Induction of Detoxification Enzymes by Quercetin in the Silkworm

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ABSTRACT Quercetin is one of the most abundant flavonoids and the defense secondary metabolites in plants. In this study, the effect of quercetin on the growth of the silkworm larvae was investigated. Cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), and carboxylesterases (COE) were assayed after exposure to different concentrations of quercetin for 3 d (short-term) and 7 d (long-term), respectively. The results showed that the weight gain of the silkworm larvae significantly decreased after the larvae were treated by different concentrations of quercetin except for the treatment with 0.5% quercetin. Activities of P450, GST, and COE were induced by 0.5 or 1% concentration of quercetin. In the midgut, the induction activity of P450s was reached to the highest level (2.3-fold) by 1% quercetin for 7 d, the highest induction activities of GSTs toward CHP and CDNB were 4.1-fold and 2.6-fold of controls by 1% quercetin after 7 d exposure, respectively. For COEs, the highest activity (2.3-fold) was induced by 0.5% quercetin for 7 d. However, P450s in whole body were higher inducible activities in short-term treatment than those in long-term treatment. The responses of eight cytochrome P450 (CYP) genes belonged to CYP6 and CYP9 families and seven GST genes were detected with real-time polymerase chain reaction. In addition, the genes induced by quercetin significantly were confirmed by qRT-PCR. *CYP6AB5*, *CYP6B29*, and *GSTe8* were identified as inducible genes, of which the highest induction levels were 10.9-fold (0.5% quercetin for 7 d), 6.2-fold (1% quercetin for 7 d), and 7.1-fold (1% quercetin for 7 d), respectively.

KEY WORDS silkworm, quercetin, cytochrome P450, glutathione transferase, carboxylesterase

In the ecological interactions of plants and insects, plants have evolved various defense mechanisms against insects. For example, to defend against herbivorous insects, plants produce and release specific odors to attract predators of the insects (Turling et al. 1990, Reinhard et al. 2009, Wen et al. 2006). Flavonoids are widely distributed in the plant kingdom with a variety of medicinal values (Naira et al. 2002), which could not only attract the predators (Wilbert et al. 1997), protect plants from ultra violet (UV) damage (Simmonds 2003), and ozone damage (Saleem et al. 2001), but also defend against microorganisms (Cushnie and Lamb 2005, Shi et al. 2001) and herbivorous insects (Abou-Zaid et al. 1993, Isman and Duffey 1982). Because of interesting chemical and biological properties, quercetin is a flavonoid studied very well, which could inhibit the growth of *Heliothis virescens* (F.), *Helicoverpa zea* (Boddie), and *Pectinophora gossypiella* (Saunders) (Shaver and Lukefahr 1969, Chan et al. 1978), reduce the pupation rate of *Helicoverpa armigera* (Hübner), and lead to larval mortality of *Spodoptera eridania* (Stoll) (Lindroth and Peterson 1988). Thus, quercetin is an important defensive secondary metabolite in plants.

Similarly, herbivorous insects have evolved a variety of mechanisms to adapt to their host plants, such as target molecule variation and the induction of detoxification enzymes, including cytochrome P450 monooxygenase (P450), UDP-glucosyl transferase (UGT), glutathione S-transferase (GST), and carboxylesterase (COE) (Li et al. 2007). Increase of detoxification enzymes in insects is the key metabolic mechanism. Both COE and GST activities were significantly induced in *Bemisia tabaci* (Gennadius) exposed to low dosage of quercetin (Mu et al. 2006). In addition, quercetin could induce P450 O-demethylase activity (Liu et al. 2006), COE (Gao et al. 1998), and GSTs in *H. armigera*. Baculovirus-mediated expression of the *H. zea* CYP6B8 protein demonstrated that the CYP6B8 could metabolize six plant allelochemicals, including quercetin (Li et al. 2004). The expressed CYP6AS1, CYP6AS3, CYP6AS4, and CYP6AS10 enzymes of *Apis mellifera* (L.) could metabolize quercetin with different metabolic activities in vitro (Mao et al. 2009). Recent research has focused on the effect of quercetin on insects at the physiological and biochemical levels, and the studies at the molecular level are commonly limited to identify the induction of a class of detoxification enzymes (Li et al. 2004, Liu et al. 2006, Mao et al. 2009, Mu et al. 2006).

