Genome-Wide Analysis of Host Responses to Four Different Types of Microorganisms in *Bombyx Mori* (Lepidoptera: Bombycidae)

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Abstract

Several pathogenic microorganisms have been used to investigate the genome-wide transcriptional responses of *Bombyx mori* to infection. However, studies have so far each focused on one microorganism, and systematic genome-wide comparison of transcriptional responses to different pathogenic microorganisms has not been undertaken. Here, we surveyed transcriptional responses of *B. mori* to its natural bacterial, viral, and fungal pathogens, *Bacillus bombyseptieus*, *B. mori* nucleopolyhedrovirus (*BmNPV*), and *Beauveria bassiana*, respectively, and to nonpathogenic *Escherichia coli*, by microarray analysis. In total, the expression of 2,436, 1,804, 1,743, and 912 *B. mori* genes was modulated by infection with *B. bombyseptieus*, *BmNPV*, *B. bassiana*, and *E. coli*, respectively. Notably, the expression of 620, 400, 177, or 165 of these genes was only modulated by infection with *B. bombyseptieus*, *BmNPV*, *B. bassiana*, or *E. coli*, respectively. In contrast to the expression of genes related to juvenile hormone synthesis and metabolism, that of genes encoding juvenile hormone binding proteins was microorganism-specific. Three basal metabolic pathways were modulated by infection with any of the four microorganisms, and 3, 14, 5, and 2 metabolic pathways were specifically modulated by infection with *B. bombyseptieus*, *BmNPV*, *B. bassiana*, and *E. coli*, respectively. Interestingly, *BmNPV* infection modulated the JAK/STAT signaling pathway, whereas both the Imd and Toll signaling pathways were modulated by infection with *B. bombyseptieus*, *B. bassiana*, or *E. coli*. These results elucidate potential molecular mechanisms of the host response to different microorganisms, and provide a foundation for further work on host–pathogen interaction.

Key words: silkworm, pathogenic microorganism, systematic response, DNA microarray
versus BmNPV-susceptible silkworm strains, 92 differentially expressed genes were identified, including 43 upregulated and 49 downregulated genes, encoding amino acid transporters, serine proteases, and serpins, which are degraded by the proteasome in insect antiviral responses (Zhou et al. 2013). In the case of N. bombycis infection of the silkworm, a genome-wide survey revealed that more than 1,000 B. mori genes showed dynamic changes in expression (Ma et al. 2013), including those related to basal metabolic pathways, JH synthetic and metabolic pathways, and immune-related Toll and JAK/STAT pathways, and that the production of antimicrobial peptides (AMPs) was triggered in response to the invasive microsporidia.

A Digital Gene Expression (DGE) approach, based on high-throughput RNA sequencing, provides an alternative strategy for analyzing genome-wide differential gene expression and dynamic changes in gene expression. By this strategy, 1,430 differentially expressed B. mori genes were identified after infection of silkworm larvae with B. bassiana, of which 960 were upregulated and 470 downregulated. Functional annotation showed that genes associated with defense and response, signal transduction, and phagocytosis pathways were responsible for the interaction between the silkworm and B. bassiana (Hou et al. 2014). Another example of DGE analysis is in CPV infection of the silkworm; 752 differentially expressed B. mori genes, comprising 649 that were upregulated and 103 downregulated, were detected, and were involved in signaling, gene expression, metabolic process, cell death, binding, and catalytic activity (Gao et al. 2014).

Although several pathogenic microorganisms have been used in genome-wide studies to investigate the responses of the silkworm to infection, each individual study has only addressed a single microorganism. In this study, we surveyed the genome-wide transcriptional response of the silkworm to four different microorganisms, including three different types of natural pathogen (bacterial, viral, and fungal), by microarray analysis. Our results provide a comprehensive comparison of the transcriptional profiles of the respective host–pathogen interaction for infection with each of the four microorganisms, and reveal that the host responses in respect of development, basal metabolism, pathogenesis, and immune defense may be common or specific to different microorganisms.

Materials and Methods

Silkworm Strain and Microorganisms

The B. mori strain, Dazao, was used in this study. The silkworm larvae were reared on an artificial diet under standard conditions. The feeding of larvae was stopped on day 3 of the fifth instar, when they were used in infection experiments. The microorganisms BmNPV, B. bassiana, and Escherichia coli were maintained under standard laboratory conditions at the Institute of Sericulture and Systems Biology, Southwest University, Chongqing, China. For B. bombyseptieus infection, the data from a previous paper were used for comparison in the present study (Huang et al. 2009).

