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REVIEW

Regulation of the innate immune responses in the silkworm, *Bombyx mori*

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Abstract

Insects possess an effective innate immune system against foreign microorganisms. Innate immunity of insects is divided into two major reaction types: humoral and cellular reactions. Humoral reactions involve soluble proteins in the hemolymph such as phenoloxidase, antimicrobial proteins (AMPs), lysozymes, and lectins, whereas hemocytes mediate cellular reactions such as phagocytosis, encapsulation and nodule formation. In *Bombyx mori*, six different families of AMPs have been identified: Cecropin, Attacin, Lebocin, Moricin, Gloverin, and Defensin. One lysozyme and three lysozyme-like proteins, one of which is involved in elimination of invading pathogens, are also found in the silkworm. Both lysine-containing peptidoglycan (Lys-PGN) and meso-diaminopimelic acid containing peptidoglycan (DAP-PGN) trigger expression of AMP genes, probably through the Toll and IMD pathways, respectively. DAP-PGN has stronger elicitor activity than Lys-PGN in *B. mori* because of the difference in transcriptional activity between BmRelishes and BmRels, which are effectors of the IMD and Toll pathways, respectively. Furthermore, two recognition proteins and a serine protease are involved in activation of prophenoloxidase for melanization, and several C-type lectins, which participated in cellular reactions, were identified in *B. mori*. Moreover, a paralytic peptide was reported to play important roles in silkworm immunity. Recent development of transgenic technologies and silkworm genome information are expected to accelerate silkworm immunity studies.

Key words: innate immunity; *Bombyx mori*; antimicrobial peptides; melanization; cellular immunity; paralytic peptide

Introduction

Insects first appeared on the earth approximately 350 - 400 million years ago; today, they account for more than 70 % of the animal species on the earth (Mayhew, 2007). Evolution of an effective innate immune system is one reason for the prosperity of insects on the earth. They have a primitive innate immunity system, but lack acquired immunity for antibody production or immunological memory similar to that present in vertebrates (Brennan and Anderson, 2004; Pinheiro and Ellar, 2006; Lemaitre and Hoffmann, 2007). Insects have developed this powerful innate immune system against invading microorganisms during their evolution. However, they belong to diverse families with different life cycles and feeding methods. Thus, recognition and elimination systems against invading pathogens may also vary among them. At present, insect immunity studies using the common vinegar fly, *Drosophila melanogaster*, are the most advanced, and a large amount of data has been accumulated about *Drosophila* immunity (Brennan and Anderson, 2004; Cherry and Silverman, 2006; Ferrandon *et al*., 2007; Lemaitre and Hoffmann, 2007; Pal and Wu, 2009). However, data from *D. melanogaster* may not always be applicable to other insect species owing to their diversity.

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). Now, the technology to construct a transgenic silkworm has

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been developed for use in functional analysis of unknown gene products and to produce recombinant proteins (Yamao *et al*., 1999; Tamura *et al*., 2000; Tomita *et al*., 2003). Furthermore, *B. mori* is the first lepidopteran insect to have an almost complete genome sequence documented (The International Silkworm Genome Consortium, 2008). Therefore, the silkworm is very useful for scientific studies as a model for Lepidoptera, which includes the most disruptive agricultural pests.

By the 1980s, studies using *B. mori* also led to the insect immunity research, such as analysis of the activation mechanism for melanization, and analysis of the morphology of hemocytes in response to invading pathogens. Although silkworm immunity studies, particularly on the molecular level, are not as advanced as those of the common vinegar fly and mosquitoes, increasing studies on silkworm immunity using silkworm genome information and transgenic technology are being published. In addition, silkworm immunity mechanisms different from those of *D. melanogaster* have been reported.

In this article, we will review research on the immune responses of *B. mori*. Some excellent review articles are available on innate immunity of the lepidopteran, *Manduca sexta* (Kanost *et al*., 2004; Ragan *et al*., 2009; Kanost *et al*., 2009).

Humoral immunity and regulation of its response

Insect immunity is divided into two major response types: humoral and cellular defense responses. Humoral responses involve phenoloxidase (PO) and immune proteins such as antimicrobial proteins (AMPs), lysozyme, and lectins. In response to microbial infection, AMPs and lysozyme are rapidly produced primarily in the fat body (FB) and hemocytes, and subsequently secreted into the hemolymph to eliminate invading pathogens. In addition, melanization induced by PO, and lectins are implicated in segregation of invaded microorganisms in the hemolymph.

