Effects of Quercetin on the Growth and Expression of Immune-Pathway-Related Genes in Silkworm (Lepidoptera: Bombycidae)

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Abstract

Quercetin is a flavonoid produced as a defense by plants. The effects of 1% quercetin on the growth and development of Bombyx mori were studied. The activities of the enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), carboxy-esterase (CarEs), and glutathione S-transferase (GST) were all measured at 24, 48, 72, and 96 h after quercetin exposure. The results show that quercetin induces the activities of antioxidant and detoxification enzymes. With longer exposure times, enzyme activity first increased and then decreased. The relative expressions of AMP (defensin, CecA), the Toll pathway (cactus, Spatzle, and Rel), the IMD pathway (Imd, Fadd, and Dorsal), the JAK-STAT pathway (STAT, HOP, and Pi3k60), and the Melanization gene (DDC and PAH) were analyzed using quantitative polymerase chain reaction (qPCR). The results indicated that long-term exposure to quercetin could inhibit the expression of immune-related pathway genes in silkworms. This suggests that it can inhibit the activities of antioxidant and detoxifying enzymes, thus inhibiting the immune system and affecting the growth and development, resulting in an increase in the death rate in silkworm. This study provides the novel conclusion that quercetin accumulation inhibits the immune system of silkworm and increases its death rate, a result that may promote the development and utilization of better biopesticides that avoid environmental pollution.

Key words: quercetin, antioxidant enzyme, detoxifying enzyme, immune response

Plants have evolved a variety of defense mechanisms against insects. Their main strategy is to essentially lower their nutritional value and induce specific secondary metabolites (De et al. 2007). Some secondary substances produced by plants cause intestinal lesions and damage to the digestive system of insects, such as the chemicals in Azadirachta Indica A.Juss. (Rutales: Meliaceae) (Nisbet 2000), which can eventually lead to death. Flavonoids are important secondary metabolites that are widely distributed in plants and have many valuable biological activities (Jiang and Xia 2014). Not only do they attract pollinators and protect plants from ultraviolet (UV) light and ozone, they also protect them from microbes and insects. In addition, flavonoids have a large and important influence on the growth and development of insects. Quercetin is an important component of flavonoids (Ferreya et al. 2012). It can inhibit Helicoverpa armigera (Lepidoptera: Gelechiidae), Helicoverpa zea (Lepidoptera: Noctuidae), and Heliothis virescens (Lepidoptera: Noctuidae), and it can also kill Spodoptera eridania (Lepidoptera: Noctuidae) larvae. Furthermore, it also inhibits the activity of polyphenol oxidase (Zhang and Zhu 2011), suggesting that it may play an important role in the development of insects and their immune defenses.

Insects have evolved mechanisms to adapt to host plants and protect themselves from chemical damage (Ferreya et al. 2012). These include the development of protective enzymes that enhance their defenses and immunity to the secondary metabolites of plants, such as antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). The key mechanism by which insects eliminate the toxicity of the secondary metabolites of plants is to increase detoxification-enzyme activity. Low-dose quercetin significantly induces carboxylesterase (CarEs) and glutathione S-transferase (GST) activity in Bemisia tabaci (Hemiptera: Aleyrodidae) (Shao-Fei et al. 2006). Bombyx mori (L.) is an oligotrophic lepidopteran insect that eats only mulberry leaves. Mulberry leaves contain a variety of defensive substances, including quercetin, and the amount of quercetin in mulberry leaves varies with the development of the leaves, reaching 0.06% at its highest concentration (Sun et al. 2001). These defensive substances are not toxic to the silkworms that grow on them. This suggests that silkworms have evolved mechanisms to eliminate the toxicity of secondary metabolites such as quercetin. However, the mechanism of detoxification of quercetin in insects remains unclear, and the possible effects on the immune
response of these insects must be explored further. The two main mechanisms of insect immunity are humoral immunity and cellular immunity (Mcgreal 2009). In an initial humoral immune response, pattern-recognition proteins recognize the invading exogenous threat and then synthesize several antimicrobial proteins (AMPs) through the Toll, IMD, and JAK-STAT signaling pathways (James and Xu 2012). The immune responses available to cells include phagocytosis, nodulation, and encapsulation, usually accompanied by melanization (Mcgreal 2009).

