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Insights Into the Antiviral Pathways of the Silkworm *Bombyx mori*

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The lepidopteran model silkworm, *Bombyx mori,* is an important economic insect. Viruses cause serious economic losses in sericulture; thus, the economic importance of these viruses heightens the need to understand the antiviral pathways of silkworm to develop antiviral strategies. Insect innate immunity pathways play a critical role in the outcome of infection. The RNA interference (RNAi), NF-kB-mediated, immune deficiency (Imd), and stimulator of interferon gene (STING) pathways, and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway are the major antiviral defense mechanisms, and these have been shown to play important roles in the antiviral immunity of silkworms. In contrast, viruses can modulate the prophenol oxidase (PPO), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), and the extracellular signal-regulated kinase (ERK) signaling pathways of the host to elevate their proliferation in silkworms. In this review, we present an overview of the current understanding of the main immune pathways in response to viruses and the signaling pathways modulated by viruses in silkworms. Elucidation of these pathways involved in the antiviral mechanism of silkworms furnishes a theoretical basis for the enhancement of virus resistance in economic insects, such as upregulating antiviral immune pathways through transgenic overexpression, RNAi of virus genes, and targeting these virus-modulated pathways by gene editing or inhibitors.

Keywords: immunity, signaling pathway, virus, antivirus, insect, silkworm

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INTRODUCTION

Virus infection poses a serious threat to human health and agricultural production. As the only fully domesticated insect, the lepidopteran model silkworm, *Bombyx mori*, is economically important for silk production. Sericulture is one of the main sources of income for farmers in many developing countries (1, 2). However, viral diseases have caused losses of nearly 16% of the potential cocoon production each year in sericulture, which are induced mainly by the *Bombyx mori nucleopolyhedrovirus* (BmNPV), *Bombyx mori cytoplasmic polyhedrosis virus* (BmCPV), or the *Bombyx mori bidensovirus* (BmBDV) (1).

Insects mainly rely on innate immunity to defend against invading pathogens, and immune pathways play an important role in this process. Although some host signaling pathways can be modulated by viruses to elevate virus proliferation, targeting these pathways can also inhibit virus infection. In this review, we present an overview of the main pathways involved in the antiviral mechanism of silkworms. Such knowledge could provide a theoretical basis for strategies for control of viral diseases in economic insects.

CHARACTERISTICS OF SILKWORM VIRUSES

Among the three major pathogenic viruses of silkworms, the BmNPV, a member of the Baculoviridae family having a circular double-stranded DNA genome (3), is the most prevalent threat to sericulture in almost all countries (1). The viral DNA combines with capsid proteins to form a nucleocapsid that is contained within an envelope (1, 3). The BmNPV replication cycle has two virion phenotypes: (1) the occlusion-derived virus that is transmitted among hosts, and packaged and protected in an occlusion body (4, 5), and (2) the budded virus that spreads throughout the host. The BmCPV belongs to the *Cypovirus* genus of the *Reoviridae* family, and its genome consists of ten discrete double-stranded RNA (dsRNA) segments (6, 7). The BmCPV particles contain nucleic acid and protein capsid, and they are non-enveloped and occluded within polyhedral bodies (6, 7). The BmBDV belongs to the *Bidensovirus* genus of *Bidnaviridae* family, and has two geographical variants, BmDNV-2 and BmDNV-Z (8–10). The BmBDV virions are non-enveloped and assembled by a protein capsid and nucleic acid, with their viral genome consisting of two linear non-homologous single-stranded DNA segments (8–10).

These viruses invade the silkworm larvae mainly via oral infection. The BmNPV can infect almost all tissues of the silkworm whereas the BmCPV and BmBDV can only infect the silkworm midgut (1). Some silkworm strains are resistant to the viruses at any viral dose (1, 9). For example, the *nsd-2* mutation is caused by a 6-kb deletion in the open reading frame of $+^{nsd-2}$ and imparts resistance to the BmDNV-2 (9). However, the receptor and major resistance genes to the BmNPV and BmCPV have not been identified in silkworm. The BmN and BmE are two cell lines commonly used in silkworm research, which are derived from the ovary and embryonic cells of silkworm, respectively. The BmNPV can infect the two cell lines, unlike the BmCPV and BmBDV; therefore, most silkworm antiviral research is focused on the BmNPV (11–17), a few on the BmCPV (18, 19), and very few on the BmBDV (20).

