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Detoxification Enzyme Activity and Gene Expression in Diafenthiuron Resistant Whitefly, *Bemisia tabaci*

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Abstract

B-biotype *Bemisia tabaci* has developed high levels of resistance to many insecticides. To investigate the risks and explore possible mechanisms of resistance to the diafenthiuron in *B. tabaci*, a 24.7-fold diafenthiuron resistant strain (TH-R) was established after selection for 20 generations compared with the susceptible strain (TH-S). Biochemical assays showed that the activity of cytochrome P450 towards *P*-NA was significantly higher (3.6-fold higher) in the TH-R strain than in the TH-S strain. Similarly, the carboxylesterase (COE) activity and Glutathione S-transferase (GST) activity were also significantly higher (2.55- and 1.65-fold higher, respectively) in the TH-R strain than in the TH-S strain. The expression of 5 of 7 P450 genes was significantly higher (>2.3-fold) in the TH-R strain than in the TH-S strain. The expression of *COE1* was significantly higher (>2.3-fold) in the TH-R strain than in the TH-S strain. Cytochrome P450, COE and GST appear to be responsible for the resistance to diafenthiuron in *B. tabaci*. It is also valuable for usage of insecticides for resistance management and control of this species.

Keywords: *Bemisia tabac*, resistance, diafenthiuron, *carboxylesterase* (*COE*), *glutathione S-transferase* (*GST*), P450 (*CYP6CM1*)

1. Introduction

The cabbage whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae), is an important pest that inflicts damage in temperate and tropical regions worldwide. It is a complex of biotypes that varies greatly involved in characteristics such as host range, fecundity, insecticide resistance ability to transmit plant viruses and induction of plant disorders (Dinsdale et al., 2010; De Barro et al., 2011), which has showed a strong ability to develop resistance to many insecticides including organophosphates, carbamates, pyrethroids, neonicotinoids and juvenile hormone mimics (Brown et al., 1995; Horowitz et al., 2004; Nauen & Denholm, 2005). Furthermore, mechanisms of resistance to these insecticides were also widely studied (Nauen et al., 2002; Rauch & Nauen, 2003; Karunker et al., 2008, 2009; Feng et al., 2009; Wang et al., 2010; Yang et al., 2013). However, the resistance to diafenthiuron and mechanisms for *B. tabaci* has not been reported.

Diafenthiuron is a thiourea derivative as a very useful entry in the available chemical insecticides against *B. tabaci*. It is also reported to be safe to parasitoids and predators (Ismail, 1997; Zuhua & Shusheng, 1998) and can fit in integrated pest management (Delbeke et al., 1997; Sun & Soo, 2000). So it is valuable to evaluate the risk of resistance development.

The most common reasons for insect resistance to most insecticides are enhanced metabolic detoxification and target-site insensitivity. It has been reported that the enhanced metabolism mediated by cytochrome P450 monooxygenase (P450) and glutathione S-transferase (GST) was reported to be involved in abamectin resistance (Wang & Wu, 2007). The resistance to neonicotinoid and thiamethoxam is mediated in part by cytochrome P450 (Nauen et al., 2002; Rauch & Nauen, 2003; Feng et al., 2009, 2010) In *B. tabaci*, a specific cytochrome P450, CYP6CM1 was involved in the resistance to imidacloprid and could metabolize this insecticide in vitro

(Karunker et al., 2008, 2009). Another cytochrome P450 gene, *CYP6CX1*, was putatively involved in imidacloprid resistance in a field population of *B. tabaci* (Zhuang et al., 2011). Furthermore, increased cytochrome P450 indicated resistance to both imidacloprid and thiamethoxam (Bass et al., 2011). Alon et al. (2008) found that organophosphate resistance in *B. tabaci* is related to a point mutation in an ace1-type acetylcholinesterase and overexpression of *COE1*.

In our study, we reared the TH-R and TH-S strain on cabbage, and then studied the biochemical characteristics and gene expression profiles of their metabolic detoxification enzymes, including carboxylesterase (COE), GST and cytochrome P450. It appears that the increased enzyme activity and multiple up-regulated COE, GST and cytochrome P450 genes in the resistant strains that may be responsible for the development of resistance to insecticide. At the same time, it also provides the theoretical basis for a better understanding of the mechanism of resistance in B-biotype *B. tabaci*, and rational usage of insecticides for resistance management and control.

2. Materials and Methods

2.1 Insect Materials

Two B-type *B. tabaci* strains, the TH-R strain and the TH-R strain, were used in the experiments and reared as previous described (Feng et al., 2009; Xie et al., 2012). The TH-R strain exhibited over 24.7-fold resistance to diafenthiuron in comparison with the TH-S strain. Samples were collected from both strains at the following life stages: one-day-old unmated adults. Samples were frozen at -80 °C and stored for downstream RNA work.

