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REVIEW

Immune strategies of silkworm, *Bombyx mori* against microbial infections

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Abstract

The silkworm, *Bombyx mori* has great economic and scientific value, as it has long been exploited as a primary silk producer and as a model system for lepidopterans and arthropod studies. This species is highly susceptible to microbial diseases that affect quality and quantity of silk, thereby causing huge economical losses. Insects have developed efficient innate immune system to fight against microbial pathogens. The innate immune system plays a crucial biological role in the limitation of microbial infections by using different immune strategies such as antimicrobial peptides production (AMPs), reactive oxygen species generation and melanin formation. So far, many studies identified different biological factors, which are considered to be involve in the regulation of these biochemical processes in *B. mori.* Here, we describe, current knowledge on the molecular patterns of various immune factors and also highlight their molecular mechanism of action in the limitation of viral, bacterial and fungal pathogens in *B. mori.* Furthermore, we discussed different strategies to improve the immune responses of silkworm species. This review will be helpful to understand the molecular aspects of immune factors, and their regulatory mechanism to control microbial diseases in the economically important insect species, *B. mori*.

Key Words: immunity; biological pathways; antimicrobial peptides; prophenoloxidase cascade; pattern recognition receptors

Introduction

The silkworm, *Bombyx mori* has great economic and scientific value, as it has long been exploited as a primary silk producer and as a model system for lepidopterans and arthropod studies (Nagaraju and Goldsmith, 2002). This species is largely cultured in many Asian countries (e.g. China, Japan, India) for silk production (Faruki, 2005). However, silk industry is gradually declining due to microbial (Viral, Bacterial, Fungal) diseases, which greatly affect the quality and quantity of silk (Xu *et al*., 2015; Abbas *et al*., 2017a). Thus, researchers have paid attention to understand the defense system of *B. mori* for better management of this economically important insect species.

Invertebrates including insects lack the adaptive immune system, therefore they rely on an innate immune system to fight against invading microbial pathogens (Dai *et al*., 2017; Wu *et al*., 2017; Chu *et al*., 2019).

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The immune system is further subdivided into cellular and humoral immune responses, which together provide an effective barrier to microbial infection (Abbas *et a*l., 2017a; Zhu *et al*., 2019). When microbial pathogens pass through host's physical barriers (e.g. epithelium of midgut or cuticle) and reach the hemocoel, the host pattern recognition receptors (PRRs) recognize pathogen associated molecular patterns (PAMPs) resided on the surface of the microbes and stimulate cellular and humoral immune responses (Kanost *et al*., 2004; Ishii *et al*., 2010; Dai *et al*., 2018). The cellular responses are mediated by various types of immune cells, hemocytes (Lavine and Strand, 2002; Zhou *et al*., 2017). While, humoral immunity largely stimulates the immune deficiency (IMD) and Toll pathway, which produce antimicrobial peptides (AMPs) through a signal transduction cascade, melanin and reactive oxygen species (ROS) (Kanost *et al*., 2004; Kausar *et al*., 2017a).

The silkworm *B. mori,* utilize innate immune system to fight against microbial pathogens during their life cycles. The larvae of this species are susceptible to viral, bacterial and fungal infection. The innate immune system comprising AMPs and

lysozymes, melanization and phagocytosis plays a crucial biological role in limiting microbial infections to a nonlethal level (Zhang *et al*., 2017; Kausar *et al*., 2018). Over the past years, many researchers studied the innate immune system of *B. mori* and reported different immune associated factors, and also described their molecular mechanism of action in this species. In this review, we demonstrate the existing knowledge on the molecular patterns of *B. mori* immune system following viral, bacterial and fungal infection. Further, this review will provide a comprehensive knowledge for researchers to further explore the immune system, and it will also help the industrialists for better management of silkworm.