In this study, quercetin was administered to silkworm larvae, and its effects on the growth, protective enzyme, detoxification-enzyme activity, and immune-related pathway gene expression of silkworm larvae were studied. We measured changes in digestion and utilization, growth and development, and spinning and cocooning of silkworm under the influence of quercetin treatment. We also measured the activities of SOD, CAT, POD, CarE, GST, and AChE after exposure. The responses of 13 immune-pathway-related genes in the silkworm larvae were detected using real-time fluorescent quantitative polymerase chain reaction (qPCR). Our results showed that quercetin had definite effects on the growth and development of the silkworm larvae, as follows. It induced activity in antioxidant and detoxification enzymes. With longer exposure times, enzyme activity first increased and then decreased. The expressions of immune-related-pathway genes were also induced to varying degrees, which indicates that the activity of the antioxidant and detoxification enzymes may be induced by quercetin (Zeitoum et al. 2019) and prompt the immune system to eliminate the toxic effects of quercetin. These results will clarify the relationship between quercetin and flavonoids in relation to the growth and development of silkworm and establish a solid foundation for biological pest control.

Materials and Methods

Artificial Food Preparation

We weighed 100 g artificial food (ZhengFeng Silkworm Food Co., Ltd, Guangxi, China), added 200 ml water, stirred it fully with a glass rod, put it into a LX-C35L High-Pressure Sterilization Pot (Hefei Huatai Medical Equipment Co., Ltd, Anhui, China), and sterilized it at 105°C for 15 min. After sterilization, when the artificial food had cooled to about 70°C, wearing disposable plastic gloves, we pressed it to remove any air bubbles remaining in the artificial food. Then we sealed it with film and kept it at 4°C until use. For compression it to remove any air bubbles remaining in the artificial food. Then we sealed it with film and kept it at 4°C until use. For compression it to remove any air bubbles remaining in the artificial food. Then we sealed it with film and kept it at 4°C until use. We also measured the activities of SOD, CAT, POD, CarE, GST, and AChE after exposure. The responses of 13 immune-pathway-related genes in the silkworm larvae were detected using real-time fluorescent quantitative polymerase chain reaction (qPCR). Our results showed that quercetin had definite effects on the growth and development of the silkworm larvae, as follows. It induced activity in antioxidant and detoxification enzymes. With longer exposure times, enzyme activity first increased and then decreased. The expressions of immune-related-pathway genes were also induced to varying degrees, which indicates that the activity of the antioxidant and detoxification enzymes may be induced by quercetin (Zeitoum et al. 2019) and prompt the immune system to eliminate the toxic effects of quercetin. These results will clarify the relationship between quercetin and flavonoids in relation to the growth and development of silkworm and establish a solid foundation for biological pest control.

Test of Quercetin Exposure

The Dazao silkworm larvae were reared on an artificial food (using a knife, artificial feed was cut into small strips for feeding) in a climate box (light/dark cycle 12 h), with a temperature of 24 ± 1°C and a humidity of 75 ± 5%. After 7 d from the onset of the fourth instar, we divided the larvae into two groups. Each group included three repeated experimental groups, each of which had 80 larvae. The silkworms were fed normal artificial food (control group) or artificial food with a quercetin content of 1% (treatment group). Changes in food intake, excretion, body weight, and body length, and any deaths were recorded for both groups, and the average values were calculated. After 24, 48, 72, and 96 h treatment, the larvae were placed on ice for dissection. The whole body and fat body of the 10 larvae were collected from each treatment and frozen at −80°C for subsequent enzyme assay and RNA extraction, and the assays were repeated three times (Zhang et al. 2012).

Sample Preparation

The fat body of the silkworm was placed in 0.05 M phosphate buffer (pH 7.5) (1:10, wt:vol) that contained 0.5 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), 1 mM dithiothreitol (Sigma, St. Louis, MO), and 0.1 mM ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, MO), and then they were centrifuged in a cryogenic ultracentrifuge at 8,000 rpm for 20 min at 4°C. The extract supernatant was stored at −80°C to analyze the activity of the enzyme. The fat body collected by dissection was added to 3 ml phosphate buffer with pH 7.0, homogenized in an ice bath, centrifuged for 30 min at 4°C and 12,000 rpm, and the supernatant was drawn to make 1 ml enzyme source solution for standby. The protein concentrations were determined using the Bradford method.