SILKWORM ANTIVIRAL IMMUNE PATHWAYS

The antiviral defense mechanism of silkworms mainly relies on innate immunity, including the RNA interference (RNAi), NF-kB-mediated pathways, and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (19, 21–24). Among these immune responses, RNAi is the major defense strategy against viruses in insects (23, 25).

RNAi Pathways

There are three RNAi-related pathways in insects, including the small interfering RNA (siRNA) pathway, microRNA (miRNA) pathway, and the PIWI-associated RNA (piRNA) pathway (26). When challenged with viruses, the siRNA pathway is activated by the dsRNA that is commonly generated as a byproduct of viral replication (27, 28). The Dicer2 enzyme recognizes viral dsRNA and processes the dsRNA into siRNAs. One strand of duplex siRNA is associated with Ago2 to form the RNA-induced silencing complex (RISC), and then directs RISC to the viral RNA target through base pairing. Subsequently, Ago2 cleaves the viral RNA, inhibiting viral replication (25, 27, 28) (**Figure 1A**). The expressions of both Ago2 and Dicer2 were not induced by silkworm viruses (21). However, the results of deep sequencing revealed that a large number of viral siNRA (∼20 nucleotides) was generated in insect hosts infected with baculovirus (29) and BmCPV (30), indicating that the RNAi response is an important antiviral defense of hosts. Overexpression of Ago2 and Dicer2 can improve the susceptibility of silkworm to dsRNA (31). Expression of dsRNA targeting the viral genes of BmNPV (13), BmCPV (18), and BmBDV (20) in transgenic silkworms substantially decreased the viral mRNA content and silkworm mortality after viral infection. The siRNA pathway is the predominant mechanism responsible for antiviral activity in insects (27, 28). For the applications and challenges of insect RNAi, please refer to the recent reviews (32, 33).

The miRNAs are small noncoding RNAs that can bind to target genes and regulate their expression (34). The miRNA pathway is involved in the interaction between silkworm and viruses (23, 35). Virus-encoded miRNA can facilitate viral multiplication. For example, BmNPV-miR-1 (35) and BmNPV-miR-3 (36) can enhance BmNPV infection via regulating the exportin-5 cofactor Ran and the viral *P6.9* expression, respectively; BmCPV-miR-1 could facilitate target gene *BmIAP* expression and BmCPV replication (37). Similarly, silkworm-encoded miRNA could be regulated to promote viral proliferation. For example, bmo-miR-274-3p, whose inhibition enhances target viral *NS5* expression and facilitates BmCPV replication, was downregulated in a BmCPV-infected silkworm midgut (38). Additionally, host miRNA can inhibit viral proliferation. For example, bmo-miR-2819 can downregulate the *ie-1* gene of BmNPV to suppress viral multiplication (39); although bmo-miR-278-3p could decrease target gene *IBP2* expression and increase BmCPV mRNA, it is downregulated and *IBP2* is upregulated in BmCPV-infected silkworms (40). The contribution of the miRNA pathway is minor in the RNAi antiviral defense of insects. In contrast to siRNAs and miRNAs, piRNAs are derived from single stranded RNA precursors (23). The role of the piRNA pathway in the antiviral response of insect models has been reviewed recently (41), however, of which the exact roles in the interaction between silkworm and its major pathogenic viruses are unclear, having few relevant reports so far (42, 43).

NF-kB-Mediated Antiviral Pathways

The Imd and Toll pathways are canonical NF-kB-dependent pathways involved in the innate immunity of insects, wherein they activate the downstream antimicrobial peptide (AMP) genes transcription mediated by two distinct orthologs of the NF-kB transcription factor (19, 25, 44). The NF-kB ortholog Relish is the terminal transcription factor for the Imd pathway, whereas the Dorsal and Dorsal-related immune factor (Dif) function in the Toll pathway (25). Toll pathway responds to Gram-positive bacteria and fungi infections, whereas Imd pathway