Immune patterns against fungal pathogens

Fungi are one of the important groups of microbial pathogens that cause various diseases in silkworm and in other insect species (Abbas *et al.,* 2019b; Zhu *et al*., 2019). The white muscardine is one of the most common disease among silkworm species, which is caused by entomopathogenic fungus, *Beauveria bassiana* (Chengxiang *et al*., 2017; Lu *et al*., 2017; Sun *et al*., 2018). The spores of this species germinate on the host's integument, and penetrate into the hemocoel to obtain nutrients, and ultimately cause larval death (Wang and Wang, 2017). Thus, it is highly important to understand the immune responses of *B. mori* for its better management. Recent technologies (e.g. transcriptome analysis and suppression subtractive hybridization) have made it possible to investigate immune responses of this species against fungal infection (Liu *et al*., 2015; Yang *et al*., 2018). Many researchers suggested that immune associated genes (Cecropin B, Moricin, lysozyme precursor, ubiquitin, and β-1,3-glucan recognition protein (βGRP)-3 precursor) greatly vary their expression after fungal infection (Fig. 1) (Hou *et al*., 2011; Sun *et al*., 2017; Wang and Wang, 2017). It seems that these immune associated genes play a crucial biological role in the limitation of fungal infection, however the detailed molecular mechanism remained to define in *B. mori.*

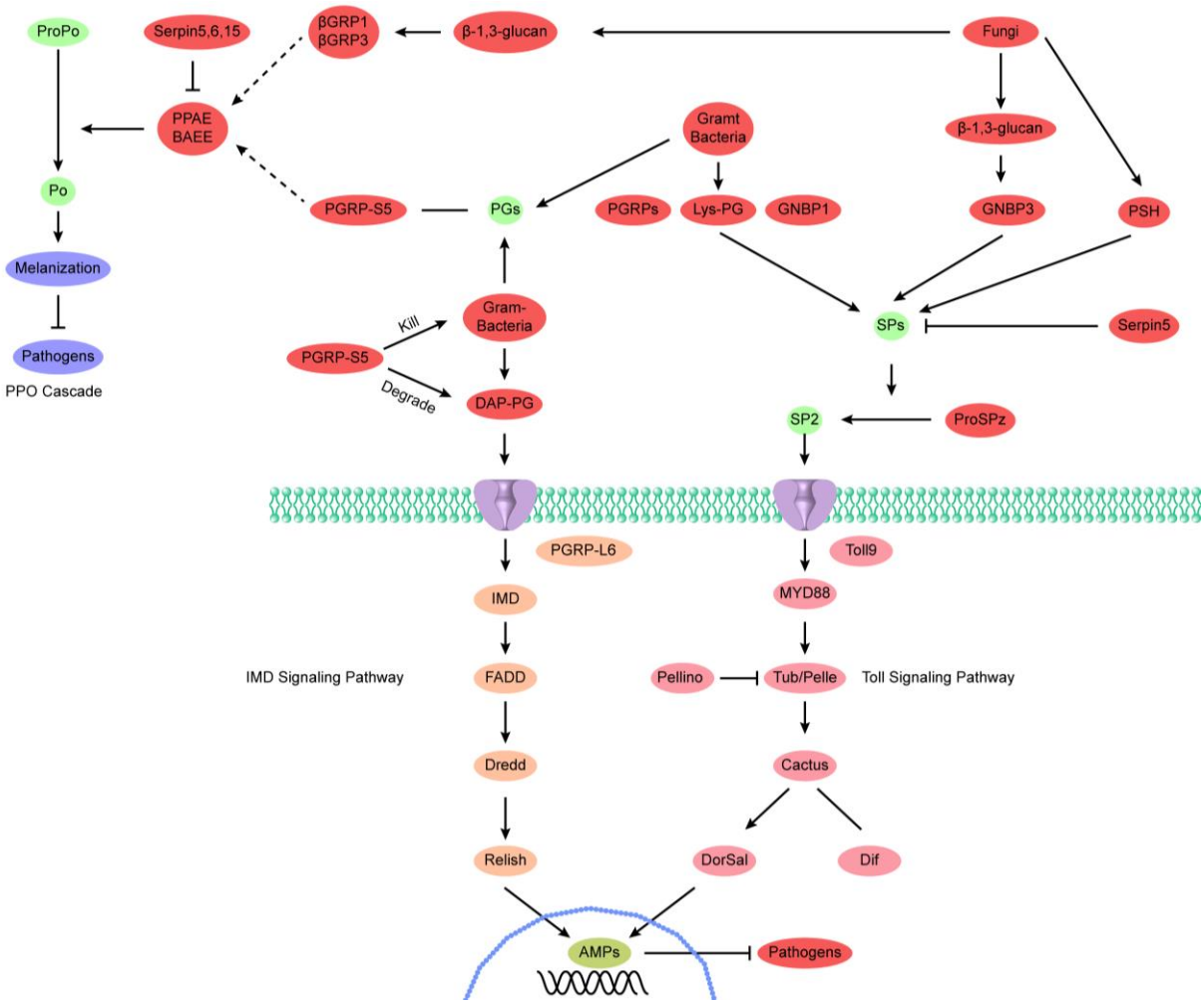

Fig. 1 The putative Toll and immune deficiency signaling pathway (immune pathways) involved in the immune responses against microbial (bacterial and fungal) infections in silkworm, *B. mori*