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The silkworm, *Bombyx mori* (L.), is an oligophagous Lepidopteran insect, specialized on mulberry leaves. Previous studies demonstrated that mulberry contains various defensive substances, such as 1, 4-dideoxy-1, 4-imino-D-arabinitol (D-AB1), 1-deoxynojirimycin (DNJ), and 1, 4-dideoxy-1, 4-imino-D-ribitol. These sugar mimic alkaloids are highly toxic to *Mamestra brassicae* (L.) (Konno et al. 2006). In addition, the concentration of the quercetin in mulberry leaves varies with development of the leaves, the highest concentration reached to 0.06% (Sun et al. 2001). However, these defense substances in mulberry are not toxic to *B. mori*, which grows on mulberry leaves very well. This indicates that the silkworm has evolved some mechanisms to eliminate the toxic effects of secondary metabolites, including quercetin. The metabolism of quercetin in insects is still unclear and requires further investigations. Because the silkworm whole genome sequence has been already available (The International Silkworm Genome Consortium 2008), this facilitates study of the detoxification mechanism of quercetin in the silkworm.

In this study, silkworm larvae were exposed to four different concentrations of quercetin for different time durations to investigate the effect of quercetin on larval growth and activities of the detoxification enzymes. We measured the activities of GSTs, COE, and P450 after exposure. The responses of eight CYP genes and seven GST genes in the silkworm after quercetin exposure were firstly examined by reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qRT-PCR). Our results indicated that quercetin could induce the activities of the GSTs, COE, and P450 differentially and the expressions of three genes *CYP6AB5*, *CYP6B29*, and *GSTe8*. These results will help us understand the mechanisms of mulberry-silkworm interaction. The identification of specific genes expressed in response to quercetin would provide the foundation for further RNA interference research aimed at pest control.

Materials and Methods

Experimental Exposure to Quercetin. Silkworm strain *Dazao* was reared on artificial diet under photoperiod of 12:12 h (L:D) at 24 ± 1°C and 75 ± 5% RH. According to the method of Li et al. (2002) and Liu et al. (2006), the long-term treatments were performed by allowing 30 newly molted fourth instar larvae with uniform size to feed on artificial diet adding 0.1, 0.5, 1, or 3% concentrations of quercetin (Sigma, St. Louis, MO), which was a solid suspension in water, for 7 d. The short-term treatments were performed by exposing the newly molted fifth instar to the four concentrations of quercetin for 3 d. Each concentration was repeated three times. The whole body and midgut of the larvae, whose peritrophic membranes were removed, were collected from each treatment and frozen at −80°C for subsequent enzyme assay and RNA extraction.

The Growth of Silkworm Exposed to Quercetin. The newly molted fourth instar larvae with uniform size were weighed, and then fed on diet adding 0.1, 0.5, 1, or 3% concentrations of quercetin for 7 d. After long-term treatment, silkworms were weighed again separately for each group with 30 larvae to calculate the average weight gain of each group (grams/larva/day). Each concentration was treated with 30 larvae, and repeated three times.

Biochemical Sample Preparation. The preparation of biochemical sample was based on the methods of Poupardin et al. (2008) and Bautista et al. (2009). The whole body and midgut were homogenized in 0.05 M ice-cold phosphate buffer (pH 7.5) (1:10, wt:vol) containing 0.5 mM phenylmethylsulphonyl fluoride (PMSF, Sigma), 1 mM dithiothreitol (DTT, Sigma), and 0.1 mM ethylenediaminetetraacetic acid (EDTA, Sigma) (Liu et al. 2006, Yu et al. 2011). Then the homogenates were centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant (500 μl) was stored at −80°C to analyze GSTs activity and COE activity. Other supernatant (1 ml) was centrifuged again at 100,000 × *g* for 60 min at 4°C. Then the resulting pellet was resuspended in 0.05 M phosphate buffer (pH 7.5) and stored at −80°C to analyze P450 activity. The protein concentration was determined by the Bradford method.

COE Activity. The COE was measured using α-naphthylacetate (α-NA, Sigma) as a substrate (Van Asperen 1962). The catalytic reactions were performed in 120 μl volumes containing 20 μl supernatant, 99 μl phosphate buffer solution (0.04 M, pH 7.0), and 1 μl 30 mM α-NA dissolved in acetone as substrate (Cui et al. 2011). The reaction solution was incubated for 30 min at 37°C. Reaction was stopped by the addition of 22 μl 1% Fast Garnett GBC Salt (Sigma) *aqueous* solution and 55 μl 5% sodium dodecyl sulfate (SDS, Sigma) aqueous solution. The reaction of Fast Garnet with α-naphthol produced chromophores, which was measured at 600 nm. COE activity was expressed as nanomole/milligram protein/minute.

GST Activity. GSTs could catalyze reaction between GSH and GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB), which is the model substrate of GSTs. The GST activity toward CDNB was measured according to the method of Habig et al. (1974). In brief, 190 μl reaction mixture contained 0.1 M phosphate buffer (pH 6.5) (Prapanthadara et al. 2000, Yu et al. 2011), 1 mM CDNB, and 1 mM GSH, was incubated for 30 min at 25°C, and followed by the addition of 10 μl supernatant. The absorbance of the reaction was measured at 340 nm for 5 min. Observations were expressed as nanomole/milligram protein/minute.

