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The Mechanisms of Silkworm Resistance to the Baculovirus and Antiviral Breeding

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Abstract

Silkworm (*Bombyx mori*) is not only an economic insect but also a model organism for life science research. *Bombyx mori* nucleopolyhedrovirus (BmNPV) disease is a major infectious disease in the world's sericulture industry. The cocoon loss caused by this disease accounts for more than 60% of the total loss caused by all silkworm diseases. To date, there has been no effective solution for preventing and treating this disease. The most effective measure is to breed disease-resistant varieties. The quickest way to breed disease-resistant varieties is to apply genetic modification. However, this requires that we obtain disease resistance genes and know the mechanism of disease resistance. Since the discovery of disease-resistant resources in 1989, scholars in the sericulture industry around the world have been inspired to search for resistance genes. In the past two decades, with the help of multi-omics technologies, screening of resistance genes, gene localization, protein modification, virus–host interactions, etc., researchers have found some candidate genes that have been proposed to function at the cellular or individual level. Several disease-resistant varieties have been obtained and used in production through hybrid breeding, RNA interference, and genetic modification. This article summarizes and reviews the discovery of and research advances related to silkworm resistance to BmNPV. It is anticipated that the review will inspire scientific researchers to continue searching for disease resistance genes, clarify the molecular mechanism of silkworm disease resistance, and promote disease-resistant silkworm breeding.

1. INTRODUCTION

Chinese mythology has it that Lei Zu (a legendary Chinese empress and wife of the Yellow Emperor) invented silkworm rearing and silk reeling, which brought well-being to humankind, approximately 5,000 years ago. The ancient Silk Road (a trade route leading from China over Asia to Europe) made an important contribution to material and spiritual communication between the Chinese and Western cultures (10). Today, the silkworm (*Bombyx mori*) is not only an economic insect but also a model organism of Lepidoptera. It has important value in many research fields, including life science, environmental science, and food toxicology (13). The silkworm life cycle is one of complete metamorphosis and includes four stages: egg, larva, pupa, and imago. The larva undergoes 4–5 instars characterized by molting (10). China is the largest producer of silkworm cocoons, and the value of the silk industry output is approximately 200 billion yuan per year (43). *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a pathogen that seriously harms sericulture, and the loss of silkworm cocoons caused by BmNPV accounts for more than 60% of the total losses caused by all silkworm diseases (13). BmNPV contains a double-stranded circular DNA of approximately 128 kb, and belongs to the baculovirus family (21, 28). There are two structurally and functionally different virus phenotypes carrying an identical genome in the baculovirus life cycle: the occlusion-derived virus (ODV) and the budded virus (BV). ODVs are embedded within crystallized protein matrix to form an occlusion body (OB). Ingested OBs are dissociated in the alkaline environment of the larval midgut, which initiates primary infection in midgut epithelial cells and spreads infection orally among larvae. BVs are produced and released from the basal surfaces of virus-infected epithelial cells into other tissues and transmit infection systemically among cells or tissues. *Bombyx mori* larvae show swollen bodies after infection with BmNPV, and OBs are released into the environment when cells lyse and larvae disintegrate or liquefy (6, 93). With the development of new technologies and tools in molecular biology, research on BmNPV resistance of silkworms has accelerated in recent years, and rapid progress has been achieved in the past two decades.

2. INTERACTIONS BETWEEN BMNPV AND THE HOST

Baculovirus infection leads to a series of changes in host energy metabolism, gene expression, signal regulation, and other biological processes, which in turn regulate host response and virus replication.

2.1. Infection and Signaling Pathway

There are three main signal transduction pathways in the insect response to viruses, Toll, Imd, and Jak-STAT (3), all of which could be activated by BmNPV infection. In the silkworm, the JaK-STAT pathway can be activated by challenge with BmNPV (60). The expression of BmToll10–3 was significantly upregulated in cells infected with BmNPV, indicating that the Toll pathway was also activated upon virus infection (76). It was found that suppressor of cytokine signaling 2 participated in the antiviral immune response against virus replication (113). In the silkworm, the ERK- and JNK-dependent signaling pathway is also involved in BmNPV infection. Inhibiting or interfering with BmErk and BmJnk significantly inhibited replication of BmNPV in BmN cells (52). BmNPV infection activated silkworm ERK as well as the PI3K/Akt signaling pathway via epidermal growth factor receptor (46). Sprouty protein is a negative regulator upstream of ERK. *Sprouty* was downregulated upon BmNPV infection to promote ERK phosphorylation and facilitate virus replication (30).

2.2. Infection and Autophagy

Autophagy is an adaptive cellular response to microbial infection or physiological changes. BmNPV infection triggers host cell autophagy, which is beneficial to virus infection (92). A highly expressed polyhedron leads to aggresome formation upon BmNPV infection. The polyhedron is colocalized with the silkworm microtubule-associated protein 1-light chain 3. The inhibition of autophagy by 3-methyladenine significantly reduced expression of polyhedrin and production of polyhedrons, indicating that the polyhedron is involved in cellular autophagy (31). Overexpression of *BmAtg13*, an autophagy-related gene, promoted the expression of viral genes and increased virus production in BmN-SWU1 cells, while knockout of the *BmAtg13* gene inhibited the replication of BmNPV. The viral protein BRO-B can directly interact with BmATG13 to mediate autophagy and promote replication of BmNPV (104).