Immune strategies of *B. mori* against fungal infection

It has been shown that melanin formation and AMPs production are most effective strategies among insects to fight against fungal infections (Kausar *et al*., 2017a). The melanin formation is initiated by PPO cascade, which is modulated by various proteins in insects (Takahasi *et al*., 2009; Kausar *et al*., 2017b). Four βGRPs (βGRP1, βGRP2, βGRP3 and βGRP4) have been identified and molecularly characterized in *B. mori*, which play a crucial biological role in the activation of PPO cascade in this species. For instance, the βGRP1 contains a β-1,3 glucan-binding domain and a glucanase domain; additionally, the α-chymotrypsin digestion analysis of this protein indicates that it contains two functional components: the first 20 kDa (first 102 amino acid residues) component can bind to fungal β-1,3-glucan, while 43 kDa (a glucanase like domain) can induce PPO cascade (Ochiai and Ashida, 2000; Tanaka *et al*., 2008; Chen *et al*., 2016). A recent study determined that fungal β-1,3 glucan fails to activate the PPO cascade in βGRP deficient plasma. However, it resumes its activity on the addition of recombinant βGRP-3 protein (Takahasi *et al*., 2009). Overall, both of these (βGRP-1 and βGRP-3) proteins are important modulator in the activation of the PPO cascade by recognizing β-1,3-glucan in *B. mori*. The PPO activation mechanism has been well-established in a model insect, e.g. *M. sexta*. Briefly, β-1,3 glucans stimulate self-association of βGRP-2 proteins and forms a complex that provides a molecular platform for the subsequent events in the PPO activation. The C-terminal of this protein interacts with low-density lipoprotein receptor class A domains of hemolymph protease 14 (HP14) to recruit HP14 zymogens, which leads to the conversion of proHP21 to its active form, and also stimulates the PPO cascade (Wang and Jiang, 2007; Dai *et al*., 2013; Takahashi *et al*., 2015). However, the molecular mechanism of PPO activation still has not been completely understood in *B. mori*. To date, only few studies demonstrated the structural features of βGRPs, and the molecular mechanism of PPO activation in this species. A recent study reported that HP6 and HP21 form a complex with serine protease inhibitor 5 (Serpin 5) during the PPO activation (Li *et al*., 2016a), however, the subsequent events still need to be elucidated. Thus, to understand the detail mechanism of PPO activation in *B. mori*, future studies should focus to determine the events that occur following the detection of β-1,3-glucan by βGRPs, the mechanism of interaction between βGRP/β-1,3-glucan complex and PPO cascade. Although, some researchers described that PGRP-S5, βGRP-1, and βGRP-3 and HP6 and HP21 play a crucial biological role in the activation of PPO cascade. The PGs following β-1,3-glucan recognition activate proBAEEase (proBzArgOEtase) in the presence of Ca2þ, and this PPO-activating protein can directly cleave PPO (Satoh *et al*., 1999).

The production of AMPs is another important strategy for the limitation of fungal infection in invertebrates (Kausar *et al*., 2017a; Min *et al*., 2017). The Toll pathway has been reported to be involved in the production of AMPs in silkworm (Abbas *et al*., 2017a; Kausar *et al*., 2017b). This pathway comprises Toll9 and BAEEase proteins, which are essential for its normal biological functions (Jang *et al*., 2006; Wu *et al*., 2010). In *B. mori*, PAMPs stimulate the conversion of the inactive proBAEEase to its active form, which seems to be a homolog of *Drosophila* Spatzle-processing protein. This protein cleaves proSpatzle to activate the Toll signaling cascade in *Drosophila* (Jang *et al*., 2006). It is assumed that Toll pathway in *B. mori* also follow the same patterns of activation like its counterpart, *Drosophila*.