Oral Infection of Silkworm Larvae by Microorganisms

Infection of day-3 fifth-instar silkworm larvae was carried out by the same oral means with a previous study (Ma et al. 2013). Briefly, for oral infection with BmNPV, the virus was separated and concentrated from an overnight culture in LB medium, and a volume of ~200 μL bacterial cells (OD_{600}≈100) was thoroughly mixed with the artificial diet for feeding of silkworm larvae for 3 h. At the end of the infection period, the silkworm larvae were incubated at 25°C in approximately 70% humidity, and the time calculation (hours post infection; hpi) was started. The silkworm larvae were collected at different time points, 3, 6, 12, and 24 hpi for BmNPV and E. coli infection, and 6, 12, 24, and 48 hpi for B. bassiana infection.

RNA Extraction

After removing the contents of the midgut, silkworm larvae were collected at different time points and immediately snap-frozen in liquid nitrogen. Three larvae were collected for each sample and at least three independent samples were prepared for each time point. Each sample containing three larvae was grinded broken fully in liquid nitrogen. And then the broken powders were dispersed into 1.5-mL centrifuge tubes (each containing approximately 0.1 g). Total RNA was extracted using TRizol reagent (Invitrogen, CA), according to the manufacturer’s instructions. RNA samples were quantified by spectrophotometry and analyzed by 1.0% formaldehyde denaturing agarose gel electrophoresis. All RNA samples were stored at −80°C until use in microarray and real-time polymerase chain reaction (PCR).

Microarray Hybridization and Data Analysis

The reverse transcription of RNA samples, and labeling and hybridization of cDNAs, was carried out by the CapitalBio Corporation (Beijing, China), as detailed in a previous paper (Xia et al. 2007). Gene expression experiments were carried out according to a standard procedure detailed on the CapitalBio website (http://www.capitalbio.com). Labeled cDNA was hybridized to the 23 k DNA chip (CapitalBio), which carries an array of 22,987 70-mer oligonucleotides representing silkworm genes and internal controls. Hybridization data were collected using a LuxScan 10KA dual-channel laser scanner (CapitalBio). Referred previous studies, two independent replicates were performed (Xia et al. 2007, Huang et al. 2009).

The microarray data from this study have been deposited in the SilkDB silkworm genome database (http://www.silkdb.org/silkdb/). The details of the analysis strategy and the microarray data for B. bombyseptieus infection were from our previous reported study (Huang et al. 2009). Briefly, any faint hybridization signals, with signal intensities less than 400 units by laser scanning, were filtered out. The signal intensities from all chips were normalized using the LOWESS method (Yang et al. 2002). Differentially expressed genes were identified by Significant Analysis of Microarrays software with a false discovery rate of <0.05 (Tusher et al. 2001). The expression of a gene was considered modulated when the relative expression showed a ≥2-fold change at P < 0.05 compared to the control sample (Xia et al. 2007, Huang et al. 2009, Ma et al. 2013).

For genes whose expression was modulated, gene ontology (GO) analysis was carried out using the Blast2go software and the CapitalBio MAS 3.0 online Molecule Annotation System (http://www.capitalbio.com). Pathway mapping of genes was carried out using the KEGG database (http://www.genome.jp/kegg/), and functional assignments to the Wnt signaling pathway, MAPK signaling pathway, p53 signaling pathway, cell cycle, and cytokine/cytokine signaling pathway were made using KEGG pathway human gene SEED assignments (http://thefeed.read/SEEDViewer_Main/KEGG/) for Blast analysis of the SilkDB database (http://silkworm. swu.edu.cn/silkdb/). The identified silkworm genes were then manually confirmed by the nr database (http://www.ncbi.nlm.nih.gov/).
KEGG metabolic pathway-related genes were directly identified from the Molecule Annotation System database (http://bioinfo.capitalbio.com/mas/; \( P < 0.05 \)). Some previously reported data were used for JH- and innate immunity-related genes (Vermunt et al. 2001, Tanaka et al. 2008). Genes related to cuticle proteins, metalloproteases, and proteolytic enzymes were identified using the corresponding Pfam domains (http://pfam.xfam.org) of the silkworm proteins.