2.3. Noncoding RNA in Virus Replication

MicroRNA (miRNA), a short noncoding RNA, regulates gene expression at the post-transcriptional level and plays an important role in eukaryotic immunity and development (2, 86). The expression of the silkworm miRNA bmo-miR-8, which targets and regulates the very early viral gene *ie-1*, is downregulated upon BmNPV infection. Silencing this miRNA can inhibit the expression of *ie-1* and increase the viral load (79). BmNPV infection also induced downregulation of other two host miRNAs, bmo-miR-390 (48) and bmo-miR-2819 (99). Overexpression of bmo-miR-390 and bmo-miR-2819 downregulated the expression of the viral genes *cg30* and *ie-1* and inhibited virus replication. In addition to host-encoded miRNAs, viral miRNAs can also regulate the host's defense response. The *B. mori* GTP-binding nucleoprotein Ran is related to the transport of small RNA from the nucleus to the cytoplasm. The BmNPV-encoded miRNA Bmnpv-miR-1 can inhibit Ran expression, resulting in a reduction of the small RNA population, thereby enhancing virus replication. Inhibition of Bmnpv-miR-1 upregulated the expression of silkworm bmo-miR-8 and inhibited virus replication (79). Bmnpv-miR-3 facilitated BmNPV escape from the host immune response by negatively regulating the expression of viral *p6.9* and other late genes (80). In addition, circular RNAs (circRNAs) (36) and long noncoding RNAs (lncRNAs) (115) were significantly differentially expressed after BmNPV infection, suggesting that circRNAs and lncRNAs also play important roles in responses to BmNPV infection by interacting with their target genes and miRNAs.

2.4. Infection and Host Metabolism

Virus replication depends on the host's metabolism to provide energy and biosynthetic raw materials. Infection with baculoviruses such as BmNPV significantly increases the host's oxygen consumption and enhances the tricarboxylic acid cycle activity (67). BmNPV infection leads to significant changes in the metabolism of BmE cells, including purine metabolism and aminoacyl-tRNA synthesis (39). One study showed that, during BmNPV infection, amino acid metabolism in the hemolymph of silkworm changed significantly, and the consumption of amino acids increased at 24 h after virus infection (24). However, there was no obvious change in central sugar metabolisms such as glycolysis and glutamine hydrolysis, and inhibition of these two processes did not affect virus replication. In contrast, studies have also shown that glycolysis regulated by the adenosine receptor signal induces and mediates the permissibility of baculovirus infection and plays an important role in the antiviral immune response of silkworms. Inhibition of glycolysis or adenosine signal pathways can reduce the host's antiviral activity and enhance the ability of the baculovirus to replicate in nonpermissive cells. In contrast, activating these pathways can improve the host's antiviral ability (59). Compared with susceptible silkworms, glycolysis and

gluconeogenesis pathways had relatively low activity in resistant silkworms, and phenylalanine and tyrosine accumulated significantly in the hemolymph of resistant silkworms (95). As a timely energy source, trehalose plays an important role in the response of insects to pathogen infection. During BmNPV infection, trehalose in the hemolymph of resistant silkworms continuously increases compared with that in susceptible silkworms. Tricarboxylic acid cycle–related metabolites were significantly upregulated in resistant silkworms throughout viral infection, whereas this upregulation was only detected in susceptible silkworms at the early stage of infection (95).

Using metabolomics analysis, Qian et al. (73) identified 451 differential metabolites in the midgut of BmNPV-infected susceptible and resistant silkworm strains, including sugars, acids, amines, and glycosides. In both strains, the main metabolic changes were associated with tryptophan metabolism, oxidative phosphorylation, β-alanine metabolism, and phenylalanine metabolic pathways. Transcriptomic analysis showed that tryptophan metabolism and oxidative phosphorylation were closely related to the resistance of silkworms to BmNPV. Lipid profiles also differed between susceptible and resistant silkworms, and lipid composition changed upon BmNPV infection, implying that lipid metabolism plays a key role in virus infection and immune response (123).

Post-translational modifications also play a role in host responses to viral infection. BmNPV infection globally impacted the acetylome in BmN cells and altered the amount of lysine acetylation sites in acetylated proteins. Lysine acetylation may regulate gene expression, energy metabolism, substance synthesis, and substance metabolism during virus infection (35).

2.5. Infection and Epigenetic Mechanism

Baculoviruses can also utilize the host's epigenetic mechanism to regulate gene expression and facilitate replication. N^6-Methyladenosine (m6A) is a ubiquitous form of RNA modification in eukaryotes that plays an important role in regulating messenger RNA transport and stability, translation efficiency, and gene expression. Employing the methylated RNA immunoprecipitation method, Zhang et al. (118) identified 9,144 and 7,384 m6A peaks in silkworms before and after BmNPV infection, respectively, of which 1,221 were differentially expressed peaks related to virus infection. These peaks were associated with signal transduction, translation, and degradation. Silencing methyltransferase-like genes in BmN cells increased the expression of the viral structural protein VP39. Overexpression of these genes reduced the expression of VP39, indicating that m6A may play an important role in regulating viral replication.

DNA viruses can hijack and manipulate the host's chromatin state to facilitate self-replication. BmNPV infection caused the host chromatin to undergo progressive recombination and enhanced the accessibility of chromatin via modification of facultative heterochromatin. Meanwhile, the accessibility of chromatin was regulated by modification of euchromatin in noninfected BmN cells (55). As another mode of epigenetic regulation, DNA methylation also plays a positive role in BmNPV infection. BmNPV infection causes a significant change in DNA methylation patterns. Inhibiting silkworm DNA methyltransferase activity can inhibit BmNPV replication by reducing the expression of apoptosis inhibitor genes (40).

