IKK), which ultimately phosphorylates the Rel protein Relish (Leulier *et al*., 2002; Naitza *et al*., 2002). Phosphorylated Relish is then cleaved by the DREDD caspase dissociating the I-B like domain from the Rel DNA binding domain, which can then translocate into the nucleus (Leulier *et al*., 2000; Stoven et al., 2003). The pattern-recognition receptor for the Imd pathway appears to be PGRP-LC, which contains a putative transmembrane domain (Choe *et al*., 2002; Gottar *et al*., 2002). There are two main families of

pattern-recognition receptors, the peptidoglycan-recognition proteins (PGRPs) and the Gram-negative binding proteins (GNPBs), which have homology to enzymes (amidase or glucanase), can bind to molecules (lipopolysaccharides, peptidoglycans and glucans) associated with microbes, then trigger signaling cascades to activated immune cells or the transcription of AMPs to isolate or kill invaders. There have another pathway which had been reported in the response of insect immunity, that is the JAK-STAT pathway, like the Toll pathway, the JAK-STAT pathway also plays dual biological function in development and immunity. The STAT protein is accumulated in the nucleus after immune challenge in the mosquito, it was first suggested that the JAK-STAT pathway was involved in insect immunity (Barillas-Mury et al., 1999). In Drosophila, the JAK-STAT pathway plays an important role in hematopoiesis, stress responses, stem cell proliferation, and antiviral immunity, but its role in the defense against natural bacterial pathogens is unknown (Agaisse and Perrimon, 2004; Lee, 2009; Singh *et al*., 2007). The silkworm were orally infected by Staphylococcus aureus and Escherichia coli, all the three JAK/STAT pathway genes, such as Dome, Hop and Stat1 were significantly up-regulated (Wu et al., 2010). The results showed that JAK-STAT pathway also participates in the antimicrobial response of the insect gut, which is consistent with the previous study results by the Buchon group (Buchon *et al*., 2009).

Pattern recognition receptors in the innate immune system

Invertebrate innate immune reactions are triggered when so-called pathogen-associated molecular patterns (PAMPs) of microorganisms are detected and recognized as "non-self" by the pattern recognition receptors (PRRs) of the host. So far, seven groups of PRRs have been identified in invertebrates, including peptidoglycanrecognition proteins (PGRP), thioester-containing proteins (TEP), Gram-negative binding proteins (GNBP), multidomain scavenger receptors (SCR), C-type lectins(CTL), galactoside-binding lectin (galectin) and fibrinogen- like domain immunolectin.

----------Table 1. Gene counts of immunity in silkworm (Gene Family: Recognition).

Gene	Count
PGRP	11
GNBP	04
Fibrinogen-related protein	03
Scavenger	12
C-type lectin	22
Galectin	02

Source: Zhengang Ma, *et al* (2013) http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3875524/

Peptidoglycan recognition proteins (PGRPs):
PGRPs have been shown to play a central role in the Wan et al. 2321 recognition of invading microorganisms in insect immunity (Hultmark, 2003; Royet *et al*., 2005; Steiner, 2004). Silkworm PGRP is a hemolymph protein that specifically binds peptidoglycan from bacterial cell walls. It is required for the peptidoglycan-mediated activation of the phenoloxidase cascade (Yoshida et al., 1986, 1996). There is 12 distinct PGRP genes with conserved PGRP domains in the genome of Bombyx. Six belong to the short subfamily, four of the S-type PGRPs (BmPGRP-S3, -S4, - S5 and -S6) are located in tandem on chromosome No.16 and predicted to be secreted proteins, because all transcripts from these genes have

putative signal peptides, BmPGRP-S1 and S2 are surmised to be secreted proteins. BmPGRP-L1, -L2, -L3, -L4 and -L5 are located in tandem within 70 kb on chromosome No. 1, BmPGRP-L6 possesses no signal peptide and transmembrane domain and is thought to be present in the cytoplasm. BmPGRP-L2, -L3 and -L5 appear to be

----------Table 2. Gene counts of immunity in silkworm (Gene Family: Signaling).

Gene	Count
Spatzle	06
Toll	13
CLIP-SP	15
Serpin	15
REL	02
Components#	17

#:The components from immune pathways including Toll, Imd, JNK and JAKSTAT pathway.

Source: Zhengang Ma, *et al* (2013) http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3875524/

----------secreted proteins, whereas BmPGRP-L1 and -L4 appear to be transmembrane proteins (Tanaka et al., 2008). Recent studies on the binding specificity of PGRP to PGN using human and Drosophila recombinant PGRPs and synthetic PGN fragments have demonstrated that the PGRPs distinguish between PGNs from different bacteria based on the differences in both the diamino amino acid at the third position of the stem peptide and the structures of the peptide bridge cross-linking the stems (Kumar et al., 2005; Swaminathan *et al*., 2006).