Antimicrobial proteins and lysozyme

In *B. mori*, six different families of AMPs have been identified: Cecropin (Morishima *et al*., 1990; Taniai *et al*., 1992; Kato *et al*., 1993; Yamano *et al*., 1994; Kim *et al*., 1998; Yang *et al*., 1999; Cheng *et al*., 2006; Hong *et al*., 2008), Attacin (Sugiyama *et al*., 1995), Lebocin (Hara and Yamakawa, 1995a; Chowdhury *et al*., 1995; Furukawa *et al*., 1997), Moricin (Hara and Yamakawa, 1995b; Furukawa *et al*., 1999), Gloverin (Cheng *et al*., 2006; Kaneko *et al*., 2007; Kawaoka *et al*., 2008), and Defensin (Kaneko *et al*., 2008; Wen *et al*., 2009) (Table 1). Cecropins, which were originally isolated from the giant moth *Hyalophora cecropia* (Steiner *et al*., 1981), consist of approximately 40 amino acids, and kill both Gram-positive and -negative bacteria by forming ion channels in the bacterial membrane (Christensen *et al*., 1988). They were discovered as a family of 13 genes including two *Cecropin A*, six *Cecropin B*, one Cecropin C, one *Cecropin D*, one *Cecropin E*, and two *Enbocin*; eleven of these genes form a cluster on the same chromosome (Tanaka *et al*., 2008). Attacin is a glycine-rich protein with a molecular mass of approximately 20 kDa (Sugiyama *et al*., 1995). In *Escherichia coli*, treatment with Attacin from *H. cecropia* led to specific inhibition of the synthesis of several outer membrane proteins, such as *OmpC*, *OmpF*, *OmpA*, and *LamB*, and subsequent elevation of its outer membrane permeability (Carlsson *et al*., 1991). Therefore, *Bombyx* Attacin also appears to suppress the growth of Gram-negative bacteria by inhibiting the production of the bacterial outer membrane proteins. Silkworms possess two copies of *Attacin*, located in tandem on chromosome 6 (Tanaka *et al*., 2008). Lebocin, a proline-rich glycosylated peptide, consists of 32 amino acid residues. Glycosylation of threonine 15 with N-acetylgalactosamine and galactose or solely with N-acetylgalactosamine is involved in its antibacterial activity (Hara and Yamakawa, 1995a). Moricin is a highly basic peptide

Table 1 Antimicrobial proteins from *B. mori*

Name	Type	Gene number	Main activity
Cecropin	Amphiphilic-alpha-helix	13	Gram-positive, Gram-negative
Attacin	Glycine-rich	2	Gram-negative
Lebocin	Proline-rich, O-glycosylated	1	Gram-negative
Moricin	Amphiphilic-alpha-helix	1	Gram-positive, Gram-negative
Gloverin	Glycine-rich	4	Gram-negative
Defensin	Six cysteine containing	2	?
Lysozyme		1	Gram-positive
Lysozyme-like		3	Gram-positive, Gram-negative (LLP1)

Their names, which types they belong to, their gene numbers, and their main activity against Gram-positive bacteria or Gram-negative bacteria are shown.

consisting of 42 amino acid residues; it shows antibacterial activity against both Gram-positive and -negative bacteria by attacking the bacterial membrane (Hara and Yamakawa, 1995b). *Lebocin* and *Moricin* genes are present as single genes (Tanaka *et al*., 2008). Gloverin is a glycine-rich protein. *B. mori* has 4 different isoforms named Gloverin 1-4 (Kaneko *et al*., 2007; Kawaoka *et al*., 2008). All Gloverins are reported to exhibit antibacterial activity against Gram-negative bacteria by using the recombinant proteins, even though their activity was weak (Kawaoka *et al*., 2008). Defensins are basic peptides consisting of 30 - 40 amino acids with six cysteine residues to form three pairs of disulfide bonds. In *B. mori*, two Defensins, designated Defensin A and Defensin B, have been identified, even though their antimicrobial activity has not yet been confirmed (Kaneko *et al*., 2008; Wen *et al*., 2009). Lysozyme catalyzes the cleavage of the β-1,4-glycosidic linkage between the N-acetylglucosamine and N-acetylmuramic acids of peptidoglycan (PGN), a major component of the bacterial cell wall. A lysozyme from *B. mori* shows higher antimicrobial activity against Gram-positive bacteria than against Gram-negative bacteria similar to that of the lysozyme from other organisms (Lee and Brey, 1995). Recently, three lysozyme-like proteins (LLPs) were reported to exist in *B. mori* (Gandhe *et al*., 2007) (Table 1). All of LLPs lack catalytic amino acid residues for muramidase activity, but LLP1 inhibits the growth of *E. coli* and *Micrococcus luteus*, probably by an unknown mechanism different from that of lysozyme (Gandhe *et al*., 2007). The function of other two LLPs has not yet been elucidated.