2.2 Bioassays

A leaf-dip bioassay was used according to the methods of Feng et al. (2009) with slight modifications. The diafenthiuron was dissolved and diluted with distilled water. Leaf discs (22 mm in diameter) from cabbage plants (medium-sized leaves) were dipped in the insecticide solutions for 10 s. After drying, the leaf discs were placed with their adaxial surface downwards onto a bed of agar (2 mL of 15 g L^{-1}) in a flat-bottomed glass tube (78 mm in length). Control groups were treated with distilled water applied to leaf discs in the same manner as the treatment groups. Each tube contained about 30 unsexed whitefly adults (within 5 days after eclosion) and was placed in an incubator at 25 °C with a 14:10 h light:dark photoperiod. Mortalities were recorded after 48 h. Each treatment included four replicates, using 5–7 concentrations based on preliminary data.

2.3 Insecticides and Chemicals

Diafenthiuron (98.1% technical crystal) was obtained from Foshan entry-exit inspection and quarantine. Fast Blue B and Coomassie Brilliant Blue (G-250) were purchased from Sigma Aldrich. Bovine serum albumin (BSA), glutathione reduced (GSH) (> 99.5%) and NADPH (tetrasodium salt) (> 98%) were obtained from Roche Diagnostics (Indianapolis, IN), EDTA (> 99%) was obtained from Amresco Inc., 1,4-dithioerythritol (DTT, > 99%) was obtained from Merck Chemicals Ltd and phenylmethanesulfonyl fluoride (PMSF) was obtained from BBI (Blockbuster Inc.). 1-Naphthyl acetate (α-NA), 1-naphthol, 4-nitroanisole (*p*-NA), p-nitrophenol (*p*-nP), 1-chloro-2,4-dinitrobenzene (CDNB). TRIzol reagent was purchased from Invitrogen (USA). Taq DNA polymerase and DNA Marker DL 2000 were purchased from Sangon Company (Shanghai, China). Agarose, DNase I and SYBR Green I were purchased from TaKaRa (Dalian, China).

2.4 Metabolic Enzyme Assays

COE activity was determined using *α*-naphthyl acetate as the substrate by measuring the OD_{600} according to the method of Van AsPeren et al. (1962) and Feng et al. (2009) with slight modification. 100 adults were homogenised in 0.2 M phosphate buffer (pH 6.0). The homogenate was centrifuged at 4 °C and 10800 × *g* for 15 min, and then the supernatant was used as the enzyme source. A standard curve was prepared with 1-naphthol. The reaction mixture (6 mL) included: *α*-NA (0.3 mM), eserine (0.1 mM), phosphate buffer (0.04 M, pH 7.0) and enzyme solution (0.1–0.5 mL).

GST activity was determined using GSH and CDNB as substrates according to Wu and Miyata (2005) with slight modification. 150 adults were homogenised in 4 mL of 0.05 M Tris–HCl buffer (pH 7.5). The homogenate was centrifuged at 4 °C and 10800 × g for 15 min, and then the supernatant was used as the enzyme source for the GST activity assay. The reaction mixture consisted of GSH (50.0 mM), CDNB (30.0 mM), and enzyme solution (0.2 mL). Changes in absorbance values were recorded at 340 nm for 5 min using a spectrophotometer (Unico, Shanghai, China).

Cytochrome P450 activity was assayed according to the method described by Yu and Nguyen (1992) and Feng et al. (2009) using *p*-nitroanisole as substrate with slight modification. 300 adults were homogenised in sodium phosphate buffer (0.1 M, pH 7.5, 1 mM of EDTA, 0.1 mM of DTT, 1 mM of PTU and 1 mM of PMSF). The

homogenates were centrifuged at 10800 × g at 4 °C for 15 min, and the supernatants were treated at 10800 × g at 4 °C for 20 min and used as the crude enzyme source. The reaction mixture consisted of *p*-nitroanisole (2 μM), NADPH (9.6 mM) and enzyme sources. The mixture was incubated at 34 °C in an air atmosphere for 30 min. The OD_{405} value was recorded, and the quantity of the product was determined from a *p*-nitrophenol standard curve; the substrate (0.02 mM) was dissolved in the same buffer used for the PNOD activity measurement. The reaction was started by adding NADPH (10 mM; 10 μL), and the reaction was followed at 34 °C for 1 min with a spectrofluorometer. The wavelengths for excitation and emission were 380 and 460 nm, and the slits were 5 and 10 nm respectively.