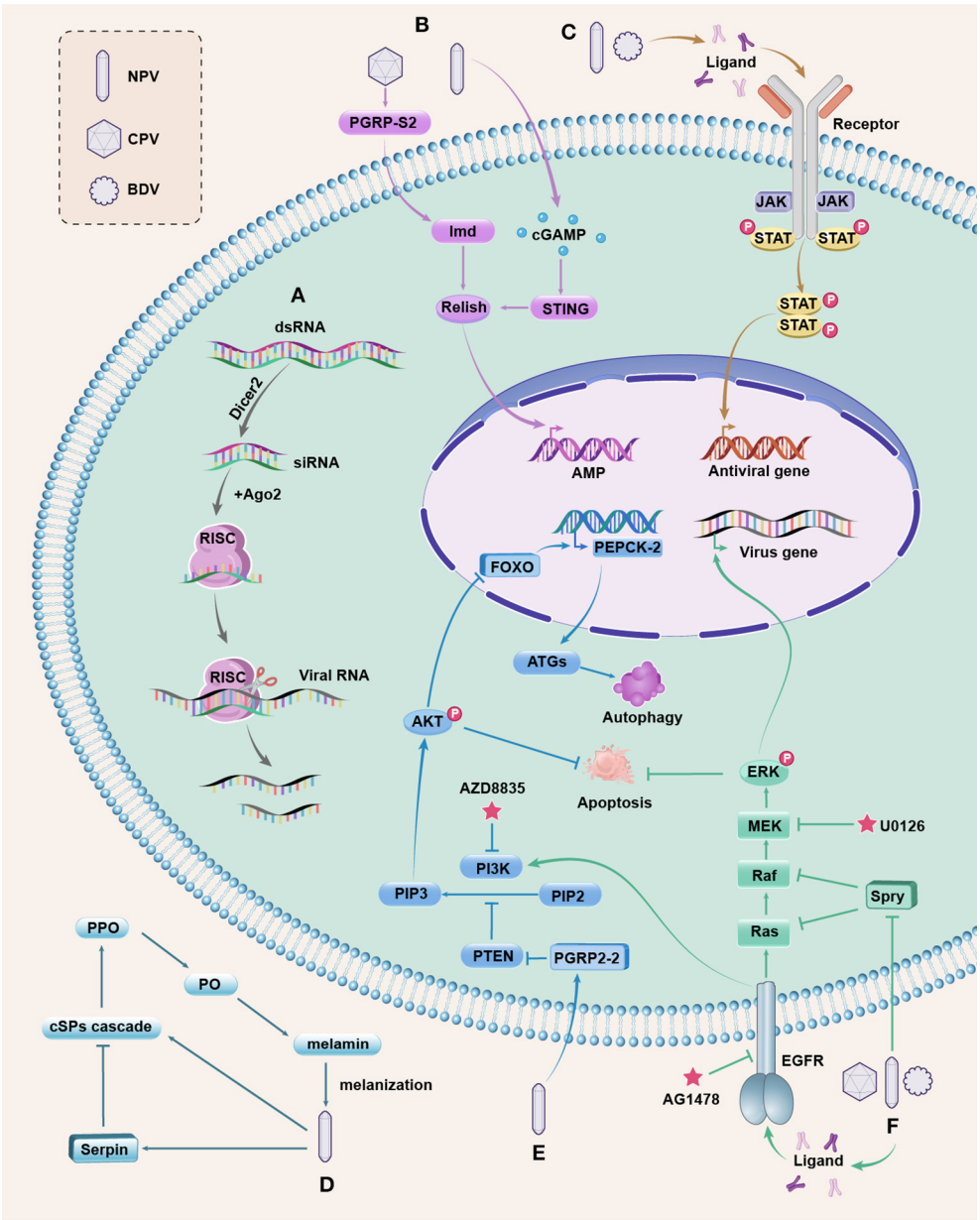

FIGURE 1 | Antiviral pathways in silkworm. **(A)** The siRNAi pathway is activated by viral dsRNA, which is cleaved into siRNAs by Dicer2. Ago2 is associated with one strand of siRNA to form RISC that can target and cleave the viral RNA to inhibit viral replication. **(B)** The NF-kB-mediated, Imd, and STING pathways. BmCPV induces the extracellular BmPGRP-S2 to active Imd and the downstream NF-kB ortholog Relish; BmNPV infection triggers the production of cGAMP to activate BmSTING for processing Relish. Activated Relish is translocated to the nucleus to initiate the transcription of AMP. Whether AMPs have antiviral function in silkworms needs further study. **(C)** The JAK/STAT pathway. The extracellular ligands bind to JAK associated receptors upon stimulation, leading to the activation of JAKs, and then cytosolic STATs are phosphorylated, forming the STAT dimers, which are translocated to the nucleus to regulate the expression of antiviral genes. **(D)** The PPO pathway is initiated by recognizing invading microbes, and then the extracellular cSP cascade is activated to convert the zymogen PPO to active PO. PO catalyzes the formation of melanin, resulting in melanization that kill the microbes. This pathway is negatively regulated by serpins, and baculovirus can induce serpins to suppress the melanization response of host insects for survival. **(E)** The PI3K/Akt pathway. Activated PI3K converts PIP2 into PIP3 to cause Akt phosphorylation (p-Akt). PTEN is a negative regulator of the PI3K/AKT pathway. BmNPV induces BmPGRP2-2 to suppress PTEN, resulting in increased p-Akt that inhibits cell apoptosis. Upregulated p-Akt also causes the inhibitory phosphorylation of the transcription factor FOXO, decreasing the expression of *BmPEPCK-2* and resulting in reduced autophagy genes (ATGs) expression, thereby blocking host autophagy. The inhibited apoptosis and autophagy are beneficial for viral replication. The PI3K inhibitor AZD8835 can decrease the mortality of silkworms infected with BmNPV. **(F)** The ERK pathway. Upon viral infection, the extracellular ligands activate EGFR (a receptor tyrosine kinase) to promote ERK phosphorylation (p-ERK) through the activation of Ras to the Raf/MEK/ERK phosphorylation cascade. p-ERK can regulate the transcription of viral genes and inhibit apoptosis. The Spry protein is a negative regulator of EGFR/ERK pathway that inhibits Ras or Raf, and both DNA and RNA viruses can downregulate Spry to increase p-ERK to ensure viral reproduction. AG1478 is a specific inhibitor of EGFR and U0126 binds to MEK to prevent p-ERK. The EGER also participates in the activation of PI3K by BmNPV. These pathways are integrated and are responsive to one another, which are complex and merit further investigation.