Growing evidence suggest that JAK-STAT pathway is also an important regulator of AMPs production (Abbas *et al*., 2017a). C-type lectin 5 act as a receptor molecule especially for fungal infection in this pathway. The suppression of this gene by RNA interference (RNAi) can reduce the production of STAT and HOP. Whereas, it enhances the expression of JAK/STAT inhibitors such as SOCS2. Furthermore, depletion of JAK/STAT inhibitors enhance the survival and hemolymph fungi clearance activity (Geng *et al*., 2016). Abbas and his co-workers noted that downregulation of JAK-STAT inhibitor (SOCS2) stimulates the production of various AMPs in *B. mori* (Abbas *et al*., 2017a). Further, it has been reported that Lebocin 5, Defensin B, Cecropin A, and Gloverin 2) have strong anti-fungal activities and show variable expression patterns (Kaneko *et al*., 2008; Lu *et al*., 2016, Ma *et al*., 2019).

Besides the Toll and JAK-STAT pathways, some cuticle proteins (e.g. SVWC) have also been reported to be involved in the limitation of *B. bassiana* infection (Han *et al*., 2017). TIL-type protease inhibitors such as SPI38 and SPI39 (serine protease inhibitors/serpins) inhibit melanization caused by CDEP-1, and also prevent the germination of *B. bassiana* spores *(*Li *et al*., 2012; Li *et al*., 2015).

Immune patterns against bacterial pathogens

In insects, bacteria are most broadly studied group of microbial pathogens. They use host metabolic machinery for their growth and reproduction. Further, they paralyze the host cellular and biochemical activities by producing PGs or by secreting proteases (Karlsson *et al*., 2012; Kong *et al*., 2015). To fight against infection, insects have developed innate immune system through the evolutionary period. The immune system initiates by the recognition of invading pathogen, and subsequently produce effectors to eliminate invading pathogens (Ochiai and Ashida, 2000; Chen *et al*., 2016). In this section, we describe different strategies being utilized by *B. mori* to fight against bacterial infection.

Bacterial detection mechanism of silkworm

The silkworm, *B. mori* contains a group of pattern recognition receptors for the recognition of bacterial pathogens. These receptors interact with PAMPs, subsequently initiate immune signaling in

Table 1 The list of peptidoglycan recognition proteins

S.no	PGRPs	Recognize	Source	Reference
1	PGRP-S1	PGs	*M. luteus*	Yang *et al*., 2017
2	PGRP-S2		Contribute in AMP production	Chen *et al*., 2018
3	PGRP-S3	-	-	-
4	PGRP-S4	PGs	*B. subtilis, S. aureus, S. marcescens*	Yang *et al*., 2017
5	PGRP-S5	PGs	*E. coli, B. megaterium, B. subtilis, M. luteus, S. aureus*	Chen *et al*., 2014; 2016
6	PGRP-S6	-	-	-
7	PGRP-L1	-	-	-
8	PGRP-L2	-	-	-
9	PGRP-L3	-	-	-
10	PGRP-L4	-	-	-
11	PGRP-L5	-	-	-
12	PGRP-L6	PGs	*S. aureus, E. coli, B. subtilis*	Tanaka and Sagisaka, 2016

host cells (Charroux *et al*., 2009; Karlsson *et al*., 2012). It has been shown that Gram-positive and Gram-negative bacteria strongly stimulate immune responses in insects e.g. *B. mori* and *Drosophila* (Lemaitre and Hoffmann, 2007; Kausar *et al*., 2018). Many in vivo and in vitro studies suggested that PAMPs (PGs and LPS) induce the production of various AMPs (e.g. cecropin B and lebocin 3) in *B. mori* (Hua *et al*., 2016; Abbas *et al*., 2017b). The PGs are recognized by peptidoglycan recognition proteins (PGRPs), So far, variety of PGRPs have been identified and characterized in animals (Christophides *et al*., 2002; Tanaka *et al*., 2008; Zhu *et al*., 2019). In *B. mori* first PGRP was identified from hemolymph during 1990s (Yoshida *et al*., 1996). Later, this number increases to twelve in this species (Tanaka *et al*., 2008). As shown in Table 1, the biological functions of only few PGRPs have been described, suggesting these proteins are primarily used to detect PGs (Chen *et al*., 2016; Yang *et al*., 2017). However, subsequent events following recognition of PGs remained unclear. Generally, LPS-binding protein (LBP) recognize LPS (a component of Gram-negative bacteria) and initiates downstream signaling. For instance, the LPS-LBP complex interacts with CD14, and delivered LPS to Toll-like receptors to stimulate downstream signaling in human (Tapping and Tobias, 2000; Ranoa *et al*., 2013). In *B. mori,* LBP protein has also been described, and suggested to be involved in the clearance of bacteria (Koizumi *et al*., 1999). But still the mechanism of action of this protein need to be illustrated.