2.5 Primer Design

The primers for Quantitative Real-time PCR (qRT-PCR) were designed using primer3 input (http://primer3.ut.ee/). The primers were designed on the basis of sequences published in NCBI. The primers used were shown in Table 1.

Table 1. Sequences of primers used for the relative expression of the target genes from *B. tabaci*

Gene Name (Accession. No)	Primer sequences (forward/reverse)	Tm (°C)	Product length (bp)
EF1α	F: TAGCCTTGTGCCAATTTCCG	55.4	110
(EE600682)	R: CCTTCAGCATTACCGTCC	56.0	
COE11	F：CCATGCTGAGTTTCCAACCC	57.5	86
(EF675184)	R：TGTGCATGTCAATAGCTGCC	55.4	
GST	F：GTGGAGGAAAAACACCCTCA	55.4	97
(EU723684)	R：AGTCGGTTTTTGGCCTCTTT	53.4	
CYP6CM1	F：CATCAAAGACCTCGTGAGCA	55.4	128
(GQ214539)	R：TGCACAGCTTTCCTCATCTG	55.4	
CYP6CX1	F：CATCAAAGACCTCGTGAGCA	55.4	114
(GQ330542)	R：TGCACAGCTTTCCTCATCTG	55.4	
CPP6CX4	F：TGTCTGCCATCACCACATTT	51.3	113
(JN165265)	R：CTTAATTTCGCCGCATGATT	55.4	
CYP6DW3	F：ATCCACGACGCTTGCTACTT	55.4	88
(JN165261)	R：CAAGACCCGTTCGATTTCAT	55.35	
CYP6DZ6	F：TGCGTTCATTTTCTTCATCG	51.3	123
(JN165260)	R：TTCACGGATTCCACCTCTTC	55.4	
CYP6A	F：TCGGAAACTACGCGGAAGTA	55.4	101
(EU723677)	R：AGACCTAGAACGGGAATGCA	55.4	
CYP9F	F：CTGTTCTGGGAAGACGTTGC	57.5	114
(EU723679)	R：ATTGCGGGTCATGATGGAGA	55.4	

2.6 Total RNA Isolation and Reverse Transcription

Total RNAs were extracted from about 30 adults of *B. tabaci* using a Trizol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. The resulting total RNA was resuspended in nuclease-free water and was quantified on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). First-strand cDNAs were synthesized with 1.0 μg μL^{-1} total RNA using the PrimeScript® RT reagent kit (Takara Biotech, Dalian, China) according to the manufacturer's protocol.

2.7 Quantitative Real-Time PCR (qRT-PCR)

The relative expression levels of *CYP6CM1*, *CYP6CX1*, *CYP6CX4*, *CYP6DW3*, *CYP6DZ6*, *CYP6A*, *CYP9F*, *COE1*, and *GST* from the adults of *B. tabaci* were examined using qRT-PCR. Each reaction was performed in a

20 μL mixture containing 1 μL of cDNA, 10 μL of SYBR Green qRT-PCR SuperMix-UDG, 0.15 μL of each primer, and 8.7 μL of H_2O. All samples, including a 'no-template' negative control, were performed in a 20 μL mixture containing 1 μL of cDNA, 10 μL of SYBR Green qRT-PCR SuperMix-UDG, 0.15 μL of each primer, and 8.7 μL of H_2O. The amplification efficiency of the target genes and housekeeping gene (*EF-1α*) were estimated using $E = 10^{-1}/\text{slope} - 1$, where the slope was derived from the plot of the cycle threshold (C_t) value versus the log of the serially diluted template concentration. The optimised qRT-PCR program consisted of an initial step at 50 °C for 2 min, 94 °C or 2 min, followed by 50 cycles of 94 °C for 15 s and 60 °C for 30 s. After the cycling protocol, melting curves were obtained by increasing the temperature from 60 to 95 °C (0.2 °C·s^{-1}) to denature the double–stranded DNA. The qRT-PCR amplifications were carried out in 96-well plates. The assays were run in an ABI 7500 system using the SDS v.1.4 application software (Applied Biosystems). Quantification of the transcript level of 9 detoxification genes mRNA was performed using the comparative $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

2.8 Statistical Analysis

Data statistics and bioassay analysis were performed on Excel (2010) and PoloPlus. Statistical analysis was performed by one-way ANOVA and Tukey's test ($P < 0.05$) to compare the expression levels of *COE1*, *GST* and cytochrome P450 genes by using the software InStat v.3.0 (GraphPad Software, San Diego, CA).