responds Gram-negative bacteria (19, 25). The transmembrane receptors peptidoglycan recognition protein (PGRP)-LC and the intracellular PGRP-LE sense the diaminopimelic acid-type peptidoglycan of Gram-negative bacteria, and transmit the signal to the adaptor molecule Imd, which is essential for the activation of Relish (25, 45). The Imd and Toll pathways have been shown to play a role in the antiviral immunity of *Drosophila* (25, 46–48). AMPs seems to have antiviral function in *Drosophila*, but their exact antiviral mechanisms are still unknown and more in-depth researches are needed (49).

Our research showed that *BmPGRP-S2* was induced by BmCPV in the silkworm midgut (7). Further experiments revealed that BmPGRP-S2 was a secreted protein, which may recognize a certain viral component and then transmit the signal to downstream molecules, and its overexpression increased the expression of *BmImd*, *BmRelish*, and *AMPs* and decreased silkworm mortality after BmCPV infection (19) (**Figure 1B**). These results indicate that the Imd pathway is involved in the defense against the RNA virus in silkworms. However, the function of this pathway in DNA virus-infected silkworms is not yet known. There have been few reports on the Toll pathway involved in antiviral immunity in silkworms. Recently, the stimulator of interferon genes (STING) has been reported to provide antiviral immunity against BmNPV in silkworms by promoting NF-kB activation (22). Production of cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) is triggered upon BmNPV infection, inducing the BmSTING activation to process BmRelish, and then the activated BmRelish is translocated to the nucleus to initiate the transcription of AMP (22) (**Figure 1B**). The aforementioned result revealed that the NF-kB-mediated, Imd, and STING pathways play important roles in silkworm antiviral defense, but the antiviral mechanisms of the two pathways are only partially elucidated and need more experimentation. Deciphering the roles of Toll pathway in silkworm antiviral immunity remains a challenging task.

JAK/STAT Pathway

JAK/STAT signaling is an important pathway involved in multiple cellular processes such as cell proliferation and immune regulation in insects (21, 25). This pathway contains a diverse family of extracellular ligands such as cytokine and growth factors, transmembrane receptors, JAK tyrosine kinases that are associated with the intracellular part of the receptor, and STAT proteins (25, 50). Following stimulation, a ligand binds to the extracellular part of the JAK-associated receptors, leading to the activation of JAKs. Subsequently, cytosolic STATs are recruited to the JAK/receptor complex, and then phosphorylated, forming the STAT dimers, which are translocated into the nucleus and bound to the DNA promoters of the target genes to regulate their expression (25, 50) (**Figure 1C**).