2.6. Interaction with Host Viral Proteins for Virus Replication

Interaction between the virus and the host proteins occurs throughout the viral life cycle and regulates virus proliferation. Baculovirus inclusion bodies are lysed in the alkaline environment of the silkworm midgut to release ODVs. ODVs first passes through the peritrophic membrane of the midgut, which is a grid-like membrane structure composed of chitin and protein. The

peritrophic membrane protects midgut epithelial cells from direct contact with food particles. Some baculoviruses, including the ODVs, encode the metalloprotease enhancin, which can digest the protein–chitin chain of the peritrophic membrane to help ODVs enter through the peritrophic membrane (81, 94). ODVs enter midgut epithelial cells by directly fusing with microvillous membranes. Although the virus receptor is not yet known, there is evidence that the nine oral infectious factors in the ODV envelope form a large protein complex, which regulates the binding and fusion of ODVs with midgut microvillous membranes (6).

Virus infection and transport of virions depend on the cytoskeleton network system (53, 90). The entry of BmNPV BVs into BmN cells was mediated by clathrin- and dynamin-dependent endocytosis (26). Internalization of budded virus of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) into host insect cells was facilitated by actin polymerization and dynamin, and nucleocapsid release occurred in early endosomes in a microtubule-dependent manner (74). Some viral proteins can manipulate the cytoskeleton to facilitate virus replication. For instance, the AcMNPV p78/83 protein can interact with the host actin-related protein 2/3 complex (Arp2/3 complex) to induce polymerization of actin, which was required for intercellular trafficking of virus and was essential for viral progeny production (27, 70). BmNPV VP39 can also interact with cellular actin and mediate actin polymerization. Amino acids 192–286 of VP39 are responsible for nuclear actin polymerization (114). Ac102 was related to nuclear accumulation of actin and participated in the multimerization of nuclear actin induced by p78/83 by regulating ubiquitination of BV/ODV-C42 (34, 119). AcMNPV VP80 and BmNPV ORF67 proteins can also interact with actin to support virus replication (9, 64). Although replication of baculoviruses relies on actin, overexpression of actin can interfere with synthesis of AcMNPV polyhedrons and assembly of the virus (88). In addition, the host heat shock protein 90 was found to promote actin polymerization and participate in BV morphogenesis during baculovirus infection (57).

Cell recognition and entry of baculovirus BVs are regulated by the viral envelope glycoproteins GP64 (in group I alphabaculoviruses) or F (in group II alphabaculoviruses) (6). Although the receptor of baculovirus is still unknown, several host proteins are considered as the binding proteins of GP64 associated with the entry of BVs. For instance, overexpression of silkworm nuclear hormone receptor 96 (BmNHR96) can increase the entry of BVs by promoting GP64-mediated membrane fusion and elevating intracellular cholesterol levels (17). The E3 ubiquitin-protein ligase SINA-like 10 (SINAL10) is a GP64 binding protein that has been shown to stimulate the proliferation of BmNPV (25). *Bombyx mori* receptor expression enhancer protein a (BmREEPa), patched domain containing protein (BmPtchd), and GP64 interacted with each other to form a complex, which facilitated BV invasion (19, 20). In addition, interference with the expression of BmNHR96 and BmREEPa can promote silkworm resistance to BmNPV (18, 108).

2.7. Infection and Host Behaviors

Baculovirus infection can lead to increased insect motility. Virus-infected larvae exhibit enhanced locomotory activity (ELA) and tree-top disease. This symptom is conducive to the spread of progeny viruses after the insects die. ELA is most obvious in the late stage of BmNPV infection. This behavior is the strongest within 12–24 h before death. The virus-encoded *tyrosine phosphatase* (*ptp*) gene is related to this behavior. *ptp*-deficient recombinant viruses cannot induce ELA. The host's *ptp* homolog can partially remedy the mutant BmNPV lacking *ptp* and lead to appearance of the ELA symptom (47). Further studies have shown that PTP is a structural protein of the BV envelope, and the induction of host ELA does not require the phosphatase function of PTP. However, the replication ability of a *ptp* knockout recombinant virus was decreased substantially in silkworm brain tissue. The *ptp* of the virus may be derived from the host (51). Knockout of

silkworm *Bmptp-h* by RNA interference can significantly inhibit BmNPV virus replication, and overexpression of *Bmptp-h* can enhance virus replication, suggesting that BmPTP-h may facilitate baculovirus infection by promoting virus replication (89). However, the molecular mechanism by which PTP regulates host ELA awaits further elucidation.