Real-Time PCR
Genes identified by microarray analysis were confirmed by real-time reverse transcription-PCR (RT-PCR) using the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China). Total RNAs were the same batch of samples for microarray analysis. Each reaction was prepared in 25 \( \mu \)l containing 70 ng cDNA (2 \( \mu \)l), SYBR Premix Ex Taq (12.5 \( \mu \)l), and 10 \( \mu \)M each of sense and antisense primers (0.5 \( \mu \)l), and was analyzed by the ABI Prism 7000 Sequence Detection System (Applied Biosystems). For internal standardization, primers for su22934 (transcription initiation factor 2 gene) were used (Supp Table 1 [online only]). The real-time RT-PCRs were performed at least three biological replicates for each gene. All primers used in this study are listed in Supp Table 1 (online only).

Results and Discussion
Modulation of Overall Gene Expression by Infection and GO Annotation
Using the criterion of a \( \geq 2 \)-fold change, 1,804, 2,436, and 1,743 silkworm genes were identified as showing altered expression after infection with the Gram-positive bacterial pathogen \( B. \) bombyseptieus, the viral pathogen BmNPV, and the fungal pathogen \( B. \) bassiana, respectively. For the Gram-negative \( E. \) coli bacterium, expression of 912 genes was altered after infection, less than for pathogenic infection. Genes whose expression was modulated in common or in a microorganism-specific manner during the time course of infection were determined by comparing data for infection with the three pathogens, \( B. \) bombyseptieus, BmNPV, and \( B. \) bassiana, and those for infection with the nonpathogen, \( E. \) coli. Considering all four microorganisms, the expression of 275 \( B. \) mori genes was modulated after infection (Fig. 1a), including 620, 400, 177, and 165 genes whose expression was uniquely modulated, for \( B. \) bombyseptieus, BmNPV, \( B. \) bassiana, and \( E. \) coli, respectively. There were 244, 177, 80, and 46 genes whose expression was modulated for two microorganisms, \( B. \) bombyseptieus and BmNPV, \( B. \) bassiana, \( B. \) bombyseptieus and \( E. \) coli, \( B. \) bombyseptieus and \( E. \) coli, respectively. These results show that pathogenic \( B. \) bombyseptieus, BmNPV, and \( B. \) bassiana between them showed modulated expression of more genes, in common and uniquely, than nonpathogenic \( E. \) coli. During the time course of infection, BmNPV elicited a stronger host response at 6 hpi than at other time points, whereas the other microorganisms all elicited their strongest host response at 24 hpi (Fig. 1b). In total, 686 and 644 genes were up- and downregulated, respectively, by BmNPV at 6 hpi. However, after infection with \( B. \) bombyseptieus, 1,063 and 980 genes were up- and downregulated, and for \( B. \) bassiana infection, 822 and 668 genes up- and downregulated, respectively, at 24 hpi. The results suggest that BmNPV triggers a host response more quickly than the other two pathogens and \( E. \) coli.

By GO annotation, genes whose expression was modulated post infection for each microorganism were found to represent very similar functional categories. There were 14 categories common to BmNPV and \( E. \) coli, and 13 categories to \( B. \) bombyseptieus and \( B. \) bassiana (Fig. 1c). The enzyme regulator activity category was common to BmNPV and \( E. \) coli, in which it contained six and eight genes, respectively. Among the microorganism-specific genes, there were 14, 16, 13, and 12 categories for \( B. \) bombyseptieus, BmNPV, \( B. \) bassiana, and \( E. \) coli, respectively (Supp Fig. 1 [online only]). The transcription-regulator-activity category was found for \( B. \) bombyseptieus infection. The structural-molecule-activity and extracellular-region categories were only found for BmNPV infection, suggesting that genes in these two categories might play very important roles in viral infection.