Immune strategies of *B. mori* against bacterial infection

The silkworm, *B. mori* fight against bacterial pathogens by utilizing different effective strategies such as reactive oxygen species (ROS), AMPs, melanization and immune cells (Tanaka *et al*., 2008; Panthee *et al*., 2017).

The production of ROS (nitric oxide and hydrogen peroxide) play a crucial biological role in the limitation of bacterial infection in insects including *B. mori* (Zhang and Lu, 2015; Kausar *et al*., 2018). Nitric oxide can directly remove bacterial pathogens, or indirectly it stimulates immune signaling to produce anti-bacterial effectors (Nappi *et al*., 2000; Liu *et al*., 2019). A study showed that LPS administration can induce the production of Nitric oxide synthase 1, leading to nitric oxide generation that stimulate the AMPs (e.g. cecropin B) production (Imamura *et al*., 2002). ROS protect host from bacterial pathogens by preventing their cellular growth. However, the increase in its concentration may harm cellular components e.g nucleic acids, proteins, and lipids (Abbas *et al*., 2019a; Chu *et al*., 2019). To neutralize excessive level of ROS, animals including *B. mori* have developed an antioxidant system (Wu *et al*., 2017; Dai *et al*., 2018; Abbas *et al*., 2019a). The variation in production of antioxidant enzymes (e.g. peroxiredoxins, catalase and other antioxidants) after bacterial infection has been demonstrated by various authors in *B. mori* (e.g. Shi *et al*., 2012; Zhang and Lu, 2015; Wang *et al*., 2016), suggesting their involvement in the limitation of bacterial infection in this species.

Production of AMPs have also been considered an important immune strategy to control bacterial infection in insects. In *D. melanogaster*, it has been shown that the Toll, IMD pathways are activated and produce AMPs following bacterial invasion to limit infection (Rutschmann *et al*., 2002; Kaneko and Silverman, 2005). In *B. mori*, many studies reported the enhancement of Cecropin, Attacin, Gloverin, Defensin, Moricin, and Lebocin after bacteria, PGs, and LPS challenge (Kaneko and Silverman, 2005; Tanaka *et al*., 2008; Ma *et al*., 2019). These AMPs have strong antibacterial activities*;* however, their expression patterns vary with different type of bacterial strains*.* For instance, *S. aureus* strongly stimulate the expression of Cecropin XJ (Xia *et al*., 2013), *E. coli* and *B. subtilis* enhance the production

of Defensin B in fat body (Kaneko *et al*., 2008). Whereas, *P. aeruginosa* can stimulate the production of Defensin, Attacin, Cecropin, Lebocin, Gloverin, and Moricin in fat body. The production of AMPs is regulated by the coordination of CPT1 (Tweed lecuticular protein), PGRP-S5 and LBP (Liang *et al*., 2015; Chen *et al*., 2016). Huang and his co-worker (2009) demonstrated that *B. bombyseptieus* (Gram- positive bacteria) induce the expression of Lebocin, Attacin, Enbocin, Moricin, and Gloverin in gut of *B. mori* (Abbas *et al*., 2017; Abbas *et al*., 2018). In addition, in vitro experiments on NISES-BoMo-Cam1 cells showed that LPS and other bacterial challenge can induce different AMPs expression (Ishii *et al*., 2010; Min *et al*., 2017). Interestingly, isopropanol limits *M. luteus* infection by stimulating Cecropin D, Gloverin 3, and Cecrop in fat body. Along with the production of AMPs, regulatory mechanism is also activated to control their excessive production in *B. mori*. For example, Serpin-5, Serpin-6 and Serpin-15 are important negative regulators of AMPS in this species. Of which Serpin-5 modulate the Toll pathway by targeting HP6 and SP21 (Fig. 1) (Liu *et al*., 2015; Li *et al*., 2017). Overall, AMPs production is an important strategy to control the bacterial infection in *B. mori.* However, future studies should address the threshold level of bacteria and PAMPs, which is required to stimulate the production AMPs in *B. mori.*