3. DISCOVERY AND GENETIC ANALYSIS OF BMNPV RESISTANCE RESOURCES IN SILKWORMS

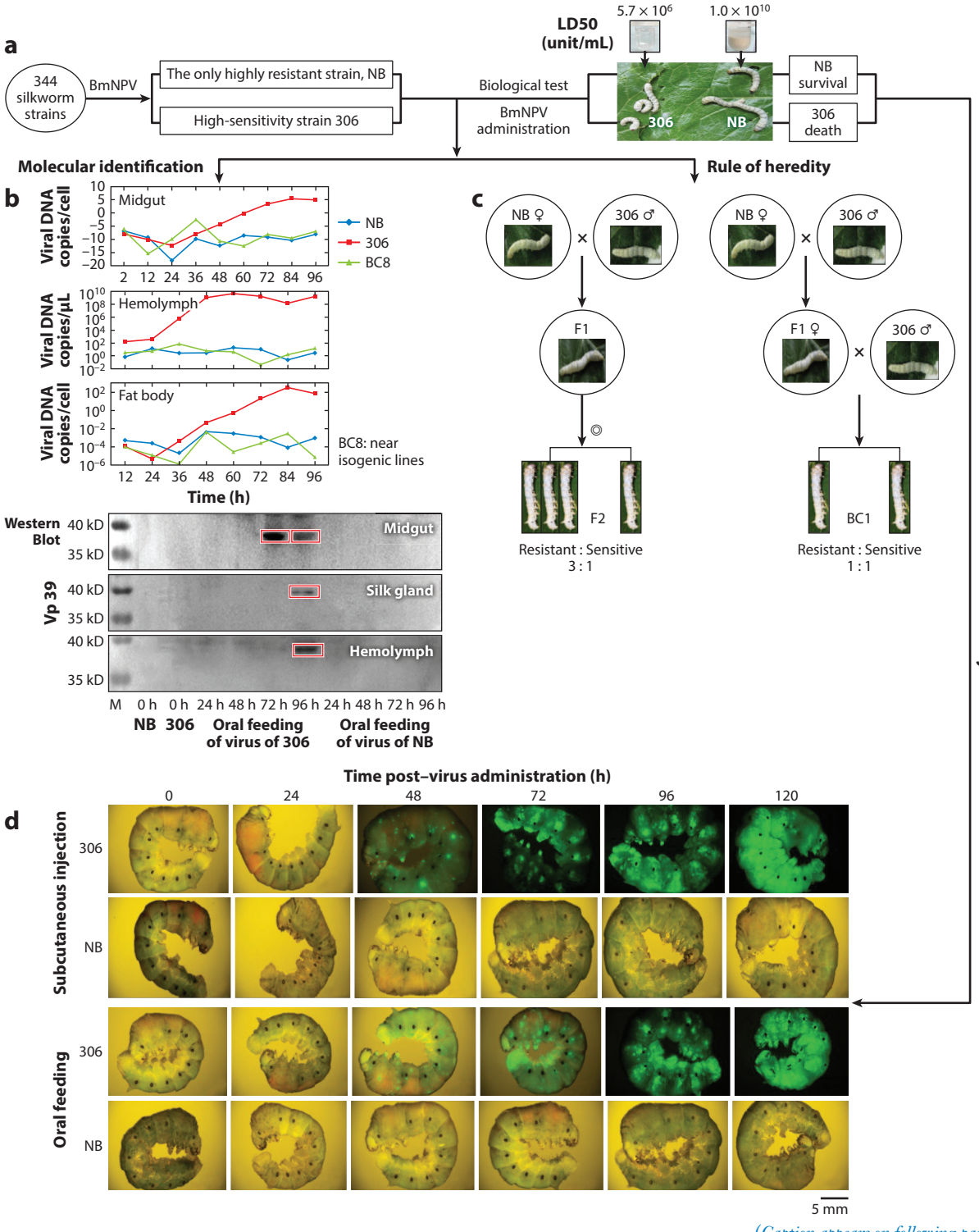
BmNPV was the first *B. mori* virus to be discovered. As early as the Chen Fu Agricultural Book in the Song Dynasty in ancient China (sixteenth to eleventh centuries BC), there were records of this silkworm disease, which was described as Swollen Feet. BmNPV is a primary pathogen that causes serious economic losses in sericulture, but there are no effective drugs to control this virus, and prevention of infection has been the major strategy to date. Screening resistant silkworm varieties and analyzing their hereditary rules are very important in sericulture.

In 1982, Zhang et al. (120) found that there were differences among silkworm varieties in the resistance to BmNPV. Watanabe (98) suggested that the silkworm has only horizontal resistance to BmNPV (control by multiple minor genes) and no vertical resistance (control by a major gene). In 1991, Chen et al. (11) conducted BmNPV resistance screening from 344 silkworm varieties in the Chinese National Silkworm Germplasm Bank (**http://www.cnsilkworm.com**). They found that the resistance of different varieties to the virus displayed a normal distribution: Some varieties were extremely sensitive, but a few were highly resistant. A highly resistant strain named NB was obtained from the Chinese local strains, and its lethal concentration at 50% (LC50) reached 6.39×10^8 polyhedrons/mL, which was nearly 1,000 times higher than that of the susceptible strain named 306. Furthermore, the gene expression of viral DNA polymerase in the midgut, hemolymph, and fat body of the susceptible silkworm strain 306 was 100,000 times higher than that in the resistant strain (109). After oral and subcutaneous inoculation with green fluorescent protein (GFP)-conjugated BmNPV, the fluorescence was densely distributed in tissues of the susceptible strains, while the resistant strains had almost no fluorescence (113), which proved that viral proliferation and gene expression were suppressed in the resistant strain.