In mammals, selenium-dependent glutathione peroxidases (SeGPx) could detoxify organic hydroperoxide, while there was no SeGPx in insects (Corona et al. 2006). However, some of the insect GSTs contained the non-SeGPx activity, which were similar to the SeGPx in mammals, and could detoxify the lipid hydroperoxid during the oxidative stress (Parkes et al. 1993, Hurst et al. 1998). To investigate the response of non-SeGPx activity of silkworm GSTs after quercetin

exposure, GPx activity of GSTs toward cumene hydroperoxide (CHP) was investigated (Ahmad and Pardini 1988). With NADPH and glutathione reductase, GSTs could catalyze the reaction between GSH and CHP; 20 μl 10 mM reduced glutathione (GSH, Sigma), 4 μL10 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH, Sigma), and 1U glutathione reductase (GR, Sigma) were added to 161.5 μl 53 mM phosphate buffer (pH7.0, containing 0.l mM EDTA), followed by incubation for 5 min at 25°C. Then 2 μl 120 mM CHP methanol solution and 1 μl supernatant as enzyme sources were added into the reaction mixture. To monitor the consumption of NADPH, the absorbance was measured at 340 nm for 4 min at 37°C. GPx activity of GSTs was expressed as nanomole/milligram protein/minute.

P450 Activity. O-demethylase activity of P450 monooxygenase was determined by the method of Hansen and Hodgson (1971), Shang and Soderlund (1984); 15 μl 9.6 mM NADPH aqueous solution, 3 μl 400 mM p-nitroanisole solution in ethanol, and 100 μl microsomal enzyme were added into 282 μl 0.14 M phosphate buffer (pH = 7.8) containing 2% BSA (wt: vol) (Liu et al. 2006), followed by incubation for 30 min at 25°C. Reaction was stopped by the addition of 100 μl 1 M HCl. Product was extracted first by 500 μl chloroform, followed by the second extraction in 350 μl 0.5 M NaOH solution. After centrifugation, absorbance of 200 μl NaOH solution was determined at 400 nm, and the product was quantified based on the p-nitrophenol (Sigma) standard curve. P450 activity was expressed as nanomole/milligram protein/minute.

Gene Expression Analysis. Total RNAs were isolated from tissues using Trizol Reagent (Invitrogen, CA) according to the manufacturer's instructions. The contaminating genomic DNA was digested with RNase-free DNase I (Promega, Madison, WI) for 15 min at 37°C. According to the manufacturer's instructions, the first strand of cDNA was synthesized using oligo (dT) and reverse transcriptase (Promega).

Eight silkworm CYP genes and seven silkworm GST genes that might participate in detoxification of xenobiotics were selected and their responses were detected by RT-PCR. In addition, the genes significantly induced by quercetin were confirmed by qRT-PCR. The silkworm CYP and GST gene sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/) and used to design the primers of RT-PCR and qRT-PCR with the software Primer 5.0 (Supp. Table S1 and Table S2 [online only]). The housekeeping gene *Bmactin3* was used as an internal control. RT-PCR in reaction volume of 15 μl was performed with the protocol consisting of an initial denaturation at 94°C for 4 min, followed by 26 cycles of 94°C for 30 s, 30 s annealing (temperatures listed in Supp. Table S1 [online only]), 72°C for 30 s, a final extension at 72°C for 10 min. The products were analyzed on 1.2% agarose gels. The reaction volume of qRT-PCR was 10 μl, containing SYBR permix Ex *Taq*II (2×), 0.3 μM of each primer, and 1 μl cDNAs. qRT-PCR was performed in a DNA Engine Option 2 thermal cycler according to the manufacturer's instructions. The qPCR protocol had an initial denaturation step of 95°C for 40 s, followed by 40 cycles of melting: 94°C for 10 s, 55°C for 30 s. Fluorescence data were collected after each amplified segment.

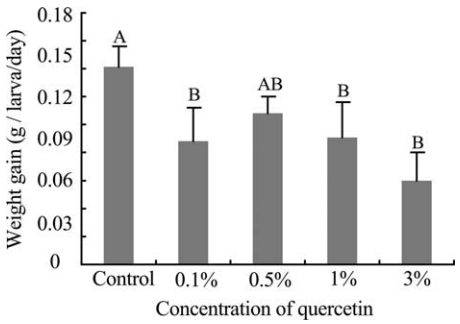

Fig. 1. Effects of quercetin on weight gain of the silkworm after 7 d. Columns topped by different letters are significantly different at probability $P \leq 0.05$ using LSD test (sample size: 30).

Statistical Analysis. The data were expressed as mean ± SD. For statistical analysis, the SPSS 12.0 package (Chicago, IL) was used. The least significant difference (LSD) test was used to examine differences among means of the treatments and control.