Gene Expression Profiles Resulting From Infection by the Different Types of Microorganisms
Hierarchical cluster analysis was used to generate gene expression profiles. Overall, two groups were identified by the RNA samples taken during the time course of silkworm infection (Fig. 2). Group I includes two subgroups, which mainly contain genes whose expression was modulated during the early stages of infection (3, 6, and 12 hpi) and group II comprises a single group, which mainly contains genes whose expression was modulated during the later stages of infection (24 and 48 hpi). From the gene expression profiles, five clusters could be defined for all genes whose expression was modulated. Among the clusters, cluster I and cluster V showed dynamic changes in gene expression. Cluster I contains genes that were significantly downregulated at 6 hpi by BmNPV and genes that were significantly upregulated at 24 and 48 hpi by all four microorganisms, whereas cluster V contains genes that were significantly upregulated at 6 hpi by BmNPV and significantly downregulated at 24 and 48 hpi by all four microorganisms. Cluster II is also notable, as it contains genes that were upregulated at almost all time points for infection by all four microorganisms. Most of the genes in cluster III were downregulated prior to 24 hpi by all four microorganisms, but many of these were upregulated at 24 and 48 hpi. Cluster IV contains genes that were upregulated after infection with \( B. \) bassiana or \( E. \) coli, and downregulated after infection with \( B. \) bombyseptieus or BmNPV. For infection of the silkworm with \( B. \) bassiana, 24 hpi was the key time point with regard to host transcriptional responses. A similar conclusion was drawn in previous reported studies (Furlong and Groden 2003, Furlong 2004). For example, when the Colorado potato beetle was infected by \( B. \) bassiana, there were significant linear relationships between larval weight gain, mortality, and sporulation. With regard to the timescale of BmNPV infection, expression of the \( Bm51 \) gene, encoding a 23-kDa structural protein located in the envelope fraction of the budded virion, was recently detected, both by RT-PCR and western blotting, from 6 to 72 hpi in BmNPV-infected BmN silkworm cells (Tian et al. 2009). This suggests that the budded virus protein is expressed at the early stage of the infection cycle, which is when transcriptional host responses to BmNPV infection were observed here.

Gene Expression Associated With Development
To investigate host regulatory networks involved in silkworm development, we undertook a comparative genomics analysis of well-characterized signaling pathways and gene networks (Fig. 3a and Supp Table 2 [online only]). Several signaling pathways, including those associated with JH, Wnt signaling, MAPK signaling, p35 signaling, and the cell cycle, that were related to host development, were generally or uniquely modulated after microorganism infection, suggesting that the silkworm mounts different responses to infection by different types of microorganism.
JH is one of the most important insect hormones for maintaining larval morphology and reproductive activities (Yamamoto et al. 2013). The expression of genes related to JH biosynthesis and metabolism, and encoding JH binding proteins, was modulated after infection of silkworm larvae with each of the four microorganisms. Genes encoding JH esterase (JHE4), JH epoxide hydrolases (JHEH1 and JHEH2), farnesoic acid O-methyltransferase 2 (FAMeT2), JH binding protein 3 (JHBP3), and JH binding protein precursor-like protein 2 (JHBPL2) were upregulated by all four microorganisms. However, genes encoding the allatostatin receptor (AlstR) and JH binding protein 5 (JHBP5) were downregulated (Fig. 3a and Supp Table 2 [online only]).

Interestingly, several silkworm genes showed highly specific transcriptional responses to infection with different microorganisms. For example, after *E. coli* infection, JHBP6 and JHBP7 (encoding JH binding proteins 6 and 7) were specifically upregulated ~3-fold, and BmNPV infection was notable for downregulating several genes at 6 hpi. Overall, these results suggest that upregulation of the silkworm JH biosynthetic pathway is a host response that is common to infection by each of the four microorganisms, whereas transcriptional responses of genes encoding JH binding proteins are specific to the different microorganisms.

The Wnt signaling pathway regulates a variety of developmental processes, including cell fate specification, proliferation, survival, migration, and adhesion (Nusse 2005). Most of the Wnt signaling pathway-related genes could be identified in the silkworm genome, and expression of 10 genes in this pathway was modulated after microorganism infection of silkworm larvae (Fig. 3a and Supp Table 2 [online only]). BmNPV could trigger upregulation of Wnt signaling genes at an early stage of infection, whereas *B. bombyseptieus* and *B. bassiana* downregulated Wnt signaling genes at a late stage. Thus, the *RAC1*, *RUVBL1*, *ALDH*, *NEAT*, *CTBPI*, and *PRKACA* genes were shown to be upregulated at 6 hpi with BmNPV, whereas all six genes were shown to be downregulated at 24 or 48 hpi after *B. bombyseptieus* or *B. bassiana* infection. Interestingly, nonpathogenic *E. coli* infection had a rather weak regulatory effect on the Wnt signaling pathway-related genes in comparison with the other three microorganisms.