Determination of Protein Concentration

Following previous research (Asryants et al. 1985), the following reaction solution was used: serum protein 0.02 ml and Coomassie Brilliant Blue G20 reagent (Nanjing JianCheng, Jiangsu, China) 5.0 ml, mixed well. The reaction took 2 min to reach equilibrium, and the standard-curve blank tube was set to zero. The OD value for optical density was measured by an FLX800 Fluorescence Microplate Reader (Berton Instruments Co., Ltd, United States) at 595 nm.

SOD Activity

SOD activity was determined using a published method (Alici and Arabaci 2016). The 3 ml reaction solution contained 1.5 ml 0.05 mol/liter phosphate buffer (pH 7.8), 0.3 ml 130 mmol/liter methionine solution, 0.3 ml 750 µmol/liter nitro-blue tetrazolium (NBT) solution, 0.3 ml 100 µmol/liter EDTA solution (EDTA-Na2), 50 µl enzyme source solution, 0.3 ml 20 µmol/liter riboflavin (VB2) solution, and 0.25 ml deionized water. The reaction was incubated at 25°C for 25 min and then measured by an FLX800 Fluorescence Microplate Reader (Berton Instruments Co., Ltd) at 550 nm.

POD Activity

POD activity was determined using the guaiacol method (Cai et al. 2019). The 5 ml reaction system contained 2.9 ml 50 mmol/liter phosphate buffer (pH 5.5), 1.0 ml 50 mmol/liter callophenol, 1.0 ml 2% H₂O₂, and 100 ml enzyme solution, with buffer as the reference. The reaction took 2 min to reach equilibrium, and the standard-curve blank tube was set to zero. The OD value for optical density was measured by an FLX800 Fluorescence Microplate Reader (Berton Instruments Co., Ltd) at 420 nm for 5 min.

CAT Activity

CAT activity was determined using an earlier method (Miao et al. 2019). Following the manufacturer’s instructions, 0.2 ml enzyme solution, 1.5 ml phosphate buffer (pH 7.8), and 1 ml distilled water were added separately. Then it was preheated in a water bath at 25°C, and 0.3 ml 0.1 mol/liter H₂O₂ was added to each tube. Started immediately after adding 0.3 ml 0.1 mol/liter H₂O₂, and it was quickly poured into a 1 cm quartz cuvette. The absorbance was measured by an FLX800 Fluorescence Microplate Reader (Berton Instruments Co., Ltd) under UV light at a wavelength of 240 nm.
The absorbance was read once every 1 min for a total of four times. CAT-specific activity was expressed as nmol/mg protein/min.

GST Activity
The 1-chloro-2,4-dinitrobenzene colorimetric method was used (Catterall et al. 2002). The reaction system consisted of 2.5 ml 0.066 mol/liter pH 7.0 phosphate buffer (containing 0.002 mol/liter EDTA), 0.3 ml 0.05 mol/liter reduced glutathione (GSH) substrate solution, 0.1 ml 0.03 mol/liter 2,4-dinitrochlorobenzene substrate solution, and 0.1 ml enzyme source. Optical density at 340 nm was measured with an FLX800 Fluorescence Microplate Reader (Berton Instruments Co., Ltd) after 5 min reaction at 25°C. Observations were expressed as nmol/mg protein/min.

CarE Activity
Following the a-acetate colorimetric method (Asperen 1962), o-naphthalene acetate (α-NA) was used as a substrate to measure CarE. 20 µl supernatant, 99 µl phosphate buffer solution (0.04 M, pH 7.0), and 1 µl 30 mM o-naphthalene acetate dissolved in acetone were mixed as substrates and placed into a 120 µl volume for the catalytic reaction. After the reaction solution was incubated at 37°C for 30 min, 22 µl 1% Fast Garnett GBC salt aqueous solution and 55 µl 5% sodium dodecyl sulfate aqueous solution were added to terminate the reaction. The reaction between fast garnet and β-naphthol was measured by an FLX800 Fluorescence Microplate Reader (Berton Instruments Co., Ltd) at 450 nm to produce chromophores. COE activity was expressed as nmol/mg protein/min.