Results

Growth Inhibition of the Silkworm by Quercetin. The weight gain of silkworm after exposure to four different concentrations of quercetin for 7 d was detected. The results indicated that 0.5% quercetin can inhibit the growth of silkworm to some extent (76% of the control), but the effect was not significant. However, the growth of silkworm was significantly inhibited by 0.1, 1, or 3% concentrations of quercetin ($P \leq 0.05$), respectively. The weight gain of silkworm exposed to 0.1 and 1% quercetin were 62 and 64% of the control, respectively. The weight gain reached the minimum after 3% quercetin treatment, which was only 42% of the control (Fig. 1).

Induction of P450 Activity in the Silkworm Exposed to Quercetin. P450s O-demethylase activity in whole body and midgut of silkworm in response to quercetin was also detected. The induction effects of P450 activity in the whole body were greater than that in the midgut in long-term treatment. In the short-term treatment, P450 activity in the whole body was augmented after exposure to 1% quercetin, which reached to the highest induction activity (9.2-fold). In addition, P450 activity only slightly increased after exposure to 0.1, 0.5, and 3% concentrations of quercetin, which all increased to ≈2.5-fold relative to the control (Fig. 2a). In the long-term treatment of quercetin, significant increase of the P450 activity in the whole body was observed at 0.1 and 0.5% concentrations of quercetin, which increased to 5.7-fold and 6.6-fold relative to the control, respectively. However, the two high concentrations (1 and 3%) of quercetin did not significantly induce P450 activity (Fig. 2b). In the

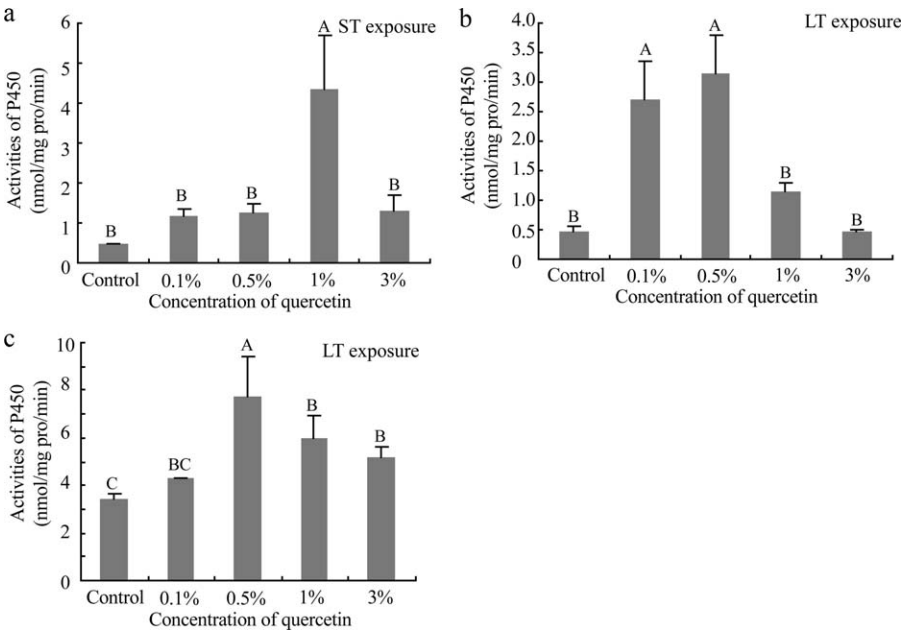

Fig. 2. Changes of P450 activities after exposure to quercetin. P450 activities in the larval whole body (a and b). P450 activities in the gut (c). Letters above the columns within the same treatment that are different indicate significant differences at probability $P \leq 0.05$ using LSD test. LT, long-time (7 d); ST, short-time (3 d).

midgut of the silkworm after long-term treatment, P450 activity induced by 0.5% quercetin reached to the highest level (2.3-fold relative to the control). The 1 and 3% quercetin resulted in an increase of 1.8-fold and 1.5-fold of P450 activity relative to the control. However, 0.1% concentration of quercetin did not significantly affect P450 activity (Fig. 2c).

Changes of GSTs Activity in the Silkworm Exposed to Quercetin. The GST activity in the midgut after quercetin exposure was detected with CHP and CDNB as the substrate. After short-term treatment, the GPx activity of GSTs was slightly but significantly induced by two high concentrations (1 and 3%) of quercetin, and the highest induced GPx activity was 1.5-fold relative to the control, observed at 1% quercetin. However, 3% quercetin was less inducible than 1% quercetin, which was 1.2-fold relative to the control. In addition, two low concentrations (0.1 and 0.5%) of quercetin did not significantly affect GPx activity of GSTs (Fig. 3a). The induction effects in long-term treatment were larger than those in short-term treatment. After long-term treatment, the highest GPx activity of GSTs was observed at 1% quercetin, which reached to 4.1-fold relative to the control. The 3% quercetin did not affect GPx activity of GSTs significantly (Fig. 3b).