The MAPK signaling pathway regulates diverse cellular programs, including embryogenesis, and cell proliferation, differentiation, and apoptosis, based on cues derived from the cell surface and extracellular environment (Raman et al. 2007). In total, the expression of 20 genes associated with this pathway was modulated after infection of...
of the PDGFRA induced regulation (Thuraisingam et al. 2007), and altered expression associated with Toll-like receptor signaling during lipopolysaccharide-(Sangha et al. 2008), the product of the ovarian serous carcinomas and co-occur with TP53 infection. In mammals, inactivation of p53-mediated signaling plays a major role in both the genesis and resistance to therapy of human cancer (Bell and Ryan 2007). For this pathway, about half of the related genes can be identified in the silkworm as orthologs of the human genes. After microorganism infection of silkworm larvae, the expression of host genes related to p53 downstream signaling was modulated, including the expression of CYCS, CCNG1, CDC2, THBS1, and CDK5 (Fig. 3a and Supp Table 2 [online only]). B. bombyseptieus infection triggered a strong modulation of the expression of p53 signaling-related genes, mainly by suppressing gene expression. The results indicate that the host silkworm mounts a weak p53 signaling response.

Expression of 17 genes related to the cell cycle was modulated and the genes divided into two clusters. One cluster included ORC, BUB1, CCNH, E2F4, ANAPC10, YWHA2, TFDP2, and CAK1; most of these genes were shown to be upregulated at the late stages of infection for all four microorganisms. The other cluster included CDC2, WEE1, SMC3, CDK5, CCNA, RBL1, and FZR1; most of these genes were shown to be upregulated by BmNPV at 6 hpi. CDK5, RBL1, and FZR1 are involved in cell division (Yamamuro et al. 2008). The data suggest that BmNPV infection might accelerate host cell division at the early stage of infection.

Gene Expression Associated With Metabolism

Using standard criteria of pathway prediction with a significance value of P < 0.05 and fold-change ≥2, basal metabolic pathways, including genetic information processing and transcription, nucleic acid metabolism, metabolism of cofactors and vitamins, xenobiotics biodegradation and metabolism, amino acid metabolism and nitrogen metabolism, and carbohydrate metabolism, were identified as being modulated after microorganism infection of silkworm larvae (Fig. 3b and Supp Tables 3–6 [online only]). Some of these pathways were modulated by infection with each of the four microbes, whereas for some of them modulation was microorganism-specific. Three distinct pathways related to the metabolism of cofactors, vitamins, and amino acids, were induced after infection with each of the four microbes, including one carbon pool by folate (one-carbon metabolism), biodegradation and metabolism, amino acid metabolism and nitrogen metabolism, and carbohydrate metabolism, were identified as being modulated after microorganism infection of silkworm larvae (Fig. 3b and Supp Tables 3–6 [online only]). The results indicated that these three pathways were very sensitive to infection and might play essential roles after invasive infection.

Seven pathways related to xenobiotics biodegradation and metabolism (benzoate degradation via hydroxylation), carbohydrate metabolism (pyruvate metabolism), nucleotide metabolism (purine metabolism), energy metabolism (nitrogen metabolism), amino acid metabolism (urea cycle and metabolism of amino groups), and metabolism of cofactors and vitamins (panthothenate and coenzyme A biosynthesis, and porphyrin and chlorophyll metabolism) were modulated by pathogenic B. bombyseptieus, BmNPV, and B. bassiana, suggesting that these pathways are both common and sensitive to infection by different pathogenic microorganisms (Fig. 3b and Supp Tables 3–6 [online only]). The expression of three genes associated with benzoate degradation via the hydroxylation pathway was modulated by infection with each pathogenic microorganism, including the genes encoding fumarylacetoacetate hydrolase...
that it is very important for viral infection and pneumococcal regard to pyruvate metabolism, a previously reported study showed to its modulation in benzoate-grown cells (Denef et al. 2004). With the modulation of this pathway after pathogenic infection is similar gene encoding fumarylacetoacetate hydrolase was upregulated. The modulation of this pathway after pathogenic infection is similar gene encoding fumarylacetoacetate hydrolase was upregulated.

Cluster analysis of development and basal metabolic pathways. (a) Gene expression of developmental associated pathways, for detailed fold changes of genes, see Supp Table 2 (online only). (b) Enriched basal metabolic pathways for modulated genes after four microorganisms’ infection (data from Supp Tables 3–6 [online only], P value < 0.05).