AMP genes are rapidly expressed in response to microbial infection in specific tissues, mainly in FB and hemocytes but not in the posterior silk gland (PSG) (Yamano *et al*., 1994; Sugiyama *et al*., 1995; Furukawa *et al*., 1997; Kaneko *et al*., 2007). However, the transcription of AMP genes was observed in an *in vitro* transcription system, using not only FB nuclear extract but also PSG nuclear extract (Tanaka *et al*., 2005a). Comparison of nucleosomal structure near the promoter regions of AMP genes indicated clear nucleosomal arrangements in the PSG but not in the FB, suggesting that regulation of tissue-specific expression of AMP genes occurs at least at the chromatin level (Tanaka *et al*., 2005a).

The induction mechanism of AMP genes has been extensively studied in *D. melanogaster*, and two distinct pathways, Toll and Immune deficiency (IMD) pathways, play important roles in the activation of AMP genes (Pinheiro and Ellar, 2006; Ferrandon *et al*., 2007; Lemaitre and Hoffmann, 2007; Aggarwal and Silverman, 2008; Valanne *et al*., 2011). The Toll pathway is known to be stimulated by lysine-containing PGN (Lys-type PGN), a cell wall component of many Gram-positive bacteria with the complex of PGN recognition protein SA (PGRP-SA) and PGRP-SD, and Gram-negative binding protein 1 (GNBP1) (Govert *et al*., 2003; Pili-Floury *et al*., 2004; Bischoff *et al*., 2004). This recognition activates the extra-cellular serine protease signaling cascade, and leads to cleavage of pro-Spätzle. The binding of Spätzle to the receptor Toll leads to the translocation of NF-κB transcription factors, Dif and Dorsal, into the nuclei to activate target AMP genes such as *Drosomycin*. IMD pathway is stimulated by meso-diaminopimelic acid containing PGN (DAP-type PGN) from Gram-negative bacteria and subclass of Gram-positive bacteria such as the *Bacillus* species, along with other PGN recognition proteins, PGRP-LC and PGRP-LE (Gottar *et al*., 2002; Rämet *et al*., 2002; Takehana *et al*., 2002; Kaneko *et al*., 2005). Initiation of the intracellular signal transduction cascade by PGRP-LC results in the cleavage of an NF-κB factor, Relish, and subsequent activation of expression of AMP genes such as *Diptericin*.

In contrast, the signal transduction pathways involved in the up-regulation of AMP genes have not been well studied in *B. mori*. In addition, recognition factors that trigger expression of AMP genes have not yet been identified. Recently, comparative genome analysis indicated that the silkworm also possesses both Toll and IMD pathways, since almost all orthologous genes encoding these intracellular components exist in the silkworm genome (Tanaka *et al*., 2008) (Fig. 1). However, 1:1 orthologous genes of *Drosophila* PGRPs involved in the recognition of PGNs and subsequent activation of both the pathways were not found in the silkworm genome by bootstrap analysis, although twelve silkworm PGRP genes were identified (Tanaka *et al*., 2008). We neither identified the 1:1 orthologous genes of serine proteases involved in extra-cellular Toll signaling such as Grass, Spirit, and Persephone (Tanaka *et al*., 2008). These data suggest that the recognition factors and serine proteases that activate intracellular Toll signaling are not well evolutionally conserved between *B. mori* and *D. melanogaster*. Although 13 or 14 genes have been identified, the Toll receptor involved in the Toll pathway in *B. mori* has not yet been identified (Cheng *et al*., 2008; Tanaka *et al*., 2008). Bootstrap analysis also failed to identify an orthologous gene of *Drosophila* Toll in *B. mori*. However, BmToll9-1 is speculated to activate the Toll pathway because *Drosophila* Toll9, an ortholog of BmToll9-1, is known to constitutively activate the Toll pathway. In fact, the expression of *BmToll9-1* is elevated in response to bacterial and fungal infection (Wu *et al*., 2010). Furthermore, *Bombyx mori* Spätzle-1 (BmSpz-1), a putative ligand for the Toll receptor, was also cloned in *B. mori* (Wang *et al*., 2007). The BmSpz-1 generated by proteolytic processing of its precursor, pro-BmSpz-1 up-regulates transcription of AMP genes, suggesting that BmSpz-1 has a similar function as the *Drosophila* Spätzle.