The GST activity toward CDNB was also detected in the midgut. They were not induced significantly after short-term treatment (Fig. 3c). After long-term treatment (Fig. 3d), the 0.5 and 1% concentrations of quecetin significantly induced GST activity toward CDNB, which resulted in an increase of 2.5- to 2.6-fold in GST activity relative to the control. The 0.1% quercetin did not induce GST activity. In contrast, 3% quercetin inhibited GST activity, ≈77% of the activity of the control.

Effect of COE Activity in the Silkworm Exposed to Quercetin. The COE activity toward α-NA was measured. After short-term treatment, quercetin did not significantly affect COE activity relative to the control (Fig. 4a). After long-term treatment, the highest induced COE activity was 2.3-fold relative to the control, which was observed in the treatments of 0.5 and 1% concentrations of quercetin. The COE activities induced by 0.1 and 3% concentrations of quercetin were 1.6-fold and 2.1-fold relative to the control, respectively (Fig. 4b).

Responses of the Silkworm CYP and GST Genes to Quercetin. Lepidopteran CYP6 and CYP9 play an important role in xenobiotic metabolism (Berenbaum 2002, Li et al. 2007, Niu et al. 2011). Thus, those silkworm genes, highly homologous to other insects' CYP6 with quercetin-metabolizing activity (Li et al. 2004, Mao et al. 2009) and abundantly expressed in detoxification tissues of silkworm, such as midgut, fat body, or malpighian tubule (Xia et al. 2007), were selected. In addition, seven GST genes induced by multiple exogenous substances or associated with insecticide resistance have been reported (Yamamoto et al. 2009a,b, 2011; Yu et al. 2011). So, these 15 candidate genes were selected to analyze the effect of quercetin by RT-PCR. Most of the CYP and GST genes expression levels did not change except for the *CYP6AB5*, *CYP6B29*, and *GSTe8* (Fig. 5). The *CYP6AB5* was induced by 0.5% quercetin in the midgut and the whole body (long-term and short-term exposure). The expression level of *CYP6B29* increased in the whole body after long-term exposure to 0.5 and 1% concentrations of quercetin. The *GSTe8* was induced by 0.5 and 1% concentrations of quercetin in the midgut.

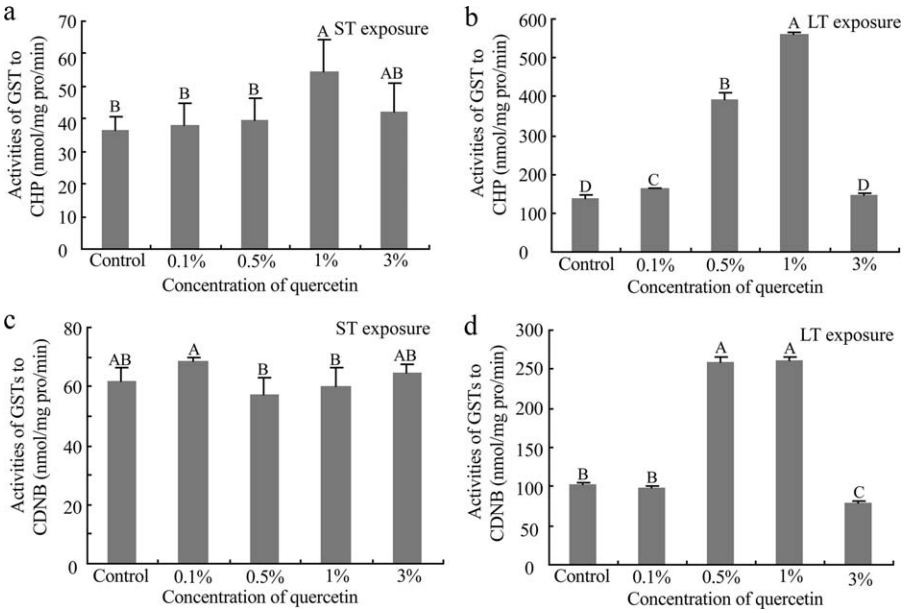

Fig. 3. Changes of GSTs activities after exposure to quercetin. GST activities toward CHP in the midgut (a and b). GST activities toward CDNB in the midgut (c and d). Letters above the columns within the same treatment that are different indicate significant differences at probability $P \leq 0.05$ using LSD test. LT, long-time (7 d); ST, short-time (3 d).