(BGIBMGA001068), trifunctional enzyme (BGIBMGA014181), and mandelate racemase (BGIBMGA003655). After infection of silkworm larvae with BmNPV, the genes encoding trifunctional enzyme and acetyl transferase were shown to be upregulated at 6 hpi, whereas after B. bombyseptieus or B. bassiana infection, the gene encoding fumarylacetoacetate hydrolase was upregulated. The modulation of this pathway after pathogenic infection is similar to its modulation in benzoate-grown cells (Denef et al. 2004). With regard to pyruvate metabolism, a previously reported study showed that it is very important for viral infection and pneumococcal fermentative metabolism (Yesilkaya et al. 2009). In total, the expression of 10, 9, and 8 genes contributing to this pathway was modulated by infection with B. bombyseptieus, BmNPV, and B. bassiana, respectively. For the purine metabolism pathway, expression of 43, 26, and 26 genes was modulated, with most of them being upregulated, by infection with B. bombyseptieus, BmNPV, and B. bassiana, respectively. From a previous study, the purine metabolism pathway was reported to be modulated after pathogenic infection (Becker 1983). For porphyrin and chlorophyll metabolism, the expression of eight, seven, and five genes was modulated by infection with
B. bombyseptieus, BmNPV, and B. bassiana, respectively, with six of the seven genes being downregulated after BmNPV infection; however, most genes were upregulated after B. bombyseptieus or B. bassiana infection. The expression pattern of genes related to the metabolism of cofactors and vitamins after infection was similar to that previously reported in mice (Bron et al. 2004).

Microorganism-specific modulation of pathways reveals the host responses to infection that are specific for a particular microorganism. BmNPV infection specifically modulated 14 pathways, more than for infection by B. bombyseptieus (three specific pathways), B. bassiana (five specific pathways), or E. coli (two specific pathways; Fig. 3b and Supp Table 4 [online only]). With regard to BmNPV-specific pathways, most of the genes encoding the 12 enzymes that contribute to the proteasomal degradation pathway were downregulated by BmNPV infection. Similarly, in humans, inhibition of proteasome function enhances the infection efficiency of the immunodeficiency virus (Wei et al. 2005). Eleven genes related to the pathway of starch and sucrose metabolism were specifically modulated by BmNPV infection, with most of them being upregulated, by 6 hpi. After cucumber mosaic virus infection, the host similarly upregulates the level of starch and sucrose metabolism, to reduce the sugar and starch content in the plant leaves (Shalitin and Wolf 2000). Thus, the data suggest that proteasomal degradation and sugar and starch metabolism are pathways that are common to the host response to viral infection in different species. After B. bombyseptieus infection, the expression of genes related to the tricarboxylic acid (TCA) cycle, benzoate degradation, and basal transcription factors was specifically modulated (Supp Table 5 [online only]). For 7 of the 10 affected genes related to the TCA cycle expression was suppressed, whereas three of four affected benzoate degradation pathway genes were upregulated, including antennal esterase (BGIBMGA004229), oxidoreductase (BGIBMGA003842),

**Fig. 4.** Cluster analysis of modulated genes associated with disease pathogenesis. For detailed fold changes of genes, see Supp Table 7 (online only).
and carboxylesterase (BGIBMGA014599). With respect to basal transcription factors, five of the seven affected genes were upregulated by *B. bombyseptieus* infection, which might accelerate nucleic acid synthesis.

### Gene Expression Associated With Disease Pathogenesis

Metalloproteases play key roles in many normal biological processes, as well as in disease pathogenesis, such as in cancer, cardiovascular diseases, and infection (Prakobwong et al. 2009). After infection with microorganisms, the expression of genes encoding members of the aminopeptidase N (APN) family, zinc carboxypeptidase dase family, and other peptidase families was modulated in the silkworm (Fig. 4 and Supp Table 7 [online only]). The APNs have been well investigated as receptors of bacterial toxins and viral particles (Miguel et al. 2002, Pigott and Ellar 2007). Here, we identified 10 APN genes whose expression was modulated after infection. After BmNPV infection, most of the affected APN genes were upregulated, as shown at 6 hpi. At the later stages of bacterial and fungal infection (24 and 48 hpi), however, most APN genes were found to be downregulated. Zinc carboxypeptidase genes were upregulated, and most of them at the later stage of bacterial infection. These results suggest that distinct types of microorganism might use different strategies for infecting host cells.