Genetic analysis showed that silkworm resistance to BmNPV is controlled by major dominant genes and several minor genes (12, 66). However, there has been a debate on whether single- or multigene control is dominant in the inheritance of silkworm resistance to BmNPV. After 10 years of research, our team first discovered that the resistant NB strain contains a heterozygous population with three genotypes (++, +–, –). After more than 20 generations of separation and purification, a completely homozygous resistant strain (++) was obtained. The genetic experiment showed that the resistance to BmNPV was controlled by single dominant gene (23). Asser-Kaiser et al. (1) used the classical genetics method to analyze resistance of *Cydia pomonella* to baculovirus insecticide [*C. pomonella* granulovirus (CpGV)]. They found that the resistance to CpGV was controlled by dominant genes that linked to the Z chromosome that remain to be identified, which further indicated that lepidopteran insects have dominant gene control for baculovirus resistance. The discovery, identification, and genetic screening of the resistant silkworm strains are summarized in **Figure 1**.

4. THE MECHANISMS OF SILKWORM RESISTANCE TO BMNPV AND RESISTANCE GENES

During the virus–host interaction, hosts develop antiviral mechanisms to eliminate viral infection. Silkworms mainly depend on innate immunity against BmNPV; some innately immune processes and antiviral proteins are involved in resistance to BmNPV in silkworms.



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

Identification and inheritance study of silkworm resistance to *Bombyx mori* nucleopolyhedrovirus (BmNPV). (*a*) Screening silkworm resistance to BmNPV from 344 varieties to obtain the highly resistant strain NB and the highly sensitive strain 306. (*b*) Detection of DNA polymerase of the virus in the midgut, hemolymph, and fat body of the 306, NB, and BC8 strains (*top*) and the expression of the viral protein VP39 in different tissues after oral feeding of BmNPV (*bottom*). The red frames indicate target protein VP39. (*c*) Inheritance study of silkworm resistance to BmNPV. (*d*) The fluorescence detection of the resistant strain NB and the susceptible strain 306 infected with enhanced green fluorescent protein (EGFP)-labeled recombinant baculovirus. The green fluorescence indicates the infection of recombinant baculovirus.

4.1. Antiviral Proteins in Midgut Juice

The midgut is the first barrier to oral viral infections. The intestinal digestive juice has been found to contain some proteins that have antiviral activities. Hayashiya et al. (32, 33) were the first to discover that silkworm intestinal juice contains an antiviral substance that emits red fluorescence. Later, a membrane protein, P252, in the silkworm midgut was found to bind with chlorophyllide, forming a red fluorescent protein (RFP) complex that exhibited significant antimicrobial activity (71). Multiple forms of RFPs had been observed in the gut juice of silkworms, and different silkworm varieties had varying numbers of RFPs. The resistant varieties had more RFPs than susceptible varieties, indicating that the number of RFPs in silkworm intestinal fluid have a positive correlation with BmNPV resistance (84, 85).

Besides RFPs, many other antiviral factors have been identified in the gut juice of silkworms. BmNOX (77), Bmlipase-1 (72), and serine protease-2 (69) purified from the digestive juice had strong antiviral activity. Jiang et al. (44) transferred *Bmlipase-1* into susceptible silkworms using transgenic methods; this procedure increased the survival rate of transgenic silkworm species by 33% after BmNPV infection and confirmed the anti-BmNPV activity of this endogenous protein. Comparative proteomics analysis showed that lipase member H-A (BmLHA) content in the digestive juice of resistant silkworms was higher than that in sensitive silkworms (116). After ODVs of BmNPV were pretreated with purified recombinant BmLHA protein, the infectivity of virus particles was significantly reduced. Overexpression of BmLHA in BmN cells could significantly inhibit the replication of BmNPV (117). However, the mechanisms by which RFPs and the other antivirus proteins resist BmNPV remain unknown.

4.2. The Role of Melanization in Resistance to BmNPV

Proteolytic activation of prophenoloxidase (PPO)- and phenoloxidase (PO)-catalyzed melanization plays an important role in insect defense response (93). Inactive precursor PPO is converted into active PO by serine protease, which is mediated by serine protease inhibitor (serpin). PO oxidizes phenols into toxic quinone substances in the presence of molecular oxygen to generate melanin. Melanin deposits on the surface of pathogens through a series of reactions and destroys pathogenic microorganisms through blackening and encapsulation (29). *Prophenoloxidase 2s* was found to be highly expressed in the resistant silkworm after BmNPV infection, in contrast to the susceptible strain (8), suggesting that the PPO cascade was involved in the anti-BmNPV process. *Bombyx mori* serine protease 142 (BmSP142) was mainly expressed in midgut tissues and differentially expressed in BmNPV-infected resistant and susceptible silkworms. Overexpression of BmSP142 in BmN cells could inhibit the replication of BmNPV; the efficiency of virus infection was significantly reduced when BmNPV was pretreated with purified BmSP142, indicating that BmSP142 had antiviral activity (56). Inhibition of melanization by serpin can promote baculovirus infection in the host (112). BmNPV infection could induce upregulation of Bmserpin2 expression in silkworms, and Bmserpin2 could regulate PO activity and inhibit melanization (87). However, the melanization pathway was inhibited in the resistant silkworm, suggesting

that the infection of BmNPV was blocked in resistant silkworms independently of melanization (15).