To determine the expression levels of the inducible genes, qRT-PCR was performed (Fig. 6). The results indicated that the *CYP6AB5* could be induced by 0.5% quercetin, the highest induction level was 10.9-fold relative to the control by 0.5% quercetin (midgut, long-term treatment). However, in long-term treatment at the whole body level, high concentration (1%) of quercetin inhibited the expression of the silkworm *CYP6AB5*, which was only 25% of the control. Contrarily, the silkworm *CYP6B29* could be induced only by 1% quercetin (from 1.4- to 3.2-fold) but not by 0.5% quercetin in the whole body. In the midgut, the expression of the *CYP6B29* was induced by both 0.5 and 1% concentrations of quercetin after long-term treatment, and the highest induction of the *CYP6B29* was 6.2-fold of control after exposure to 1% quercetin. For the silkworm *GSTe8*, the inducible expression could not be detected in the whole body but in the midgut, 4.7-fold by 0.5% quercetin and 7.1-fold by 1% quercetin. Totally, in the midgut of the silkworm of long-term treatment, the *CYP6AB5*, *CYP6B29*, and *GSTe8* were all induced significantly by different concentrations of quercetin. In the whole body, the change of each gene varied with the concentration of quercetin and the exposure time.

Discussion

In this study, we found that quercetin has effect on the weight gain of silkworm after long-term treatment. Usually, the mulberry leaves contain ≈0.06% quercetin (Sun et al. 2001). When we used the 0.5% quercetin to treat the silkworm, this concentration of quercetin did not inhibit the development of the silkworm. However, much higher concentrations of quercetin could obviously affect the growth of the silkworm. Thus, quercetin is also toxic to the silkworm, the silkworm might have evolved a series of mechanisms to adapt to it. After exposure to quercetin, the activities of GSTs,

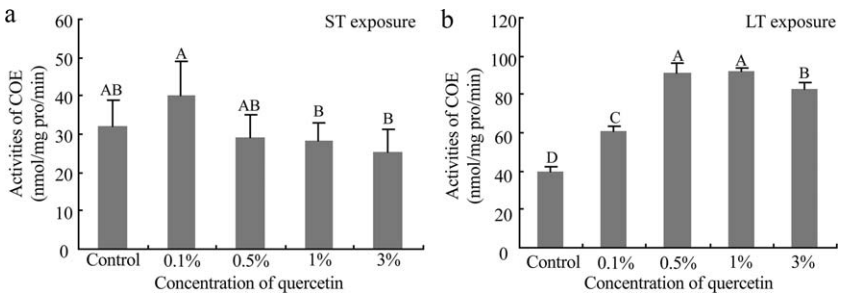

Fig. 4. Changes of COE activities after exposure to quercetin. COE activities in the midgut (a and b). Letters above the columns within the same treatment that are different indicate significant differences at probability $P \leq 0.05$ using LSD test. LT, long-time (7 d); ST, short-time (3 d).

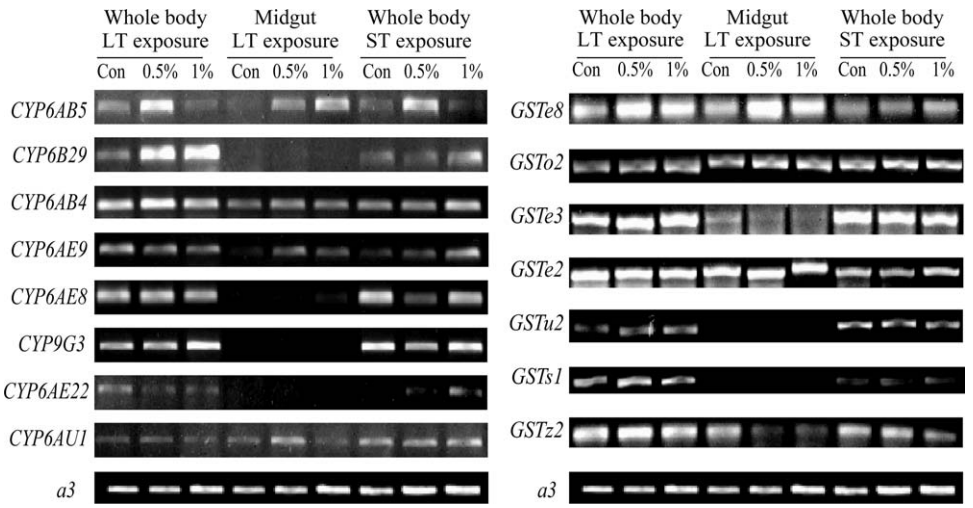

Fig. 5. Expression profiles of P450 and GST genes in response to quercetin stress. *Bmactin3* (*a3*) was used as an internal control, three replications were performed.