The melanin formation is used by insects as an important strategy to limit bacterial infection. Pattern recognition receptors recognize the invading bacteria, subsequently stimulate the PPO cascade, leading to melanin formation. Melanin is deposited on the bacterial surface to prevent their cellular growth and movement, and finally cause bacterial death. The PPO1 and PPO2 genes are activated after bacterial infection in *B. mori* (Kawabata *et al*., 1995; Clark and Strand, 2013). Additionally, in hemolymph of this species, PO and its associated proteins form a complex, which is essential to initiate melanin formation (Clark and Strand, 2013). A recent study suggested that hindgut of *B. mori* express PPO gene, which activate the PPO cascade, leads to melanin formation and ultimately reduces the bacterial load (Shao *et al*., 2012). Furthermore, PGRP-S1, PGRP-S4, and PGRP-S5 also play a crucial biological role in the activation of the PPO cascade (Chen *et al*., 2016; Yang *et al*., 2017). To prevent excessive melanization process various negative regulators are also produced in insects. The negative regulators (Serpin-5, Serpin-6, and Serpin-15) inhibit melanin formation by suppressing the activities of serine proteases in *B. mori* (Liu *et al*., 2015; Li *et al*., 2017).

Many studies demonstrated that hemocytes also play a key biological role in the suppression of bacterial infection in silkworm. *B. mori* contains five different types of hemocyte cells (plasmatocytes, prohemocytes, granulocytes, oenocytoids, and spherulocytes) Of which Plasmatocytes comprise phagocytosis activity and granulocytes play a role in the encapsulation of small particles (Ling *et al*., 2003; Zhang *et al*., 2014). However, the detailed mechanisms underlying encapsulation, phagocytosis, and nodulation remained unclear in this species.

Immune strategies of *B. mori* against viral infection

Viral infection is considered a serious threat to living organisms and their diseases cause approximately 20 % losses of *B. mori* cocoons each year. So far, there is no effective strategy to control viral infection in this species. Thus, the viral studies have great importance for better management of silkworm species. A recent report suggests that use of transgenic silkworms with strong antiviral capacity to reduce its larvae mortality would provide new strains for sericulture (Jiang, 2014; Gupta *et al*., 2015).

The *B. mori* viruses, especially nucleopolyhedrovirus (BmNPV) is a notorious pathogen in the silk industry. Researchers fail to develop potential strategy to control this infectious agent in *B. mori* (Hao *et al*., 2015; Nie *et al*., 2017; Wang *et al*., 2017; Gao *et al.*, 2018). It has been shown that the red fluorescent proteins (RFPs) is an effective protein against BmNPV infection. This protein is specifically produced in the midgut of *B. mori.* This protein effectively disrupts the NPV nucleocapsid or limits the NPV multiplication or agglutinates the virus and is excreted along with fecal material. However, still there is need to explore exact biological antiviral mechanism of this protein (Yao *et al*., 2006; Gupta *et al*., 2015; Zhang *et al*., 2018).

Many authors reported the involvement of serine proteases and lipases in viral immune response (e.g. Ponnuvel *et al*., 2003). Several studies described that lipases greatly contribute in the removal of viral pathogens. Lipase-1, purified from the digestive juice of *B. mori* larvae was found to have great antiviral activity particularly against BmNPV. This gene (Bmlipase-1) is produced only in the midgut of *B. mori.* Ponnuvel and his co-workers (2003) examined the oral administration of pre-treated BmNPV-ODV (ODV incubated with Bmlipase) in 5th instar larvae of *B. mori*, these larvae displayed strong resistance to viral infection and successfully entered the pupal stage, suggesting it might be due to the suppression of viral proliferation by midgut lipase1 (Ponnuvel *et al*., 2003).

It has been shown that serine proteases modulate different defense responses such as AMPs production, melanization and hemolymph coagulation in invertebrates (Gorman and Paskewitz, 2001; Lekha *et al*., 2015). The presence of serine protease in *B. mori* larvae display strong activity against BmNPV (Nakazawa *et al*., 2004; Kausar *et al*., 2017a). Further, some recent studies suggested the enhancement of lepidopteran-specific AMPs (lebocin, gloverin-1, 2, 3, attacin, cecropin) and lysozyme after BmNPV infection silkworm (Bao *et al*., 2009; Ma *et al*., 2019). Interestingly, gloverin-4 has also been reported to be upregulated in *B. mori* and BmN cells suggesting it is specific biological role in the limitation of viral infection (Bao *et al*., 2009).