4.3. Proteins Inhibiting Viral Replication by Apoptosis

Cell apoptosis plays an important role in the development and immune response of multicellular organisms. Baculovirus infection triggers host cell apoptosis to eliminate infected cells (54, 62). Several silkworm genes have been considered to be involved in resistance to BmNPV through apoptotic pathways. For example, the expression level of *cytochrome c* (*cytc*) was stable in selected tissues of the resistant silkworm after BmNPV infection, while it was downregulated in the susceptible strain. Knockdown of *Bmcytc* significantly promoted the in vitro infection process of BmNPV. *Bmcytc* plays a vital role in BmNPV infection by regulating the mitochondrial apoptotic pathway (96). As an inhibitor of apoptosis protein (IAP) antagonist, silkworm IAP-binding motif protein 1 (BmIBM1) can bind silkworm IAP and BmNPV IAP2 and induce apoptosis in insect cells (7, 101). The expression of BmIBM1 was upregulated rapidly at the early stage to suppress virus infection in BmNPV-infected cells (101). Silkworm β-1,3-glucan recognition protein 4 (BmβGRP4) could also inhibit BmNPV replication by activating apoptosis (91). In addition, interference with the expression of silkworm Nedd2-like caspase (BmNc) in BmN cells could significantly increase the replication of BmNPV, and overexpression of BmNc could reduce virus replication. The antiviral activity of BmNc was related to the mitochondrial apoptosis pathway (82). These antiviral molecules act in the apoptotic pathways and can promote apoptosis and inhibit the spread and replication of viruses.

4.4. RNA Interference

RNA interference (RNAi) mediated by Dicer plays an important role in host defense against baculovirus infection (50, 65). After infection with BmNPV, the expression of Dicer-2 (Dcr2) mRNA in silkworm midgut and blood cells increased significantly. The use of specific double-stranded RNA (dsRNA) in silkworms to reduce the expression level of Dcr2 moderately enhanced the production of viral genomic DNA, indicating that the Dcr2 gene in silkworm plays an important role in resisting BmNPV invasion (58).

4.5. Differentially Expressed Genes or Proteins Related to Resistance

Baculovirus infection leads to a global change in host gene expression (16, 78). A large number of studies focused on differentially expressed genes (DEGs) and differentially expressed proteins (DEPs) between the susceptible and resistant strains infected with BmNPV have provided valuable clues to understanding silkworm resistance to the virus (22, 42, 63, 76, 83, 105, 106, 122). For example, many DEGs, including *gloverin*, *cytochrome c oxidase*, and *serpin* (4, 5), were isolated from resistant and susceptible silkworms upon BmNPV infection via suppression subtractive hybridization. Proteomic analysis showed that β-N-acetylglucosaminidase 2 and aminoacylase expression were higher in resistant silkworms than in susceptible silkworms after BmNPV inoculation (61). Comparative proteomic analysis also revealed that caspase-1 and serine protease were related to silkworm resistance to BmNPV, suggesting that the apoptosis and PPO cascades participate in the antiviral process of silkworms (75). Comparative subcellular proteomics analysis showed that DEPs were primarily located in the cytosol and microsomes of the silkworm midgut. Sixteen proteins were identified to be potentially involved in resistance to BmNPV infection, including PEBP, NADH dehydrogenase (ubiquinone) Fe-S protein 8, and tudor staphylococcal/micrococcal nuclease (97). These DEGs and DEPs were associated with host response to viral infection, and

they either benefit host survival or facilitate replication of the virus. However, whether they are antiviral molecules remains to be further verified.

4.6. The Location of Silkworm Anti-BmNPV Genes

Genetic analysis has indicated that a single dominant gene is responsible for resistance to BmNPV in silkworms (23). Researchers have obtained different candidate genes related to resistance through various methods (**Table 1**), but the exact resistance genes have not yet been confirmed. Silkworms have 28 chromosomes and a mid-range genome size of ~432 Mb encoding approximately 18,510 genes (102, 103). Locating the candidate genes in the silkworm genome is helpful to correctly identify the resistant genes. Recent studies have suggested that the major BmNPV resistance gene is located in the 27th linkage group of the resistant strain; these studies used simple sequence repeats molecular markers and high-resolution melting curve analysis, from which a genetic linkage map of the major resistance gene with a total genetic distance of 29.1 cM

Table 1 Genes and proteins related to anti-BmNPV activity

Resistance gene/protein name	Property and function	Reference(s)
Red fluorescent protein	A chlorophyllide-protein complex with anti-BmNPV activity in midgut juice	32, 33, 85
Soluble NADH-oxidoreductase-like protein	A 26.5-kDa soluble protein in midgut juice with broad-spectrum antiviral activity	77
Bmlipase-1	Strong antiviral activity in midgut juice	44, 72
Serine protease-2	Strong antiviral activity in midgut juice	69
Prophenoloxidase 2s	Differential expression related to anti-BmNPV activity	8
Lipase member H-A	Anti-BmNPV activity in midgut juice	117
Ferritin	Differential expression proteins related to anti-BmNPV activity	22
Ribosomal protein s3a		105
Suppressor of profilin		106
PP-BP		37
Amino acid transporter and K^+-coupled amino acid transporter		122
β-N-acetylglucosaminidase 2		61
Bmcaspase-1 and serine proteinase		75
Cytochrome c	Inhibition of BmNPV replication by apoptosis	96
IBM1	Inhibition of BmNPV replication by apoptosis	101
Dicer-2	Inhibition of BmNPV replication by RNAi	58
Phosphatidylethanolamine binding protein isoform 2, NADH dehydrogenase (ubiquinone) Fe-S protein 8, and tudor staphylococcal/micrococcal nuclease	Differential expression proteins related to anti-BmNPV activity	97
Gloverin, cytochrome c oxidase, and serpin	Differential expression gene related to anti-BmNPV activity	4, 5
Serine protease 142	Anti-BmNPV activity	56
β-1,3-glucan recognition proteins	Inhibition of BmNPV replication by activation of apoptosis	91
V-ATPase	Anti-BmNPV activity	63
Ser/Thr protein phosphatase 2A	Anti-BmNPV activity	38
Bm123	Differential expression gene related to anti-BmNPV activity	83