COE, and P450 in the silkworm were also assayed. Our data showed that the inductions of the GSTs and COE activities in the midgut after long-term exposure were higher than those after short-term treatment, while P450 activities were just the opposite in the whole body. In the midgut, the induction levels of detoxification enzymes were P450 (2.3-fold), GST activity toward CHP (4.1-fold), GST activity toward CDNB (2.6-fold), and COE (2.3-fold). The cytochrome P450 monooxygenases participate in detoxification of many toxic substances. For example, P450 detoxified alpha- and beta-thujones in *Drosophila melanogaster* (Hold et al. 2001). Thus, we speculated that these detoxification enzymes in the silkworm might participate in the transformation of quercetin and the corresponding metabolic intermediates.

The responses of eight CYP and seven GST genes to quercetin were investigated by RT-PCR and qRT-PCR. The results suggested that the silkworm *CYP6AB5*, *CYP6B29*, and *GSTe8* were significantly induced. In all treatments, *CYP6AB5* showed higher inductions in 0.5% quercetin than that in 1% quercetin (Fig. 6). On the contrary, for the *CYP6B29*, 1% quercetin showed much more inducible. In addition, the *CYP6B29* was more inducible in the whole body after short-term treatment than long-term treatment. Thus, inductions of CYP genes varied with concentration of quercetin (Fig. 6). The *GSTe8* was more inducible in the midgut exposed to 1% quercetin (7.1-fold) than 0.5% quercetin (4.7-fold), which is consistent with the change of GST activity to CHP. This suggested that the *GSTe8* might be responsible for the activity of GPx (Yu et al. 2011). In conclusion, the expression levels of the *CYP6AB5*, *CYP6B29*, and *GSTe8* were significantly induced by quercetin in the midgut of the silkworm. Because midgut is very important detoxification tissue and the first location where exogenous

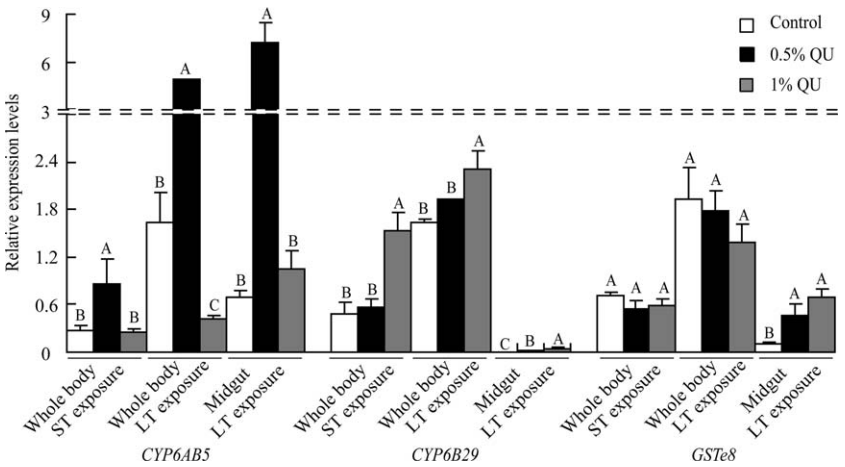

Fig. 6. The quantitative PCR analysis of the differentially expressed genes. Letters above the columns within the same treatment that are different indicate significant differences at probability $P \leq 0.05$ using LSD test. Con, control.

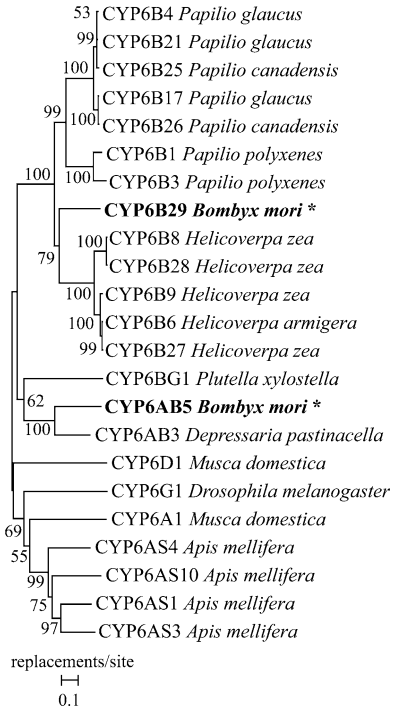

Fig. 7. Phylogenetic relationship of 23 CYP6 genes from 11 species. The phylogenetic tree was constructed in MEGA 4.0 using the neighbor-joining method. The bootstrap value indicated for each branch was percentage for 1,000 trails. The *B. mori* P450s were shown in blue.