Malondialdehyde Activity
Malondialdehyde (MDA), a product of lipid peroxide degradation, can condense with thiobarbituric acid (TBA) to form a red product (Schmedes and Ga 1989). A volume of 0.1 ml was absorbed, 0.1 ml 0.5% TBA solution was added, and the mixture was reacted in a boiling water bath for 30 min. It was quickly cooled and centrifuged again. The supernatant was taken to determine absorbance using an FLX800 Fluorescence Microplate Reader (Berton Instruments Co., Ltd) at 532 nm, and the results were expressed as nanomole/milligram protein/min.

AchE Activity
The supernatant was prepared into a 10 ml enzyme source for analyses of AchE inhibition. Acetylthiocholine iodide (ATChI) was used as the substrate (Chai et al. 2007). We dissolved 0.0101 g ATchI with 0.1 M pH 7.5 PBS in a constant volume of 50 ml and dissolved 0.040 g DTNB in 50 ml 0.1 M pH 7.5 PBS. After this, we added 125 ml ethanol to a constant volume of 250 ml with deionized water. Under the action of AchE, ATChI was hydrolyzed into thiocholine and acetic acid, thiocholine, and DTNB to produce a color reaction that turned the reaction solution yellow. Absorbance was measured at 412 nm on an FLX800 Fluorescence Microplate Reader (Berton Instruments Co., Ltd), and its activity was expressed as nmol/mg protein/min. All the reagents used in the above enzymatic reactions were analytically pure and were obtained from Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China.

Gene Expression Analyses
Total RNA in the whole body was extracted using Trizol reagent (Invitrogen, California, United States) and treated with DNase (Promega, Wisconsin, United States) to remove possible contamination by genomic DNA from the samples. The quality of the extracted RNA was evaluated using formaldehyde-agarose gel electrophoresis and was quantified by a Nanodrop 2000 Ultramicro Spectrophotometer (Thermo, MA, United States). Following the manufacturer’s instructions, the first cDNA chain was synthesized with an oligonucleotide (dT) and reverse transcriptase (Promega), and then it was amplified by PCR, using cDNA as a template to acquire the target gene. Using actin3 as an internal-reference gene, the reaction volume for qPCR was 10 µl, and SYBR permix Ex TaqII (TaKaRa, Japan) was used with 0.3 µM each primer (listed in Supp Table 1 [online only]) and 1 µl cDNA to detect the fluorescence data for AMP (defensin, CecA), the Toll pathway (cactus, Spatzle, Rel), IMD pathway (IMD, Fadd, Dorsal), the JAK-STAT pathway (STAT, HOP, PI3k60), and the Melanization gene (DDC, PAH), following the manufacturer’s instructions of Step One Plus Fluorescent Quantitative PCR instrument (Applied Biosystems, MA, USA). The initial denaturation step of the qPCR protocol was done at 95°C for 40 s. Then 40 cycles of melting were performed at 94°C for 10 s and 55°C for 30 s, and annealing temperatures were set in the range 56–59°C according to the needs of each gene.

Statistical Analyses
Statistical analyses were conducted using SPSS 26.0 software with data expressed as means ± standard deviations. We statistically analyzed data using one-factor analysis of variance (one-way ANOVA), and evaluated differences among means of the treatment and control by the least significant difference (LSD) test. The data with P values equal to or lower than 0.05 were considered statistically significant.

Results
Effects of Quercetin on the Growth and Development of Silkworm
The survival rate of the silkworms was detected after 9 d exposure to 1% quercetin. The results showed that 1% quercetin had significant (df = 1, f = 18.215, P = 0.013) effect on the survival rate of silkworm (67.5% of the control) (Fig. 1A). The weight of the silkworm larva increased with the time of quercetin exposure. For the group with 1–4 d exposure, the weight of the silkworm in the control group was almost the same as that in the treatment group, while in the group with 5–9 d exposure, the weight of the silkworms in the control group was higher than that in the treatment group (Fig. 1B). The body length of the larva also increased with exposure time. For 2–9 d exposure, controls had greater body lengths treated larvae (Fig. 1C). Similarly, the silkworm intake increased first and then decreased slowly after the fifth instar, but the intake of the quercetin group was higher than that of the control group (Fig. 1D). The same trend was observed in the excretions, which also decreased slowly after the fifth instar, such that the excretion of the quercetin-exposed group was higher than that of the control group (Fig. 1E). The quercetin-exposed group produced fewer cocoons than controls, and there were no differences in the number of silkworm pupae between the two groups, so the amount of silk was lower in the quercetin-exposed group than controls (Fig. 1F).