Abbreviations: BmNPV, *Bombyx mori* nucleopolyhedrovirus; PP-BP, paralytic peptide binding protein; RNAi, RNA interference.

was drawn. However, there is a gap of unknown size in this linkage region. Further analysis implied that there were several minor resistance genes distributed in other chromosomes (100). Our team screened a large number of single-nucleotide polymorphism and INDEL markers possibly related to the resistance gene using specific-locus amplified fragment sequencing, and the closely linked markers of the resistance gene were then obtained via high-throughput genome sequencing and bulked segregant analysis. Our results suggested that the major resistance gene was located on chromosomes 27 and 23 (K. Chen, unpublished data). There are many candidate resistance genes in these two linkage groups, which remain to be verified. To date, the decisive genes for resistance to BmNPV have not been identified in silkworms.

5. METHODS AND STRATEGIES FOR ENHANCING SILKWORM RESISTANCE TO BMNPV

Preventing viral infection by disinfection and strict breeding operation is currently the fundamental strategy for protection from BmNPV in sericulture. Resistant breeding, transgenic modification, and antiviral drugs could help silkworms resist BmNPV infection. *Adoxophyes fasciata* can develop antiviral activity after only three generations of resistance selection by feeding *Adoxophyes honmai* nucleopolyhedrovirus every generation. The antiviral ability continued to increase with selection and the passage of generations, and the resistance level could be increased by nearly 400,000 times (68). These findings indicate that the resistance of silkworms to BmNPV may be increased significantly by multiple generations of virus administration. However, virus administration only screens out disease-resistant individuals and eliminates nonresistant individuals. We believe that the existence of resistance genes in certain populations or strains is a prerequisite to improving disease resistance.

Molecular marker–assisted breeding is an effective strategy to obtain varieties resistant to BmNPV when the resistance genes are unknown. This method selects from the genetic level and avoids casual performance error during viral screening. Our research team used 150 random amplified polymorphism DNA (RAPD) primers to screen from the resistant parent NB; the susceptible parent 306; the F1 generation of reciprocal crosses; and individuals from each backcross of BC1, BC2, BC3, BC4, and BC5. An effective resistance-linked molecular marker was obtained for molecular marker–assisted breeding (110). Subsequently, the selected RAPD marker sequences were converted into sequence-characterized amplified region markers and applied to the assisted breeding of new NPV-resistant silkworm varieties. A new BmNPV-resistant silkworm variety was successfully obtained for the first time (111), which proved the existence of resistance genes in practice (**Figure 2**).

Endowing the silkworm with BmNPV-resistant traits through transgenic technology is an effective way to break through traditional breeding barriers and modify traits of the silkworm. As the silkworm disease resistance gene is still not clear, breeders employ transgenic strategies mainly targeting the BmNPV genes. Isobe et al. (41) transferred the dsRNA fragment of BmNPV *lef-1* into the silkworm genome so that viral replication was well controlled by RNAi in the transgenic silkworm. Although silkworms infected with the virus died in the end, this method offered new ideas for studying silkworm resistance to BmNPV. Subsequently, transgenic silkworms targeting BmNPV *ie-1* were constructed using the same method, which achieved a 40% protection effect (49). Jiang et al. (45) used the viral *ie-1*, *helicase*, *gp64*, and *vp39* genes as target suppressor genes to construct transgenic silkworms and found that the transgenic silkworms that inhibited BmNPV *ie-1* showed the best antiviral effects. CRISPR/Cas9-mediated disruption of the viral genome is a potential approach against infectious diseases in silkworms. Transgenic silkworms generated using