The insect JAK/STAT pathway activation mechanism has been well-established in *Drosophila* and mosquito (25, 51–53). There has been growing evidence that the JAK/STAT pathway may be functionally analogous to the mammalian interferon system (51). The JAK/STAT pathway has been shown to respond to viral infections in *Drosophila* by regulating the production of downstream effector molecules, including the AMPs (25, 53). The BmNPV and BmBDV, unlike the BmCPV, induce the expression of *BmSTAT* in silkworms, implying that the JAK/STAT pathway could be activated by the DNA viruses in silkworms (21). Overexpression of *BmSTAT* in BmN cells increased the number of cells in the G2 phase of the cell cycle (54) and host resistance to BmNPV, but not to BmCPV (55). Additionally, inhibition of Hsp90 can cause upregulation of *BmSTAT* expression and suppression of BmNPV replication in the BmN cell (56), but it is not clear how Hsp90 can be linked to JAK/STAT. The extracellular ligand and effector molecules of this pathway in response to viral infection in silkworms have not been clearly identified and merit further investigation.

VIRUS-MODULATED HOST SIGNALING PATHWAYS

During the interaction between the insects and viruses, several host signaling pathways including the prophenol oxidase (PPO), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), and the extracellular signal-regulated kinase (ERK) pathways have been reported to be modulated by viruses to elevate viral proliferation. For example, baculovirus induces *Bmserpin2* to inhibit the melanization reaction mediated by the PPO pathway, which also induces *BmPGRP2-2* to suppress *PTEN*, resulting in increased p-Akt that can inhibit cell apoptosis and autophagy. Meanwhile, silkworm viruses usurp the ERK pathway by downregulating *BmSpry* (57–60). It is noteworthy that targeting these hijacked host pathways can inhibit viral proliferation in silkworm.

PPO Pathway

Melanization reaction, mediated by the PPO pathway, is an important immune response in insect plasma and plays an essential role in the wound healing and killing of microbes (61, 62). This process is initiated by the recognition of invading microbes, and then the extracellular clip-domain serine protease (cSP) cascade is activated to convert the zymogen PPO to active phenoloxidase (PO). PO catalyzes the oxidation of phenols to form quinones and melanin, wherein the rapid polymerization of melanin at infection sites can kill and immobilize microbes (61–63) (**Figure 1D**). The melanization can kill baculovirus *in vitro* (64, 65). However, the PPO pathway is negatively regulated by serpins, and baculovirus can induce serpins to suppress the melanization response of host insects for survival (57, 64). *Bmserpin2* was upregulated in silkworms after BmNPV infection. Furthermore, knockdown of *Bmserpin2* can increase PO activity and decrease viral multiplication (57). The mechanism by which melanization contributes to the killing of pathogens remains elusive.

PI3K/Akt Pathway

The PI3K /Akt pathway plays an important role in regulating a number of cellular processes (66–68). Activation of PI3K can occur through the binding of a variety of ligands, including several growth factors to the receptor tyrosine kinases (RTKs). Activated PI3K then converts the substrate phosphatidylinositol

4, 5-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3), and PIP3 causes the phosphorylation of Akt (p-Akt). Akt is considered a central mediator of the PI3K pathway. Active Akt drives cell proliferation, survival, apoptosis, and metabolism through the inhibitory phosphorylation of several substrates, including related kinases, signaling proteins, and the transcription factor forkhead box O (FOXO) (66, 69–71). BmFOXO directly upregulates *BmPEPCK-2*, and overexpression of *BmFOXO* and *BmPEPCK-2* can increase the expression of autophagy genes *ATG6/7/8* (17, 72). In addition, phosphatase and tensin homolog (PTEN) protein causes the dephosphorylation of PIP3, resulting in the suppression of the PI3K/AKT pathway (73).

A number of studies have demonstrated that many viruses can activate the PI3K/AKT pathway for their efficient proliferation (58, 66, 74, 75). The BmNPV induces the peptidoglycan recognition protein BmPGRP2-2 to suppress *PTEN*, resulting in increased p-Akt that can inhibit cell apoptosis (58). Meanwhile, the upregulation of p-Akt attenuates the activity of FOXO and decreases the expression of *BmPEPCK-2* and *ATG6/7/8*, thereby blocking host autophagy (17, 58, 72) (**Figure 1E**). The inhibited apoptosis and autophagy are beneficial for viral replication. However, which viral components are recognized by BmPGRP2-2 is unclear and needs further study. The PI3K/AKT pathway is a target for the treatment of many diseases (68, 70). The PI3K inhibitor AZD8835 can decrease the mortality of silkworms infected with BmNPV by blocking the p-Akt and suppressing viral proliferation (76), implying a promising antiviral strategy for silkworms.