Effects of Quercetin on Antioxidant-Enzyme Activity
Figure 2 shows activity changes in antioxidant enzymes (SOD, CAT, and POD) at 24, 48, 72, and 96 h quercetin treatment. The activity of SOD in the quercetin-exposed group increased at first and then decreased. At 72 h, the activity reached its highest value. At 48 h after treatment, it was significantly greater in the treatment group.
than in the control group (the degree of freedom \( df = 1, f = 9.613, P = 0.036 \)). However, its activity reached its lowest level, lower than that in controls at 96 h (Fig. 2A). CAT and POD showed the same trends, with both peaking at 72 h and then decreasing at 96 h (Fig. 2B and C).

**Effects of Quercetin on Detoxification-Enzyme Activity**

The changes in the activities of the detoxification enzymes GST and CarE are represented in Fig. 3. GST increased significantly after 48 h (\( df = 1, f = 221.620, P < 0.001 \)) of treatment, while CarE was most significant and reached its highest level at 72 h (\( df = 1, f = 26.118, P = 0.029 \)). AChE activity in the experimental group was 0.40-fold, 0.84-fold, 0.72-fold, and 1.57-fold that of the control group at the four-time points, respectively. The activity was significantly less than that of the control group at 24 h (\( df = 1, f = 145.190, P < 0.001 \)), and reached its highest level at 96 h (\( df = 1, f = 16.301, P = 0.021 \)). MDA levels after quercetin treatment decreased significantly at 24 h (\( df = 1, f = 77.766, P = 0.001 \)), and then significantly increased at 48 h (\( df = 1, f = 14.782, P = 0.037 \)) and 72 h (\( df = 1, f = 22.788, P = 0.033 \)) (Fig. 3D).

**Effects of Quercetin on Immunity Genes in Silkworm**

Figures 4 and 5 show the relative expression levels of the immune gene pathways AMP (defensin and CecA), Toll pathway (cactus, Spatzle, and Rel), IMD pathway (Imd, Fadd, and Dorsal), JAK-STAT pathway (STAT, HOP, and Pi3k60), and melanization (DDC and PAH).

First, we conducted qPCR analyses of the AMP and Toll pathway genes. The expression levels of the defensin gene after 24, 48, 72, and 96 h quercetin treatment were 0.98-fold, 0.65-fold, 2.16-fold, and 0.60-fold those of the control group, respectively, and they reached their highest level at 72 h (\( df = 1, f = 44.186, P = 0.003 \)) and then
decreased significantly at 96 h (df = 1, f = 28.955, P = 0.006). The expression levels for the CecA gene in the quercetin group were 0.69-fold, 2.64-fold, 0.23-fold, and 0.81-fold those of the control group, respectively. The relative changes in expression levels of genes on the Toll pathway (Cactus, Spz, and Rel) are shown in Fig. 4B. Spz was significantly increased at 72 h (df = 1, f = 17.469, P = 0.014) relative to the control group, at 1.53-fold higher than the control group. The relative expression levels for Cactus (df = 1, f = 6.056, P = 0.07) and Rel (df = 1, f = 6533.157, P < 0.001) significantly increased and reached their highest level after 24 h quercetin treatment and then decreased. At 96 h treatment in particular, the expression level of Cactus (df = 1, f = 35.504, P = 0.004), Spz (df = 1, f = 22.730, P = 0.009), and Rel (df = 1, f = 252.389, P < 0.001) was significantly lower than that in the control group.