We found that DAP-type PGN induces expression of AMP genes more strongly than Lys-type PGN in the FB of silkworm larvae (Tanaka *et al*., 2009b). We further confirmed that bacteria possessing DAP-type PGN elicited stronger expression of AMP genes than those bacteria that possess Lys-type PGN; this suggests that the apparent bacterial difference in the influence on the induction levels of these genes depends on the type of PGN (Tanaka *et al*., 2009b). Transgenic knockdown analysis in silkworm showed that the Dorsal and Relish orthologs, BmRels and BmRelishes, respectively, participated in the activation

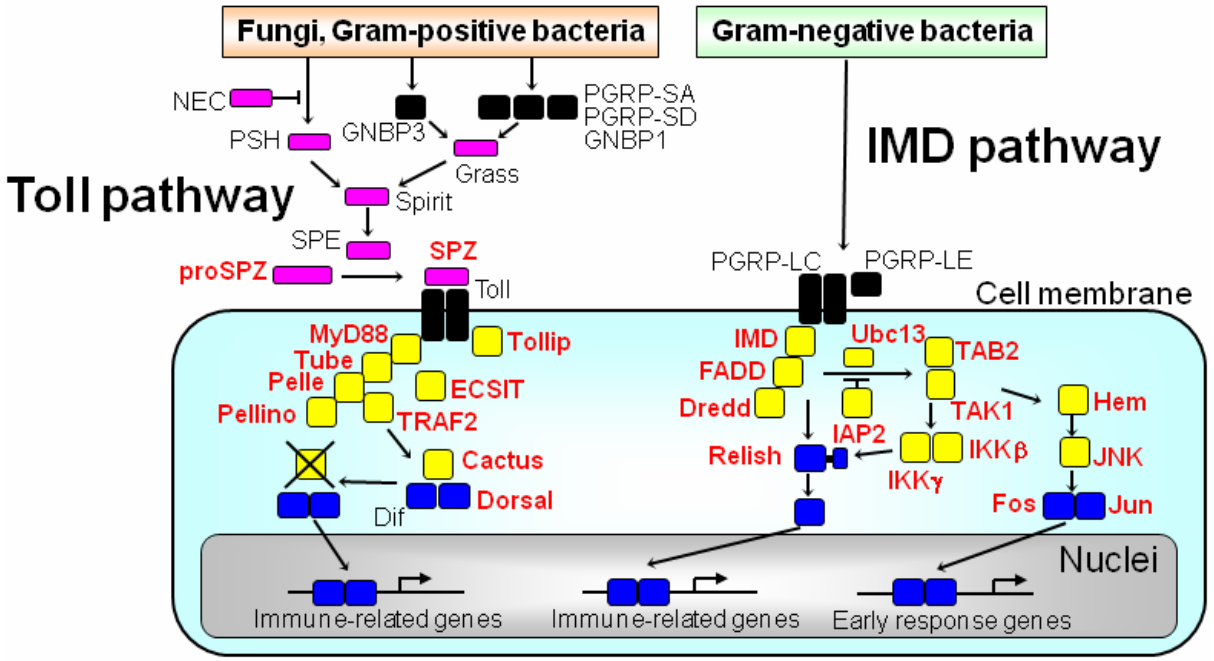

Fig. 1 Factors involved in Toll and IMD pathway in *D.* melanogaster, and *B. mori* orthologs corresponding to these factors. The factors represented by red letters indicate that *B. mori* also has 1:1 ortholog of genes encoding these factors. The factors represented by black letters indicate that *B. mori* appears to not have 1:1 ortholog of them. Black boxes indicate proteins involved in recognition, red boxes indicate proteins involved in the extra-cellular Toll pathway, blue boxes indicate transcription factors, and yellow boxes indicate proteins involved in the intracellular pathways. Spirit does not appear to cleave SPE directly, although it is located up-stream of SPZ. PSH and Grass also do not appear to cleave Spirit directly. PGRP; peptidoglycan recognition protein, GNBP; Gram-negative bacteria-binding protein, NEC; Necrotic, PSH; Persephone, SPE; Spätzle-precessing enzyme, SPZ; Spätzle, ECSIT; Evolutionarily conserved intermediate in Toll pathways, TRAF2; Tumor necrosis factor receptor-associated factor 2, FADD; Fas associated protein with death domain, IAP2; Inhibitor of apoptosis 2, TAK1; Transforming growth factor-activated kinase 1, TAB2; TAK1 binding protein 2, IKKβ; IκB kinase β, IKKγ; IκB kinase γ, Hem; Hemipterous, JNK; c-Jun NH_2-terminal kinase.

of expression of AMP genes triggered by Gram-positive and -negative bacteria, respectively (Tanaka *et al*., 2005b; Tanaka *et al*., 2007; see below in detail). This suggests that Toll and IMD pathways are also activated by Lys-type and DAP-type PGNs, respectively, in *B. mori*. These results and suggestions lead us to further speculate that expression of AMP genes in silkworm is up-regulated by both pathways, but that the activation of these genes by the Toll pathway is lower than that by the IMD pathway. We demonstrated two mechanisms for this differential activation (Tanaka *et al*., 2009b). One is that the quantity of BmRelishes, effector of the IMD pathway, is greater than that of BmRels, effector of the Toll pathway. The other is that BmRelishes have a greater ability to enhance the promoter activity of AMP genes than BmRels, and this difference is at least because of the difference in the binding affinity of the Rel family proteins to the target sites.