Heat shock proteins (HSPs) are a group of molecules, which are enhanced following stress conditions as we as they are involved in the folding and unfolding of proteins. HSP70 and HSP90 members of this family have been reported to

greatly express after BmBDV, BmNPV and BmCPV treatment (Bao *et al*., 2009; Yin *et al*., 2016). Moreover, HSP19.5, HSP 23.7 and HSP 27 are strongly increased to limit viral infection (Liang *et al*., 2007). Therefore, it is supposed that HSPs are involved in anti-viral immune responses and may activate the downstream signaling following detection of viruses.

The piRNA pathway has widely been studied in vertebrates and invertebrates. This pathway has been recognized as the crucial protection mechanism against the activity of transposable elements in genome of animals. The piRNAs production is Dicer-independent and depends on the Piwi proteins activity, a subclass of the Argonaute family (Siomi *et al*., 2011). Primary piRNAs are processed from single stranded RNA precursors that are usually transcribed from chromosomal loci primarily comprising remnants of transposable element sequences, named as piRNA clusters (Aravin *et al*., 2007). The processing of primary piRNA precursors in *D. melanogaster* and production of mature piRNAs have been linked to activity of Zucchini endonuclease (Ipsaro *et al*., 2012; Han *et al*., 2015; Mohn *et al*., 2015). The processed precursor is loaded into Piwi family Argonaute proteins Piwi or Aubergine and then cleaved by nuclease to reach its final length that range from 24 to 30 nucleotides, which vary in different insects such as fruit fly (25 nucleotides) mosquitoes (28 nucleotides). The trimmed piRNAs undergo a final 3′ end 2′-O-methyl nucleotide modification induced by the methyltransferase Hen1 (Saito *et al*., 2007) to become mature piRNAs. Primary piRNAs contain a 5′ uridine bias and are generally antisense to transposable element transcripts (Saito *et al*., 2006). The cleavage of complementary active transposon RNA by primary piRNAs loaded into Aubergine proteins starts the second biogenesis round and leads to the generation of secondary piRNAs that are loaded in Argonaute-3. During this amplification cycle, Aubergine and Argonaute-3proteins loaded with secondary piRNAs mediate the cleavage of complementary RNA to produce new secondary piRNAs similar to the piRNA that started the cycle. Since target slicing by Piwi proteins happens between 10 and 11 nucleotides, the complementary secondary piRNAs have a 10 nucleotide overlap and comprise an adenine at position 10 (Aravin *et al*., 2007).

Recently, it has been described that the piRNA pathway is involved in in antiviral defense of insects. The piRNA pathway antiviral defense activity was first reported in 2010, when small RNAs of virus with the sequence length of piRNAs were observed in *D. melanogaster* ovarian somatic sheet cells (Wu *et al*., 2010). Since then, this pathway involvement in the antiviral defense of insects has attained attention of researchers, and many studies on this subject has performed using mosquito-arbovirus experimental systems. In cell lines and Aedes mosquitoes, an expanded family of Piwi proteins is transcribed in somatic cells/tissues and viral-derived piRNAs are generated from the genomes of many arboviruses (Brackney *et al*., 2010; Vodovar *et al*., 2010). Furthermore, functional links among the piRNA pathway, arbovirus replication, and vpiRNA generation have also been reported. Suppression of Piwi-4 protein has been found to increase replication of Semliki Forest virus [SFV; (+) ssRNA, Togaviridae] without interfering with vpiRNA expression in Aag2 cells (Schnettler *et al*., 2013), whereas both Piwi-5 and Argonaute-3 have reported to be needed for the biogenesis of piRNAs from Sindbis virus [SINV; (+) ssRNA, Togaviridae] in the same cell line (Miesen *et al*., 2015). However, in vivo experimental information are scarce, and further studies are required to completely understand the extent to which the piRNA pathway participate to antiviral defense in insects.

Collectively, in recent years, the piRNA pathway has been widely studied in insects particularly in mosquitoes (e.g *Aedes Aegypti*) that broaden our knowledge on the complex picture of this pathway. This has resulted in the clarification of more and more details of this fascinating biological pathway and its variation and similarities to piRNA pathways in other invertebrates and vertebrates, such as *D. melanogaster*. A more detailed knowledge of the piRNA pathway in insects, particularly its potential participation in heritable immune system memory and likely effect on virus infection, will help us to understand the variations in vector competence among different species of insects and the spread of the pathogen.