The relative expression levels of immunity genes in the IMD pathway and the JAK-STAT pathway were also detected. We found that after 24 h treatment, the relative expression levels for Imd increased at 48 h, and Dorsal significantly increased at 24 h, respectively. While Fadd showed significant differences at 24 h (df = 1, f = 60.187, P = 0.001), 48 h (df = 1, f = 25.544, P = 0.007) and 72 h (df = 1, f = 1217.048, P < 0.001), and the relative expressions levels of Imd (df = 1, f = 12.227, P = 0.025), Fadd (df = 1, f = 1466.188, P < 0.001), and Dorsal (df = 1, f = 114.122, P < 0.001) decreased at 96 h and were smaller than those of the control group (Fig. 5A). Figure 5B shows changes in the relative expression levels of the JAK-STAT pathway genes (STAT, HOP, and Pi3k60). The relative expression levels of STAT showed a decreasing trend and significantly decreased at 96 h (df = 1, f = 33.330, P = 0.005). However, Pi3k60 (df = 1, f = 111.659, P = 0.027) and HOP (df = 1, f = 8.238, P = 0.045) were significantly increased at 72 h, at 1.93-fold, and 2.00-fold higher than in the control group, respectively. And Pi3k60 decreased at 96 h (df = 1, f = 15.155, P = 0.018) and were lower than those of the control group. The relative expression levels of melanization-related genes (PAH and DDC) are shown in Fig. 5C. At 24 h and 72 h, the relative expression levels of PAH (24 h: df = 1, f = 38.24, P = 0.003; 72 h: df = 1, f = 17.15, P = 0.015) and DDC (24 h: df = 1, f = 10.45, P = 0.032; 72 h: df = 1, f = 17.34, P = 0.014) showed significant increases, and the overall trend was one of increase at first, followed by decrease. Particularly at 96 h treatment, the expression levels of PAH (df = 1, f = 71.095, P = 0.01) and DDC (df = 1, f = 285.856, P < 0.001) were significantly decreased compared to the control group.

Discussion

The results of this study indicate that quercetin had effects on the body weight, body length, food intake, excretion, and silk production of silkworm larvae after long-term treatment. Similar results have been found in previous studies, where relatively low concentrations of quercetin (0.500, 0.250, and 125 μg/ml) have produced a negative effect on newborn Helicoverpa armigera, which increased as exposure time increased (Jadhav et al. 2012). Thus, quercetin inhibited the growth and development of herbivores, particularly after long-term exposure. Although the role of flavonoids in plant–insect interactions has been widely recognized, the mechanisms through which flavonoids inhibit herbivore growth have not yet been clearly explained. Previous studies have found that quercetin has a significant effect on the expression and activity of various detoxifying
enzymes, such as insect cytochrome P450, GST, and COE (Zhang et al. 2012). Therefore, flavonoid impacts on detoxification enzymes are candidate mechanisms through which flavonoids confer plant resistance to insect herbivores. Following this consideration, we analyzed the relationships among the activities of SOD, CAT, POD, GST, and CarE in silkworm and quercetin. Quercetin had a significant effect on the activities of protective enzymes in silkworm, and with increases in exposure time, their activities first increased and then decreased, reaching a peak at 72 h. These results showed that quercetin, a secondary metabolite, could induce the initiation of a self-protection mechanism in silkworm. However, with longer exposure times, the protective mechanism gradually decreased. This mechanism is harmful to the normal growth and development of silkworm. It was also found that the MDA content increased first and then decreased after quercetin treatment, and the change in MDA content was consistent with the change in the activity of protective enzymes and detoxification enzymes, indicating that after long-term exposure to quercetin, tolerance of the protective enzyme
reached its limit, and the original balance of the protective enzyme system was destroyed, resulting in a disorder of the free-radical scavenging system, increased contents of cytotoxic substances, and severe damage to cells. The integrity of silkworm cell membrane and the loss of its permeability affect normal development and the physiological metabolism of silkworm.

Flavonoids have complex effects on insect growth and development. Due to the particularities of immunity in insects, adverse environmental stimulation may affect their intrinsic immune system and thus impair their antibacterial and antiviral properties of insects (Dolezal et al. 2019). Herein, we explored different responses to innate immune pathways in silkworm AMP genes, Toll, ImD, JaK-STAT, and melanization-related genes in response to quercetin. The relative expression levels of 13 genes related to immune response were detected. The expression levels of almost all genes in short-term exposure to quercetin were higher than those in the control group, but their expression levels were decreased in response to long-term quercetin exposure, indicating that long-term exposure to quercetin has adverse effects on the immune system in silkworm.