3. Results

3.1 Determination of Resistance Levels to Insecticides in B. tabaci

To investigate the risks and to determine possible mechanisms of resistance to diafenthiuron in *B. tabaci*, The TH-R strain was selected in the laboratory. A 24.7-fold TH-R strain was established after selection for 20 generations compared with the TH-S strain (Table 2).

3.2 COE Activity, GST Activity and Cytochrome P450 Activity in the TH-R and TH-S Strain

CarE activity was 2.55-fold higher for the TH-R strain than for the TH-S strain (Figure 1A). GST activity was 1.65-fold higher for the TH-R strain than for the TH-S strain (Figure 1B). Cytochrome P450 activity towards PNA was 3.56-fold higher for the TH-R strain than for the TH-S strain (Figure 1C). Cytochrome P450 activity towards *P*-NA was highest for the TH-R strain than for the TH-S strain in the three of them.

3.3 Gene Expression Profiles for COE, GST and Cytochrome P450 Genes in the TH-R and TH-S Strain

To investigate the expression profiles of putative detoxification genes (one GST, one COE1 and 7 cytochrome P450 genes) in the TH-R and TH-S strain, qRT-PCR analyses were used. The relative expression of all P450 genes in the TH-R strain relative to the TH-S strain is shown in Figure 4. Of the7 P450 genes, 2 (*CYP6CM1* and *CYP6A*) had > 1-fold higher expression in the TH-R strain than in the TH-S strain, five (*CYP6CX1*, *CYP6CX4*, *CYP6DW3*, *CYP6DZ6*, and *CYP9F*) had > 2.3-fold higher expression in the TH-R strain than in the TH-S strain. In addition, the expression level of *GST* was 2.1-fold higher in the TH-R strain than in the TH-S strain (Figure 2), while the expression of *COE1* did not significantly differ between the TH-R strain and the TH-S strain (Figure 3).

Table 2. Toxicity of detoxification to *B. tabaci* strains

Strains	Slope ±S.E	LC_{50} (μg.mL^{-1}) (95% FL)	χ^2 (df)	RF
TH-S	2.71±0.27	12.43 (10.15-15.30)	23.43 (17)	1
TH-R	3.12±0.30	307.47(249.81-378.91)	28.87 (17)	24.73

Note. Resistance Factor (RF) = LC_{50} of the TH-R strain / LC_{50} of the TH-S strain.

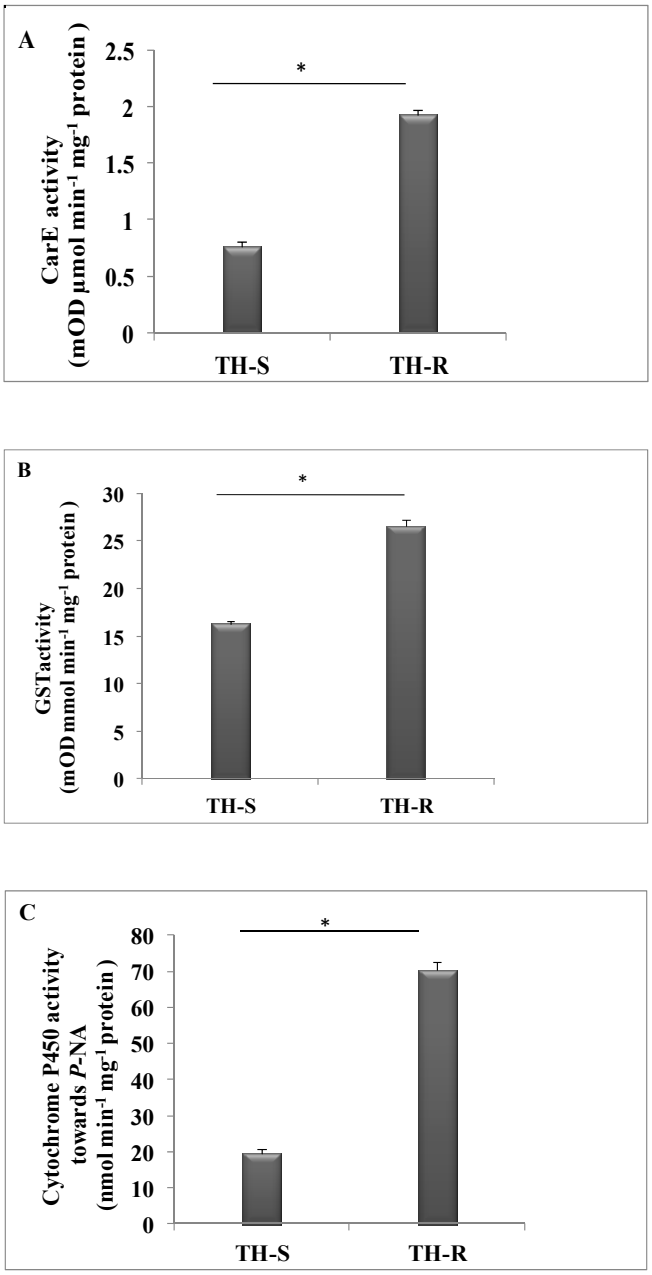

Figure 1. Activities of detoxification enzymes of the TH-R strain and the TH-S strain. (A) COE; (B) GST; (C) cytochrome P450 towards *P*-NA. Significant differences are indicated by an asterisk (one-way ANOVA, $P < 0.05$; LSD test)