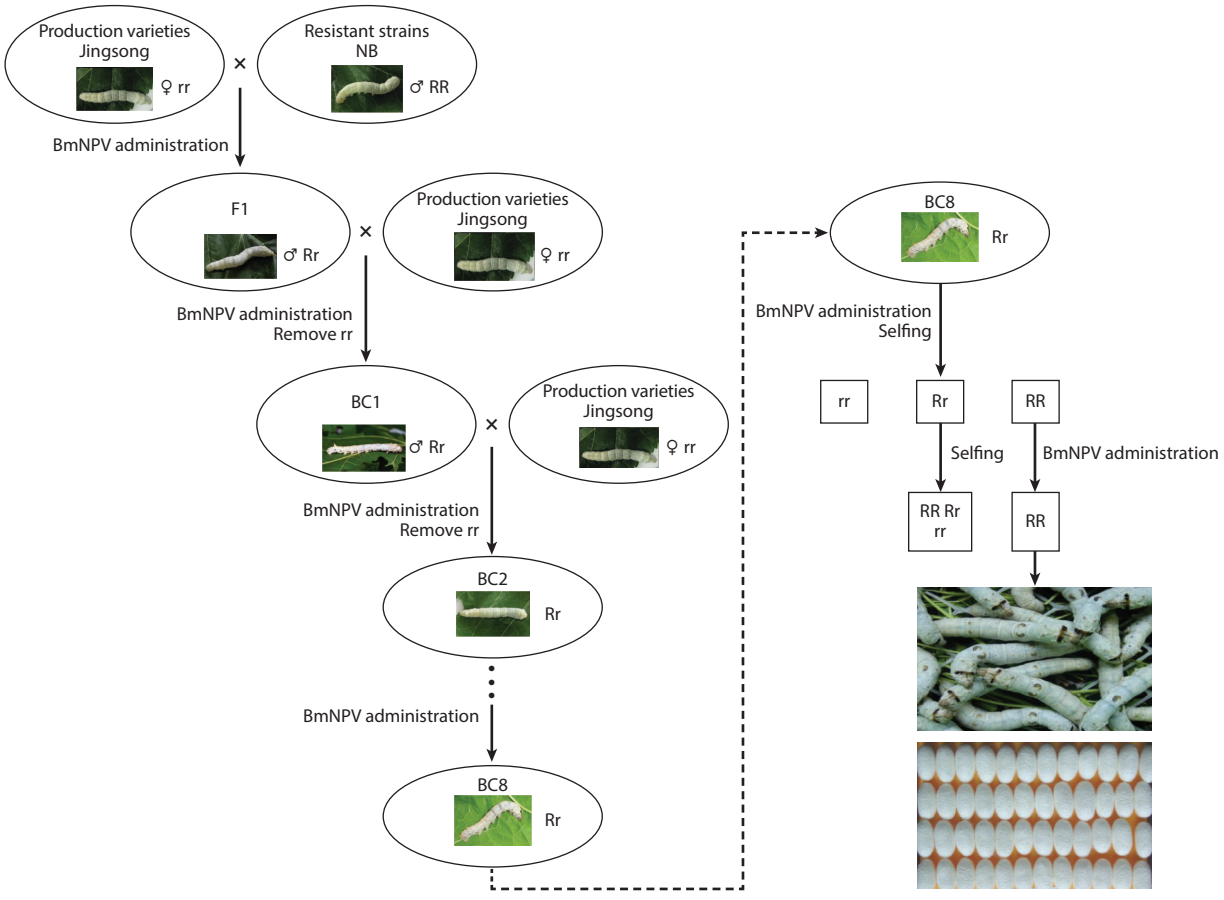


Figure 2

Breeding methods for silkworm varieties that are resistant to *Bombyx mori* nucleopolyhedrovirus (BmNPV). RR represents the resistant homozygous genotype, Rr represents the resistant heterozygous genotype, and rr represents the susceptible homozygous genotype. The F1 generation from the industrial production strain Jingsong and the highly resistant strain NB was crossed with the production strain to produce BC1 (backcross 1), and BmNPV was inoculated to screen for resistant strains among the BC1 silkworms. The resistant heterozygous BC1 was then crossed with the production strain to obtain BC2 and similarly inoculated with BmNPV to screen for heterozygous silkworms. This backcross and viral screening procedure was repeated until BC8 was produced. The resistant heterozygous BC8 was self-crossed and then inoculated with BmNPV to obtain the nonsegregating resistant homozygous silkworm strain.

the CRISPR/Cas9 system targeting viral *ie-1* and *me53* exhibited robust suppression of BmNPV proliferation (14). In addition, overexpression of silkworm *Bmlipase-1* (44) or RNA interference of *BmNHR96* (108) and *BmREEPa* (18) can also enhance the resistance to BmNPV in transgenic silkworms.

Studies have shown that some drugs have anti-BmNPV activity. Titanium dioxide nanoparticles can inhibit replication of BmNPV in the silkworm midgut and enhance the silkworm's resistance to the virus (107). In BmE cells infected with BmNPV, production of 5-pyridoxolactone, which can strongly inhibit virus replication, continually increases (39). The bacterial secondary metabolite prodigiosin can selectively kill BmNPV-infected silkworm cells, inhibit virus gene expression and virus-mediated membrane fusion, and significantly inhibit virus replication (121).

These studies suggest that specific and efficient antiviral drugs can be utilized for silkworm disease control in the future.

6. CONCLUSION AND PROSPECTS

Interactions between BmNPV and silkworms affect host gene expression, signal transduction, autophagy, epigenetics, and metabolism and regulate virus replication and host antiviral defense. Silkworm resistance to BmNPV relates to the midgut barrier, RNAi, apoptosis, and melanization. To date, many resistance-related genes have been discovered (**Table 1**), but no major resistance genes have been confirmed. Genetic experiments demonstrated that resistance was controlled by a dominant single gene, and genetic linkage mapping located the candidate gene in the 27th chromosome. However, the reference genome information was derived from susceptible strains, and there are many gaps. Acquiring an accurate resistance gene is the basis for antiviral breeding and elucidating resistance mechanisms; the best way to identify resistance genes in the future is whole-genome sequencing based on resistant strains.

Hosts and viruses are in an evolutionary arms race: Hosts evolve antiviral pathways, and viruses develop immune evasion mechanisms during coevolution. BmNPV and *Bombyx mori* are important biological models for understanding baculovirus–insect interactions. BmNPV may acquire the ability to counteract resistant silkworms in long-term evolution. However, the mutated virus will also be subject to more evolutionary pressure, and infectivity rate and mortality rate can be countered via disinfection and strict breeding operation. In addition, baculoviruses are widely used to control lepidopteran pests, but the development of resistance to the viruses is a serious problem affecting the use of baculovirus pesticides. In this regard, a highly virulent baculovirus can be good news for insect pest control.

DISCLOSURE STATEMENT

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