ERK Pathway

ERKs are serine/threonine kinases activated by a variety of extracellular stimuli such as growth factors, environmental stresses, and microbial infections, and can transduce downstream cellular responses, including cell differentiation, survival, and apoptosis (77–80). Activation of the ERK pathway is required for efficient infection by many viruses (59, 80). One major class of ERK regulators is the RTK family. Upon stimulation, the extracellular ligands activate RTKs to promote the phosphorylation of ERK (p-ERK) by the activation of the small GTPase Ras to the Raf (MAP3K)/MEK (MAP2K)/ERK (MAPK) phosphorylation cascade. The ERKs then control transcription by phosphorylating various transcription factors in the nucleus or control targets in the cytoplasm (77, 78, 81, 82).

The epidermal growth factor receptor (EGFR) belongs to the RTK family (78, 81). The BmEGFR plays an important role in BmNPV infection, which participates in the activation of ERK and PI3K/Akt pathways by the virus. Moreover, activated ERK regulates the transcription of late viral genes and inhibits apoptosis (83). Additionally, Spry is a negative regulator of the EGFR/ERK pathway through the inhibition of Ras or Raf, and the overexpression of *BmSpry* suppressed p-ERK and BmNPV replication in BmE cells (84) (**Figure 1F**). Further research has found that *BmSpry* was decreased and p-ERK was increased in silkworms after infection with BmNPV, BmCPV, or BmBDV, and the knockdown of *BmSpry* in transgenic silkworms caused increased p-ERK, viral content, and mortality after infection with the three viruses, revealing that both DNA and RNA viruses usurp the ERK pathway to ensure viral reproduction (60). AG1478 is a specific inhibitor of EGFR tyrosine kinase activity (85) and the inhibitor U0126 binds to MEK to prevent p-ERK (86). The two inhibitors can inhibit p-ERK and BmNPV in BmE cells (83), but the inhibitory effect in silkworm larvae needs further test. The ERK pathway plays important roles in regulating the outcome of viral infection in silkworms, and the mechanisms remain to be fully elucidated.

CONCLUSIONS AND FUTURE PROSPECTS

Antiviral mechanisms are a worldwide problem and research hotspot. Insect-virus interactions may provide information on a vast repertoire of antiviral immune mechanisms (27). Results from the silkworm-virus model clearly show that there are multiple layers of antiviral defense that rely on conserved but also divergent pathways. For example, RNAi is a conserved antiviral mechanism among different insects, and it is the major antiviral response against both DNA and RNA viruses in silkworms. Meanwhile, NF-kB-mediated pathways are involved in antiviral immunity in silkworms but divergent responses to different viruses, such as BmCPV induces *BmPGRP-S2* and *Imd* to activate Relish whereas BmNPV activates cGAMP and STING to process Relish. Additionally, RNAi inhibits viral replication by cleaving the viral RNA while NF-kB-dependent antiviral immunity may based on AMPs. The multi-level response is beneficial to antiviral defense of host.

It is now apparent that these antiviral pathways are integrated and are responsive to one another, providing a pathogen-specific response. For example, the ERK and PI3K/Akt pathways have all been reported to interact with the JAK/STAT pathway (25), and the melanization and Toll pathways have also been found to interact (63). However, the integrated mechanisms of these pathways are complex, that is, the mechanisms by which baculovirus activate the ERK and PI3K/Akt pathways through EGFR may be different (83) and merit further investigation. Meanwhile, some mechanisms are tissue-specific or virus-specific, highlighting the importance of the investigation of virus–host interactions in the right context.

Coevolution between hosts and viruses favors the development of immune evasion mechanisms through modulation of the host signaling pathways by the pathogen (87). Targeting these hijacked pathways using inhibitors and knocking out their key regulators via gene editing would be a promising strategy to improve silkworm resistance. Meanwhile, RNAi of viral genes and overexpression of antiviral genes can enhance antiviral capacity of transgenic silkworms (1). Additionally, upregulation of antiviral immune pathways in transgenic silkworms is an available antiviral strategy. For the enhancement of host antiviral capacity and major issues in silkworm antiviral studies, please refer to our other review (87). These studies on antiviral pathways would be very instructive as they would reveal original antiviral strategies for the protection of beneficial insects and the target pathways hijacked by viruses for pest control.

AUTHOR CONTRIBUTIONS

LJ: drew figure, wrote the article, and supervision.

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