Regulation of promoter activity of AMP genes has been analyzed relatively well in *B. mori*. κB elements, which are known as regulatory elements of AMP genes in other insects, are also conserved in the 5′ up-stream regions of silkworm AMP genes (Yamakawa and Tanaka, 1999; Cheng *et al*., 2006). Rel family transcription factors, BmRels and BmRelishes binding to these sequences have already been cloned from *B. mori* (Tanaka *et al*., 2005b; Tanaka *et al*., 2007). A single *BmRel* gene produces two alternative splicing isoforms, BmRelA and BmRelB. The two Rel proteins BmRelA and BmRelB have identical amino acid sequences with a Rel homology domain (RHD), except that BmRelA possesses extra 52 amino acids at the N-terminus. These Rel proteins appeared to have different functions: BmRelB activates *Cecropin B1*, *Attacin*, *Enbocin2*, *Gloverin2*, and *Groverin4* more strongly than BmRelA, whereas BmRelA activates *Lebocin4*, a polymorphic gene of *Lebocin*, more strongly than BmRelB. The fact that a minor structural change in a Rel protein can induce considerably different activation of AMP genes has been reported only in *B. mori*, suggests that *B. mori* has a novel regulatory mechanism for expression of AMP genes. *BmRelish* also produces two alternative splicing products, BmRelish1 and BmRelish2. BmRelish1 has a RHD at the N-terminus, and ankyrin repeats at the

C-terminus. BmRelish1 is assumed to be activated by endoproteolytic cleavage and subsequent removal of the C-terminal ankyrin repeats as seen in *Drosophila* Relish. BmRelish2 lacks a putative transcriptional activation domain and an ankyrin repeat; BmRelish2 seems to be a dominant negative factor against BmRelish1 (Tanaka *et al*., 2007). The function of BmRelish2 *in vivo* remains unknown because the amount of BmRelish2 mRNA in the *B. mori* larval FB is extremely low compared to that of BmRelish1 mRNA. Another nucleotide sequence, the "CATTT/A" motif located on the 5′ region of *CecropinB1* and *Attacin* is also reported to be involved in promoter activation by crude lipopolysaccharide (LPS), in which PGN is probably contaminated (Taniai and Tomita, 2000; Tanaka *et al*., 2005a). Further electro mobility shift assay revealed that the binding factors are contained in the FB nuclear extract (Taniai and Tomita, 2000; Tanaka *et al*., 2005a), but they have not yet been identified. GATA motif, which has been reported to play an important role in expression of AMP genes in *D. melanogaster*, seems to have no effect on promoter activity in *B. mori* (Taniai and Tomita, 2000).

Unlike PGN, LPS, which is a major cell surface component of Gram-negative bacteria, does not activate the Toll or IMD pathway in *D. melanogaster*. On the contrary, we found that LPS elicits expression of AMP genes in the FB of *B. mori* larvae using highly purified LPS, although the expression level is lower than that elicited by crude LPS and PGN (Tanaka *et al*., 2009a). This signal transduction pathway for transcriptional activation of AMP genes by LPS remains unknown.

Some other molecules have also been shown to elicit the synthesis of immune-related proteins in *B. mori*. Eicosanoids were shown to mediate induction of AMP genes in the FB of silkworm by treatment with eicosanoid biosynthesis inhibitors (Morishima *et al*., 1997). In addition, nitric oxide (NO) produced by *B. mori* nitric oxide synthase seems to be involved in a intracellular signaling to induce expression of AMP genes, since the gene expression of *Cecropin B* is induced in the FB of *B. mori* larvae injected with NO donors (Imamura *et al*., 2002). Furthermore, Tian *et al*. (2010) reported that juvenile hormone (JH) up-regulated expression of AMP genes, whereas 20-hydroxyecdysone (20E) suppressed it in the FB of silkworm larvae. They further reported that the 20E receptor complex, ecdysone receptor, and ultraspiracle were involved in the inhibition of AMP gene expression by 20E. The regulation of AMP gene expression by these two hormones in *B. mori* is contrary to that observed in *D. melanogaster*, in which 20E induces the expression of AMP genes and JH inhibits 20E-dependent up-regulation of AMP genes (Flatt *et al*., 2008). Further analysis will be necessary to clarify the molecular mechanism of JH- and 20E-mediated transcriptional regulation of AMP genes in *B. mori* and *D. melanogaster*.