Comparison of *B. mori* immune responses with other insect species

Researchers use virus, fungi, bacteria and its wall component (e.g. LPS, PGN) as immune elicitors to understand immune responses in different insect species. Many immune studies are available on various economically important insects such as *B. mori, A. pernyi, Actia selene* and *M. sexta* etc, which have described the molecular mechanisms of immune responses (Tokura *et al*., 2013; Abbas *et al*., 2017a; Kausar *et al*., 2017b). This section will provide a comprehensive overview of comparison of immune responses between *B. mori* and other insect species.

The differences in the susceptibility of different insect species to viral, bacterial and fungal invasion may be because of their immune potencies (Seyedtalebi *et al*., 2017). However, following microbial challenge, despite of the variation in experimental methods and species in the immune studies, different species approximately show similar immune response as innate immune system in insects remain conserved during evolutionary period (Wang *et al*., 2019). However, some difference may exist that may occur in the molecular mechanism of the insect species.

B. mori and *M. sexta* approximately follow the same mechanism of PPO activation following microbial infection. Many of their molecules are similar in function with approximately same molecular mechanism. Only difference has been reported at the final step of PPO activation in these species (Sakamoto *et al*., 2011; Tokura *et al*., 2013). In *M. sexta* serine protease homolog 1 and 2 are associated loosely with PPO and PAP1 or PAP3 to form a large complex. These proteins are also

needed for proteolytic cleavage to gain function that leads to their association into the active, high M_r cofactor required in the molecular reaction with PAP and PPO to produce high levels of PO activity (Gupta *et al*., 2005). Interestingly, this molecular interaction seems to be not needed for *B. mori* PPAE (Wang and Jiang, 2004), therefore further research is required to understand this interesting phenomenon.

Strategies to improve immunity of silkworm against microbial infection

To improve the management of silkworm, attempts have been made to improve the immune response of silkworm species against microbial infection. The immune responses could be improved by optimizing and integrating antimicrobial strategies, improvement to antimicrobial silkworm strains, and generating transgenic silkworm species, which have increased resistance to microbial pathogens. Using these strategies silkworm species can be generated that have improved immune responses against pathogens infection.

The best example is the improvement antiviral immunity in silkworm species. RNA interference and overexpression of antiviral proteins that efficiently targets viral genes are two greatly effective antiviral strategies. Additionally, combining these methods using transgenic technique can further improves host resistance (Jiang *et al*., 2013b). The silkworm strain SW-H is the first transgenic animal that have ability to reduce viral infection at its different stages. Bmlipase-1 is regulated through the B. mori midgut-specific, highly activity P2 promoter in this transgenic species (Jiang *et al*., 2013a) and double stranded RNA for the tandem BmNPV genes such as gp64, ie-1, lef-2, lef-1, and dnapol is derived from hr3þIE1P (Jiang *et al*., 2013b). Furthermore, by combining the different anti-viral strategies such as Bmlipase-1 overexpression, suppression of different viral genes, hycu-ep32 overexpression, and RNA interreference of BmPGRP2 could generate a transgenic silkworm species with greater antiviral resistance , which can suppress viral infection at initial stages of infection and affects the expression of viral genes and synthesis of proteins as well as host immune responses. Additionally, several antimicrobial including antibacterial, antiviral and antifungal agents (e.g. seroin) have been described in silkworm that can be used as potent candidates for use in development of transgene-based disease resistant silkworm strains (Singh *et al*., 2014).

Conclusion and future perspectives

In the past years, many researchers investigated the immune responses of *B. mori* against microbial pathogens. The whole genome sequencing of this species has enhanced the resource essential to systematically identify and characterize putative immune genes. To date many genes have been demonstrated to be involve in the immune responses of *B. mori*. Our knowledge of *B. mori* antimicrobial immunity has also been greatly expanded. Many studies suggested that the canonical immune signaling pathways are involved in antimicrobial immune responses of *B. mori*. However, there are still various questions that require to be addressed in the future studies. For instance, Demonstrating the detailed molecular mechanism of anti-microbial (virus, bacteria, and fungus) immunity, and identifying new immune associated genes will be a greatly important field of future research.

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