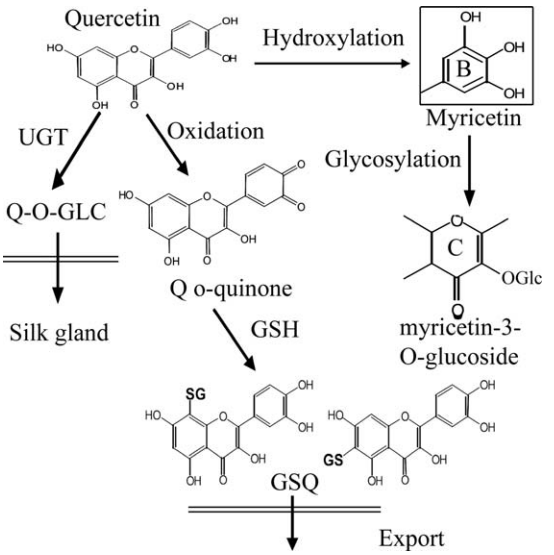

Fig. 8. Proposed metabolism mechanism of quercetin in the silkworm. The pathway was also made reference to the results of Gutzeit et al. (2005) and Boots et al. (2005).

substances are metabolized in insect (Ivie et al. 1983), the silkworm *CYP6AB5*, *CYP6B29*, and *GSTe8* might play very important roles in detoxifying quercetin.

The inductions of the detoxification enzymes in the silkworms exposed to four concentrations of quercetin were detected. The results indicated that all the activities of the P450, GST, and COE reached to the highest levels at 0.5 or 1% quercetin. Thus, 0.5 and 1% might be optimal concentrations to identify the key genes in detoxification of quercetin. At expression level, we only detected the responses of the putative detoxification genes at 0.5 and 1% quercetin. While, at higher concentration of quercetin, the detoxification enzymes were not induced and even inhibited in almost all of the treatments (Figs. 2–4). This is consistent with the results in *B. tabaci* (Mu et al. 2006). In addition, 3% quercetin could inhibit ≈50% the growth of the silkworms after long-term exposure (Fig. 1) but we found a little change in activity of enzymes investigated in this study. Currently, it is difficult to find a plausible explanation for this. Combined with the responses of detoxification enzyme genes (Figs. 5 and 6), we could speculate that those inducible detoxification genes at lower concentrations of quercetin might be inhibited at 3% level, meanwhile, some other detoxification genes might be induced. This is an interesting subject that deserves further study.

To determine the phylogenetic relationship of the CYP6 sequences, we downloaded the 23 CYP sequences related to xenobiotic metabolism in 11 insect species (Wheelock and Scott 1992; Andersen et al. 1994; Petersen et al. 2001; Li et al. 2001, 2003; Daborn et al. 2002; McDonnell et al. 2004) from GenBank (Supp. Table S3 [online only]). The phylogenetic tree was reconstructed using the neighbor-joining algorithm in MEGA4.0 with a bootstrap of 1000 replicates (Tamura et al. 2007). The obtained phylogenetic tree indicated that the *B. mori* CYP6AB5 was closely related to the *Depressaria pastinacella* (Duponchel) CYP6AB3, which shared 56% amino acid identity (Fig. 7). CYP6AB3 can be induced by furocoumarin and has the activity of metabolizing imperatorin to an epoxide metabolite (Mao et al. 2006). Thus, the *B. mori* CYP6AB5 might play important roles in detoxifications of secondary metabolites. Furthermore, the *B. mori* CYP6B29 was closely related to four CYP6B sequences of *H. zea* and CYP6B6 of *H. armigera*, which shared 58–61% amino acid identities. The study about baculovirus-mediated expression of the *H. zea* CYP6B8 proteins demonstrated that the CYP6B8 could metabolize three insecticides and six plant allelochemicals including quercetin (Li et al. 2004). Thus, we speculated that the *B. mori* CYP6B29 might be related to detoxification of quercetin.

The complex metabolism pathway of quercetin in mammals has been speculated (Gutzeit et al. 2005), but the metabolism of quercetin in insects is unclear. Daimon et al. (2010) showed that the *UGT10286* gene is the sole source of UGT activity toward the 5-*O* position of quercetin in *B. mori*, and the product of this gene increased the UV-shielding activity of cocoons. In addition, the activities of P450 and GSTs were significantly induced by quercetin. Previous study indicated that P450 might participate in the hydroxylation of flavonoids (Rice-Evans 2001). Some GST genes also showed the

activity of GPx (Parkes et al. 1993). Thus, we proposed the partial metabolism pathway of quercetin in the silkworm (Fig. 8). In the pathway, the inducible P450 and GST genes may participate in the hydroxylation of quercetin and detoxification of quinine formed by oxidation reaction of quercetin, respectively.

In conclusion, quercetin could induce the activities of GSTs, COEs, and P450s and increase the expression levels of three genes: the *CYP6AB5*, *CYP6B29*, and *GSTe8*, respectively. However, our results cannot completely rule out the possibility that other genes could be induced by quercetin. Further studies on the inducible genes in vitro are needed to help us understand how these genes detoxify quercetin and what the corresponding products are. The identification of specific genes response to quercetin would provide the foundation for further RNA interference research aimed at pest control.

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