Melanization

Invasion of microorganisms into the hemocoel triggers rapid synthesis of polymeric melanin from phenolic substances to encapsulate invading foreign organisms (Cerenius and Söderhäll, 2004). A key enzyme involved in melanization is PO. More than 40 years ago, Ashida *et al*. were the first to demonstrate that silkworm PO exists as a zymogen, proPO (Ashida and Ohnishi, 1967), and then it was found that infection of pathogens leads to its proteolytic activation of proPO through a stepwise process called the proPO cascade, composed of several serine proteases in the silkworm (Ashida *et al*., 1974; Katsumi *et al*., 1995; Johansson and Söderhäll, 1996). Ashida *et al.* also identified in 1983 that PGN and β-1,3-glucan were elicitors to activate the silkworm proPO cascade (Ashida *et al*., 1983). Subsequently, they purified for the first time a PGRP and a β-1,3-glucan recognition protein (βGRP) from larval hemolymph as proteins to recognize PGN and β-1,3-glucan, respectively (Ochiai and Ashida, 1988; Yoshida *et al*., 1996). By now, the activated proPO cascade is known to cleave proPO activating enzyme (proPPAE) to active PPAE, which is required for conversion from proPO to PO (Satoh *et al*., 1999). An unknown serine protease to process proPPAE also converts proBAEEase to BAEEase, whose target substrate in silkworm is not yet known (Katsumi *et al*., 1995). Recently, Lipid A, a component of LPS, has been reported as an elicitor to activate melanization (Kaneko *et al*., 2005).

In another lepidopteran, *Manduca sexta*, two branches of the proPO cascade/system have been identified (Kanost *et al*., 2009; Cerenius *et al*., 2010). One consists of the serine proteases, hemolymph protein 6 (HP6) and proPO activating proteinase 1 (PAP1), and the other consists of HP14, HP21, PAP2 and PAP3. The serine protease homolog (SPH) 1 and 2 are also reported to be involved in the cleavage of proPO. A genome-wide analysis of silkworm showed that silkworm has at least 15 clip domain serine protease genes (BmCLIP1 to 15). Among them, BmCLIP1 and BmCLIP2 correspond to PPAE and BAEE, respectively (Tanaka *et al*., 2008). Bootstrap analysis demonstrated that BmCLIP8 and BmCLIP11 (equal to BmSPH1) are definitive 1:1 orthologs of PAP1, and SPH1, respectively, but none has a 1:1 ortholog relationship with PAP2, PAP3, HP6, HP14, HP21 or SPH2. This comparative genomic analysis suggests that the proPO cascade between *B. mori* and *M. sexta* is not well conserved, although the two insects are Lepidoptera.

Production of reactive oxygen species (ROS) is also reported in the course of the melanization reaction for antimicrobial defense (Nappi, *et al*., 2009). Recently, experiments of injecting PGN from *Porphyromanas gingivalis* into silkworm larvae demonstrated that overproduction of ROS generated during the melanization reaction has lethal effects on the silkworm (Ishii *et al*., 2010).

Cellular immunity and regulation of its response

Hemocytes play an important role in host cellular defense mechanisms such as phagocytosis, encapsulation, and nodule formation (Lavine and Strand, 2002; Lemaitre and Hoffmann, 2007). Silkworm hemocytes are classified into five types based on their morphology and function: granulocytes, plasmatocytes, oenocytoids, prohemocytes and spherulocytes (Akai and Sato, 1973, 1976; Nakahara *et al*., 2009). Among them,

granulocytes and plasmatocytes, shared similarity with mammalian neutrophils and macrophage, respectively are involved in cellular immunity (Wago, 1980). Additionally, oenocytoids produce proPO in response to microbial infection (Iwama and Ashida, 1986). Phagocytosis is the engulfing of particles smaller than own cells such as bacteria, viruses, and latex beads by hemocytes, and it proceeds in three phases: attachment, filopodial elongation, and actual internalization by the veil-like membrane processes (Wago, 1982, 1983). In *B. mori*, granulocytes are primarily involved in phagocytosis (Wago, 1982, 1983). Larger foreign materials such as wasp eggs or larvae are encapsulated by granulocytes in cooperation with plasmatocytes (encapsulation) (Sato *et al*., 1976). In addition, granulocytes and plasmatocytes can surround and isolate aggregated foreign materials when many materials are incorporated into the hemocoel by septic infection (nodule formation) (Ratcliffe and Gagen, 1977; Koizumi *et al*., 1999; Sakamoto *et al*., 2011). In nodule formation, melanization occurs in the nodule matrix, which consists of aggregated bacteria and hemocytes (Koizumi *et al*., 1999; Sakamoto *et al*., 2011). Recently, Sakamoto *et al.* (2011) demonstrated that the precursor of SPH1 from *B. mori* (BmSPH1) was cleaved to an active species in the nodule but not in the plasma in bacteria-injected silkworm larvae. Additionally, they found that anti-BmSPH1 inhibited melanization in nodules. These results suggest that BmSPH1 is involved in melanization in nodules but not in the hemolymph. In contrast, *M. sexta* SPH-1, a putative ortholog of BmSPH1 appears to regulate the initiation of melanization in the hemolymph (Yu *et al*., 2003). More detailed analysis is needed to elucidate the different functions of SPH-1 between *B. mori* and *M. sexta*.