Proteolytic enzymes play key roles in the activation of pathogenic virulence factors in the host midgut (Ohara-Nemoto et al. 1994). Three types of proteolytic enzyme, cysteine proteases, serine proteases, and trypsins were modulated after infection with microorganisms (Fig. 4 and Supp Table 7 [online only]). One cathespin L-like gene (BGIBMGA006893) was significantly upregulated by all four microorganisms, and four were more strongly upregulated by *B. bombyseptieus* or *B. bassiana* than BmNPV or *E. coli*, as observed at 24 hpi. In respect of serine protease genes, 4 of 13 whose expression was modulated by infection were specifically upregulated by BmNPV, 4 were upregulated by both *B. bombyseptieus* and *B. bassiana*, and 2 were specifically upregulated by *E. coli*. Among the trypsin-family genes, most were upregulated at a late stage after *B. bombyseptieus*, *B. bassiana*, and *E. coli* infection. Although the mechanism by which the expression of host proteolytic enzymes is modulated by oral viral infection is unknown, our results indicate that many proteolytic enzymes are induced in response to BmNPV and may indeed mitigate viral infection in the silkworm.

The cuticle and peritrophic membrane are the crucial defense barriers of insects, but pathogenic microorganisms can produce proteases that damage their structure (Niu et al. 2006, Quhong et al. 2006). Here, the expression of 13 genes encoding peritrophic matrix proteins was modulated after infection of silkworm larvae with microorganisms; most of these were upregulated at the early stage of infection by BmNPV or *B. bombyseptieus*. Interestingly, the expression of 30 cuticle-protein genes was modulated; for most of them, gene expression was very sensitive to oral infection by *B. bombyseptieus*, *B. bassiana*, and *E. coli*. For *B. bassiana* infection, cuticle-protein genes were strongly upregulated at the later stages of infection (24 and 48 hpi). The results suggest that the host can overexpress its peritrophic matrix protein- and cuticle protein-related genes to counteract the degradation and penetration of these barriers by bacterial, viral, and fungal agents.

Pathogenic microorganisms can modulate the shape and function of the host cytoskeleton to their own benefit and thus lead to disease (Tilney et al. 1992, Tsarfaty et al. 2000). Here, the expression of 5 tubulin genes, 10 cytoskeleton-signaling-associated genes, and 13 muscle protein-encoding genes was modulated after infection of silkworm larvae with microorganisms (Fig. 4 and Supp Table 7 [online only]). The beta-tubulin gene (BGIBMGA009132) was upregulated after infection by each of the four microorganisms. Three other tubulin genes (BGIBMGA004542, BGIBMGA004681, and BGIBMGA009131) were specifically upregulated by *E. coli*, and downregulated by both *B. bombyseptieus* and BmNPV, after infection. Cytoskeleton-signaling-associated genes such as actin genes (BGIBMGA002082 and BGIBMGA002083) and SOS (BGIBMGA011102) were upregulated after infection, whereas most cytoskeleton-signaling-associated genes were downregulated at a late stage of both *B. bombyseptieus* and *B. bassiana* infection. Muscle-protein-associated genes such as muscle myosin heavy chain (BGIBMGA014227), myosin (BGIBMGA014526), paramyosins (BGIBMGA000612 and BGIBMGA000613), titin 1, 2, and 6 (BGIBMGA000623, BGIBMGA000624, and BGIBMGA013531), and kinectin 1 (BGIBMGA004932) were specifically upregulated prior to 24 hpi by BmNPV. In contrast, most of these genes were downregulated at 24 hpi by *B. bombyseptieus*, *B. bassiana*, and *E. coli*. The results demonstrate that BmNPV can harness the host’s muscle-protein synthesis for its efficient infection at an early stage (between 3 and 12 hpi), which is consistent with previous reports (Wei et al. 2008, Root-Bernstein et al. 2009). The other three microbes, *B. bombyseptieus*, *B. bassiana*, and *E. coli*, exert their effects at a late stage of infection, slowing the host’s activity through the suppression of muscle-related genes, which is also consistent with previous reports (Ridgway et al. 2008).