In this study, after short-term exposure to quercetin, the transcriptional levels of AMP genes, defensin, and CecA were significantly higher than those in the control group, and they were lower after long-term exposure to quercetin. Previous reports showed that the antimicrobial peptide genes defensin and Ceca were related to the resistance to exogenous microorganisms (Diamond et al. 2009), and defensin was a cationic AMP with antimicrobial activity against Gram-positive bacteria (Wen et al. 2009). These results suggest that short-term exposure to quercetin may activate the antimicrobial peptide gene, thus triggering the innate immune system of silkworm to resist pathogen infection and maintain normal functioning. The innate immune system of silkworms may be inhibited after long-term exposure to quercetin. Furthermore, the transcriptional levels of the Toll signaling-pathway genes Cactus and Rel significantly increased after 24 h of treatment, then decreased, and were lower than the control group. Because the activation of the Toll pathway is strictly dependent on the product of the Spz gene (Habig et al. 1974), we speculate that the trend in the Toll pathway is consistent with the trend of expression of Spz, and the relative expression level of Spz significantly decreases at 96 h. The corresponding AMP genes also showed significant decreases at long-term exposure to quercetin, inhibiting the innate immune system of the silkworm, which is consistent with the conclusions based on AMP gene expression. These results may also be due to the regulation of AMP gene expression along the Toll/IMD signal pathways. The Toll pathway is also activated as a result of infection by Gram-positive bacteria and fungus. These results indicate that quercetin may increase silkworm susceptibility to Gram-positive bacteria.

By contrast, the IMD pathway mediates the expressions of AMPs, which are activated by Gram-negative bacterial infections (Lhocine 2009). Early studies have shown that the protein encoded by the IMD gene in Drosophila melanogaster (Diptera: Drosophilidae) can form a ligand–receptor complex with Fadd, thereby regulating the expression of AMP genes (Corbo and Levine 1996, Leulier et al. 2002). In this study, the transcription levels of the Toll signaling-pathway genes Imd, Dorsal, and Fadd were significantly reduced after 96 h quercetin treatment, which may reduce the binding ability of the
ligand–receptor complex to Fadd and thus downregulate the expression of the AMP genes. The downregulation of the antimicrobial peptide gene also confirmed that the IMD signaling pathway was inhibited. These results suggest that quercetin had an inhibitory effect on the silkworm IMD pathway, which may increase the risk for infection by Gram-negative bacteria.

The transcription factors STAT, JAK, HOP, and cytokine receptor Domeless are the main members of the JAK-STAT pathway (Peng et al. 2012), and JAK-STAT is involved in the antiviral immune response of silkworm (Chen et al. 2018). Our results indicate that HOP and Pi3k60 significantly decreased after quercetin treatment for 96 h. A comparison of experimental data leads us to speculate that the JAK-STAT pathway may show the same pattern as the AMP, Toll, and IMD pathways in response to quercetin. Furthermore, the gene expression levels of the AMP, Toll, IMD, and JAK-STAT pathways all showed a downward trend after 96 h of treatment and were smaller than those of the control group. Therefore, we speculate that continuous exposure to quercetin can inhibit the JAK-STAT pathway and thus inhibit the innate immune response of silkworm, making it more susceptible to viral infection. Similarly, the expression levels of the melanization-related genes, PAH, and DDC in silkworm were significantly higher than the control group after 24 and 72 h. They showed a similar trend with other immune signal pathway genes that have previously been detected, and the expression levels were significantly decreased after 96 h treatment. Previous studies have shown that melanization plays a key role in innate immune defense mechanisms in insects (Lakaye et al. 2009). The expressions of PAH and DDC genes directly affect the formation of melanization (Liu et al. 1997). Therefore, we speculated that long-term exposure of silkworm to quercetin would inhibit the expression of melanization-related genes of silkworm.

In conclusion, we used quercetin at a concentration of 1% to treat silkworms, and we found that it significantly inhibited the growth and development of the silkworms. Quercetin can induce the activities of antioxidant enzymes (SOD, CAT, and POD) and detoxification enzymes (GST and CarE) over a short period of time and increase the expression levels of immune-related genes (defensin, CecA, Spatzle, Imad, Fadd, HOP, Pi3k60, DDC, and PAH). When exposed to quercetin for a long time, the five pathways of AMP, the Toll pathway, the IMD pathway, the JAK-STAT pathway, and the Melanization gene of silkworm are all inhibited. At this point, the innate immune system of the silkworm is reduced, leading to an increase in its mortality rate, and its growth and development are also significantly reduced. These results lay a solid foundation for the biological control of silkworm pests.

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

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Author Contributions
GS conceived and designed the experiments, analyzed the data. FR analyzed the data. YZ and PG performed the experiments.

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