The recognition of invading pathogens by pattern recognition receptors elicits cellular immunity responses. In *D. melanogaster*, several kinds of receptors have been identified, such as the PGRPs (Kurata, 2004; Aggrawal and Silverman 2007), Eater (Ertürk-Hasdemir and Silverman, 2005), Nimrod family proteins (Kurucz *et al*., 2007), scavenger-receptor family proteins (Rämet *et al*., 2001), and an immunoglobulin superfamily domain protein, Dscam (Watson *et al*., 2005). Soluble proteins, thioester-containing proteins (Blandin and Levashina, 2004) and C-type lectins (Ao *et al.*, 2007) are also known to act as opsonins, which promote the cellular immunity reactions. A genome-wide analysis revealed that the gene families encoding these recognition receptors and soluble proteins also exist in the silkworm genome except for Eater, which seems to be present only in dipteran insects (Tanaka *et al*., 2008). However, receptors that elicit cellular immunity reactions have not yet been identified in *B. mori* except for C-type lectins, such as the *B. mori* LPS-binding protein (BmLBP) (Koizumi *et al*., 1997, 1999) and *B. mori* multiple saccharide-binding protein (BmMBP) (Watanabe *et al*., 2006). Both BmLBP and BmMBP are investigated to be involved in the elimination of invading pathogens from the hemolymph. BmLBP recognizes Gram-negative bacteria through LPS, whereas BmMBP recognizes Gram-positive bacteria and yeast through lipoteichoic acid and mannose, respectively. Most recently, other C-type lectins—*B. mori* low-expression lectin 1 (BmLEL1) and BmLEL2—have been reported to bind to rough and smooth strains of Gram-negative bacteria, respectively, indicating that they also act as recognition molecules to induce immunity reactions (Takase *et al*., 2009). Hemolin, which belongs to the immunoglobulin superfamily, is also a soluble recognition molecule involved in humoral and cellular immunity (Sun *et al*., 1990; Ladendorff and Kanost 1991; Eleftherianos *et al*., 2007). *Hemolin* is detected exclusively in lepidopterans, including *B. mori*, suggesting that the gene has evolved as a lineage-specific gene for Lepidoptera (Schmidt *et al*., 1993; Tanaka *et al*., 2008).

Immune response by paralytic peptide

A cytokine-like factor, *B. mori* paralytic peptide (BmPP), which was purified from the silkworm hemolymph (Ha *et al*., 1999), belongs to the ENF peptide family. This family shares a similar amino acid sequence particularly at the C-terminal region, and is named after the N-terminal-conserved three amino acids (Glu-Asn-Phe) (Strand *et al*., 2000). ENF family proteins are synthesized as inactive precursors and are activated by a serine protease cleavage. Mature ENF family proteins, including BmPP, have been reported to show multiple effects, such as induction of morphological changes of plasmatocytes, inhibition of larval growth, promotion of cell growth, and local muscle contraction (Ha *et al*., 1999; Sasagawa *et al*., 2001; Miura *et al*., 2002; Nakahara *et al*., 2003). Recently, Ishii *et al*. (2008) reported that PGN and β-glucan-dependent ROS production in hemocytes stimulates the activation of serine proteases that lead to proteolytic activation of BmPP in the hemolymph. They also showed that BmPP activation enhances the immunity against bacterial infection, and that the enhancing host immunity against invading pathogens by active BmPP is because of the activation of hemocytic phagocytosis and up-regulation of expression of AMP genes through activation of p38 MAP kinase signaling (Ishii *et al*., 2010). Nevertheless, it still remains unclear whether the serine proteases leading to proteolytic activation of BmPP are the same as those involved in the activation of the Toll pathway or proPO cascade, and whether the p38 MAP kinase signaling pathway is implicated with the Toll and Imd pathways. It would be interesting to know whether these phenomena specifically occur in silkworm (or lepidopteran insects) or not.