### Gene Expression Associated With Immune-Related Genes and Pathways

Genome-wide bioinformatics has identified immune-related genes and pathways (Tanaka et al. 2008). The products of several gene
families, including peptidoglycan recognition proteins (PGRPs), scavenger receptors (SCRs), and C-type lectins (CTLs), function as recognition molecules (Tanaka et al. 2008). The expression of five PGRP genes, BmPGRP-S1, BmPGRP-S2, BmPGRP-S5, BmPGRP-L1, and BmPGRP-L2, was modulated after infection with microorganisms (Fig. 5 and Supp Table 8 [online only]). Interestingly, these PGRP genes were downregulated at some stages after BmNPV infection, whereas, two, three, and three PGRP genes were upregulated after infection with B. bombyseptieus, B. bassiana, and E. coli, respectively. BmPGRP-S2 and BmPGRP-S5 showed strong upregulation of 12.79-fold, specifically after E. coli infection, and BmPGRP-L2 expression was also specifically induced by E. coli infection, at 24 hpi. In regard to SCR genes, BmSCR-B10 was upregulated after B. bombyseptieus infection (by ≈2-fold at 3 hpi), seven genes were downregulated after both bacterial and fungal infection, and four genes, BmSCR-B3, BmSCR-B10, BmSCR-B11, and BmSCR-C, were upregulated at some stages after BmNPV infection (Fig. 5 and Supp Table 8 [online only]). In the CTL gene family, three genes were upregulated after infection by microorganisms; BmCTL12 was induced by infection with each microorganism, BmCTL11 was upregulated by B. bombyseptieus and B. bassiana infection, and BmCTL16 was specifically induced by B. bassiana infection. These results suggest that modulated levels of recognition molecules might play important roles in the host response to infection by different microorganisms.

Immune effectors are produced to combat invasive microorganisms. AMPs are important immune effectors in insects, and can
types of microorganism in
activated by Gram-negative bacteria, Gram-positive bacteria, and fungi, the results show that both the Imd and Toll signaling pathways were significantly induced by all of E. coli (Gram-negative bacterium), B. bombyseptieus (Gram-positive bacterium), and the B. bassiana fungus (Fig. 6). For BmNPV, the expression levels of the BmSpz1, BmImd, BmRel, and BmRelish mRNAs showed no distinct change between 3 and 24 hpi (Supp Fig. 2 [online only]); this suggests that the Imd and Toll signaling pathways do not respond to viral infection in the silkworm. In the Imd signaling pathway, the expression levels of the BmImd and BmRelish mRNAs were markedly increased (two- to fivefold at 6 and 12 hpi) after E. coli (Gram-negative bacterial), B. bombyseptieus (Gram-positive bacterial), and B. bassiana (fungal) infection (Figs. 6A and B). In the Toll pathway, the BmSpz1 and BmRel genes were significantly upregulated at 6 and 12 hpi after E. coli (Gram-negative bacterial), B. bombyseptieus (Gram-positive bacterial), and B. bassiana (fungal) infection (Figs. 6C and D). The results show that both the Imd and Toll signaling pathways are activated by Gram-negative bacteria, Gram-positive bacteria, and fungi, suggesting that the mechanism of immune response to different types of microorganism in B. mori might differ from that in D. melanogaster. In the JAK/STAT signaling pathway, the expression level of BmStat was significantly increased from 3 to 24 hpi after BmNPV infection, whereas BmDome was downregulated at 3 hpi by BmNPV infection (Figs. 6E and F), suggesting that the JAK/STAT signaling pathway also plays a role in the host response to viral infection in the silkworm.

Conclusions
In this study, the systematic comparison of transcriptional profiles provided an opportunity to reveal the mechanisms of the silkworm’s response to four types of microorganism. For each microorganism, the host response to infection was strong and complex, and this suggests the involvement of several mechanisms, such as the immune response, development, basal metabolism, and pathogenesis. The expression of hundreds of genes was generally or specifically modulated among the four microorganisms. Some basal metabolic pathways were modulated by infection in a microorganism-specific manner. Although all four microorganisms modulated the pathways of JH synthesis and metabolism, the expression of JH binding proteins was shown to be specific for different microorganisms.

The general or microorganism-specific modulation for pattern recognition proteins and effectors revealed different recognition and action mechanisms in the immune response to each microorganism; moreover, both Imd and Toll signaling pathways are activated by Gram-negative bacteria, Gram-positive bacteria, or fungi in the silkworm. The data from our systematic comparison will elucidate potential molecular mechanisms of the host response to different microorganisms, and provide a foundation for further work on host-pathogen interaction.

Supplementary Data
Supplementary data are available at Journal of Insect Science online.

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