β-Glucan recognition protein:
(βGRP) βGRP contains two functionally different proteins, one of which binds to β-1,3-glucan and the other dubbed Gramnegative binding protein (GNBP) binds to Gram-negative bacteria or Gram-positive bacteria. The previous studies showed that B. mori has four βGRP genes including a gene designated BmβGRP1 encoding the first βGRP isolated by Yoshida group (Yoshida et al., 1986), a gene designated BmβGRP2 encoding GNBP isolated by Lee group (Lee et al., 1996) and two novel genes designated BmβGRP3 and 4, all BmβGRPs are assumed to be secreted proteins because they have putative signal peptides (Tanaka *et al*., 2008). A cDNA of βGRP was cloned from the silkworm B. mori, was concluded Glucanases and the current pattern-recognition proteins that contain a glucanase-like region seem to have a common origin in their molecular evolution (Ochiai and Ashida, 2000).
Fibrinogen-related proteins (FREPs):
FREPs contain FBG (fibrinogen-like domain) domains in their C-terminal region, are found universally in vertebrates and invertebrates. These proteins are known to participate in recognition of bacteria and parasites in invertebrates (Gokudan et al., 1999; Schroder *et al*., 2003).

In invertebrates, FREPs are involved in cell-cell interaction, bacterial recognition, and antimicrobial responses (Gokudan et al., 1999; Schroder et al., 2003; Wang et al., 2004). There were only three FREP

genes in the genome of B. mori, and species-specific gene expansion seen in Anopheles, Aedes, Drosophila and Tribolium was not found in Bombyx (Tanaka *et al.,* 2008).

The multidomain scavenger receptor (SCR):
SCR family contains eight subfamilies from A to H depending on the amino acid sequences. SCRs recognize multiple ligands and serve to remove apoptotic cells and bacteria (Peiser et al., 2002). Four genes of encoding SRCR were detected in the genome of Bombyx, they encode BmSCRASP2 (Corin-like), BmSCRAL1 (Lox2- like), BmSCRAC1 (CG3921-like) and BmSCRASP4 proteins (Tanaka *et al*., 2008).

C-type lectins (CTLs):
CTLs can act as pattern recognition receptors in innate immunity, which are Ca2+-dependent proteins, and outside of cells, they function as secreted proteins or membranebound proteins (Drickamer and Taylor, 1993). CTLs play an important role as recognition proteins for microorganisms in immune reactions during the early phase of microbial infection (Watanabe et al., 2006). Invertebrate CTLs are involved in immune responses including PPO activation (Yu and Kanost, 2000), hemocyte nodule formation (Koizumi et al., 1999), opsonization and microbial clearance (Jomori and Natori, 1992; Yu and Kanost, 2003). Twenty one CTL genes (BmCTL1–21) were detected in the Bombyx genome (Tanaka et al., 2008). In the lepidopteran insects, a group of C-type lectins that contain two tandem CRDs has been suggested to have PRR functions. Two dual-CRD C-type lectins were isolated from the silkworm B. mori, which were designated as B. mori LPS-binding protein (BmLBP) and B. mori multiple saccharide-binding protein (BmMBP). BmLBP and BmMBP have distinct pathogen recognition characteristics; BmLBP binds to Gram-negative bacteria, whereas BmMBP primarily binds to Gram-positive bacteria and yeast (Koizumi et al., 1997; Watanabe et al., 2006). Three novel C-type lectins cDNAs were obtained from the B. mori, which were designated BmLEL-1, -2, and -3. They concluded that the novel C-type lectins might play a role in the innate immunity in these tissues (Takase et al., 2009).

Galectin:
Galectin is a lectin that specifically binds to β-galactoside sugar and contains evolutionary conserved CRDs. Galectin is thought to participate in microbial recognition or phagocytosis in flies and mosquitoes (Pace and Baum, 2004). There are four genes (BmGalectin1–4) encoding Galectin in the Bombyx genome (Tanaka et al., 2008).

Thioester-containing protein (TEP):
TEP is a secretory protein and related to mammalian α2- macroglobulin. AgTEP1 (ca.160 kDa) is processed upon infection with Gram-negative bacteria and the resultant Cterminal 80 kDa polypeptide binds to the surface of Gramnegative bacteria via the γ-glutamyl ketone group of the peptide, promoting phagocytosis by plasmatocytes (Levashina et al., 2001). Three TEP genes (BmTEP1–3) were identified in the B. mori genome during Bacillus bombyseptieus infection, which is unlike A. gambiae contains 15 TEPs (Huang *et al*., 2009).