Perspective

In this review, we have described the regulation of the innate immune response in the silkworm, *B. mori* (Fig. 2). Although a recognition factor to activate the immune systems was first identified in *B. mori*, recent silkworm immunity studies, particularly at the molecular level, are less advanced than those on the common vinegar fly and mosquitoes. Recently, the entire genome analysis of *B. mori* was almost completed (The International Silkworm Genome Consortium, 2008), and a subsequent

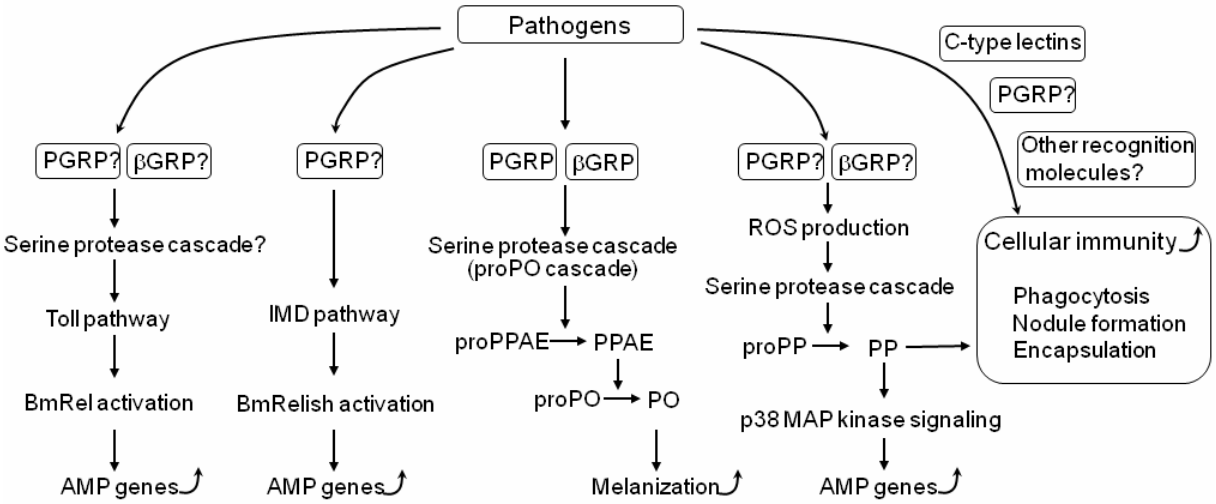

Fig. 2 Overview of innate immune responses in *B. mori*. PGRP; peptidoglycan recognition protein, βGRP; β glucan recognition protein, PPAE; prophenol oxidase activating enzyme, PO; phenol oxidase, PP; paralytic peptide.

genome-wide analysis of immune-related genes was performed (Tanaka *et al*., 2008). This analysis revealed that the factors involved in intracellular signal transduction pathways are well conserved between silkworm and non-lepidopteran insects, whereas the recognition proteins and effectors are structurally diverse among them. As for genes encoding recognition proteins and effectors, a dynamic lineage-specific gene evolution probably occurred in Lepidoptera to adapt the silkworm or lepidopteran insects to the pathogens that preferentially infect them. Unfortunately, the function of most genes encoding recognition proteins in *B. mori* is unknown. Nonetheless, they can be elucidated by the development of functional analyses such as RNA interference (RNAi) and overproduction of proteins using silkworm cell lines and/or transgenic silkworms. DNA-based RNAi in cultured silkworm cells is now available (Isobe *et al*., 2002; Fujita *et al*., 2009; Tanaka *et al*., 2009c). In addition, the established GAL4/UAS system allows a target gene or hairpin RNA corresponding to a target gene to be expressed in appropriate tissues and developmental stages in *B. mori* (Imamura *et al*., 2003; Tatematsu *et al*., 2010; Kobayashi *et al*., 2011). Moreover, the following two methods for gene targeting mutagenesis have been established, homologous recombination using *Autographa californica* nucleopolyhedrovirus (NPV) (Yamao *et al*., 1999) and zinc-finger nuclease (Takasu *et al*., 2010). Further studies on the functional analyses of immune-related genes using these techniques are expected in the near future.

Although we did not describe it in this review, it is also important to study immune systems against viruses. In contrast to studies of antiviral immunity against RNA viruses, which are proceeded mainly in Diptera (Lemaitre and Hoffmann, 2007; Kemp *et al*., 2009; Sabin *et al*., 2010), antiviral immunity against DNA viruses is poorly understood in insects. The silkworm is surely a good model for studies of immunity against DNA viruses because of the thorough characterizations of the NPV, an enveloped DNA virus that infects silkworms (Yao *et al*., 2006). Recently, we identified up-regulated and down-regulated host genes from a silkworm cell line in response to *B. mori* NPV infection (Sagisaka *et al*., 2010). We believe that elucidating of the function of these results will contribute to better understanding of the molecular aspects of insect immunity against DNA viruses.

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