The immunoglobulin (Ig)-superfamily:
The immunoglobulin proteins are known for their abilities to specifically recognize and adhere to cells. In

mammals immune system, Igs are present on the B-lymphocyte surface and play crucial roles in transducing the signals of cytoplasmic and nuclear effectors and delivering antigens to the cellular compartment where these are degraded (Litman *et al*., 1999). To date, several reports have shown that IgSF members play crucial roles in invertebrate immune response. This was the first insect IgSF molecule to be considered a PRR, since it has broad specificity for lipopolysaccharide and lipotechoic acid (Daffre and Faye, 1997; Gokudan *et al*., 1999; Yu and Kanost, 2002) and is a major player during Gram-negative bacterial infections (Eleftherianos *et al*., 2006a, b; 2007). Hemolin gene is the only one player in the immunoglobulin superfamily of invertebrate, which was found only from lepidopteran insects (Bao *et al*., 2007). Which is induced and expressed after baculovirus and dsRNA introduction, suggesting that hemolin is used in antiviral defense (Hirai et al., 2004). The hemolin gene was cloned by our group from the fat body of B. mori, the result showed that the recombinant hemolin obtained from in vitro expression by using baculovirus expression system had antibacterial activity, being an effective inhibitor to the growth of *Bacillus thuringiensis*. Hemolin is down-regulated rather than up-regulated in most cases of infection in the silkworm fat body over the time course of the infection with the Gram-negative bacteria E. coli and also the Gram positive bacteria B. thuringiensis (Huang *et al*., 2009). It was speculated because the immune response kinetic is very sensitive. 133 IgSF proteins were predicted in the silkworm genome, Comparison with similar proteins in other model organisms indicated that IgSF proteins are conserved but have rapidly evolved from worms to human beings (Huang *et al*., 2009).

Discussion

The silkworm is agriculturally very important for silk production, so their pathological and genetic studies on diseases have been very significantly and extensively carried on. Severe economic losses caused by the pathologies, such as virus, bacterium and fungus so on, so the comprehensive understanding the innate immunity pathway and host-pathogen interaction will attribute for us defending economic losses and benefit from the silk industry. In the past several years, the studies on the innate immunity of B. mori have gained significant results, such as much recognition, modulation, signaling, effectors and other immune molecules (Table 1). The comparison between Toll and Imd pathway enable us to further understand the mechanisms of innate immune responses. Recent years, phylogenitic analysis that many immunity members in the invertebrate immunity have very similar defending genes to the mammals, this result illustrate the similarities between some of the strategies used both by insects and mammals to sense infection and amplify the information (Hoffmann, 2003). Even though, some paper published that the structural and function similarities between the Toll and the TLR dependent activation of NF-κB has been interpreted as evidence for the existence of a common ancestor and shared mechanisms between the vertebrate and invertebrate innate immune systems (Royet and Dziarski, 2007). Many reports revealed that stem cell is involved in the regeneration by destroy and in maintain the homeostasis in the Drosophila intestinal. So we speculated that some kinds of stem cell are also involved in the intestinal homeostasis of silkworm. Recently, a genome-wide analysis of immune-related genes of B. mori revealed that the factors associated with the signal transduction pathways are conserved in B. mori and non-lepidopteran insects (Tanaka et al., 2008). However, the function of most genes encoding recognition proteins in B. mori is still unknown. Nonetheless, in the near future, the immune-related genes can be elucidated by the development of functional analyses such as RNA interference (RNAi) (Fujita *et al.*, 2009), transgenic technology, GAL4/UAS system (Kobayashi *et al*., 2011) and zinc-finger nuclease technique (Takasu *et*

al., 2010). Comprehensively exploring the mechanism of innate immunity between insect will give us much information about the host and pathogen interaction, defensive mechanisms evolved in the host in response to infection and anti-defensive/immunosuppression molecules released by pathogen, all of these will give us more references about the immunity mechanisms in the mammals and understand the defense mechanism in the insect, and also which will contribute to the sericultural field by establishing transgenic non-susceptible strains of silkworm and to agriculture for better control of lepidopteran pests.

ACKNOWLEDGEMENT
Author would like to express his gratitude towards the dignitaries of International Journal of Innovative Research for kind co-operation and encouragement, which served a lot to orchestrate the progression of publication of present research work.

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