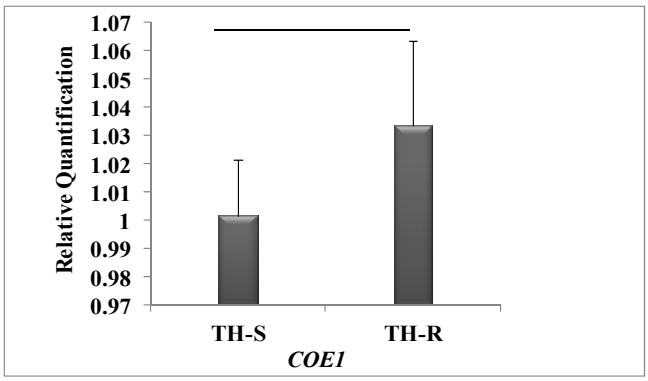

Figure 2. Expression profiles of *COE1* in the TH-R strain relative to the TH-S strain. Significant differences are indicated by an asterisk (one-way ANOVA, $P < 0.05$; LSD test)

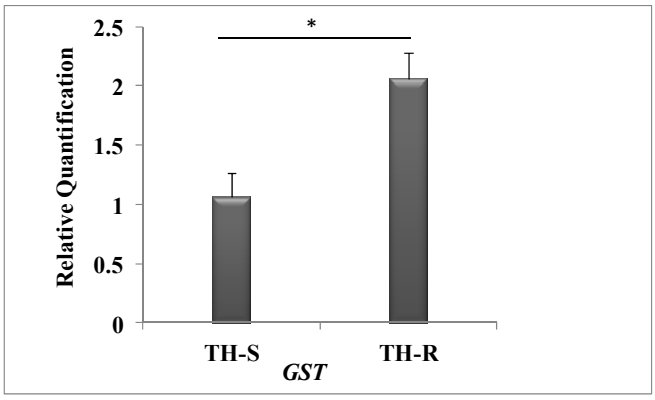

Figure 3. Expression profiles of *GST* in the TH-R strain relative to the TH-S strain. Significant differences are indicated by an asterisk (one-way ANOVA, $P < 0.05$; LSD test)

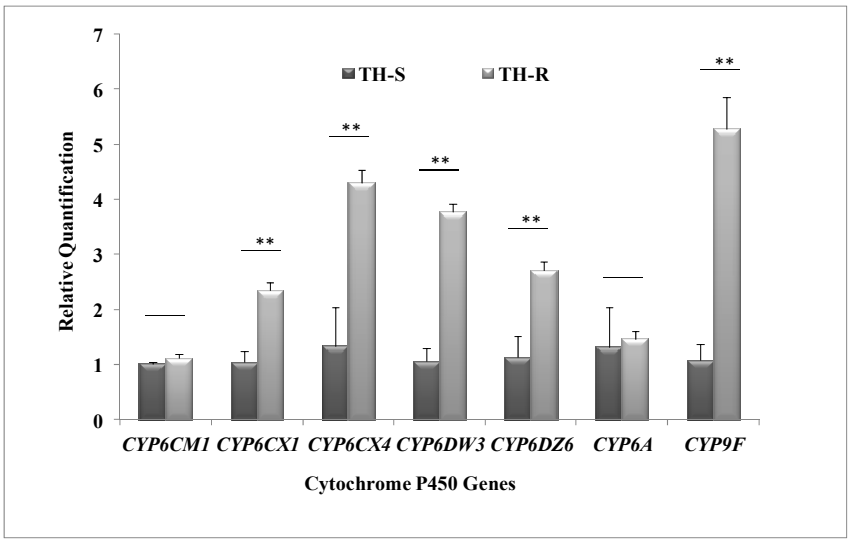

Figure 4. Expression profiles of P450 genes in the TH-R strain relative to the TH-S strain. Significant differences are indicated by an asterisk in the same gene (one-way ANOVA, $P < 0.05$; LSD test)

4. Discussion

B. tabaci has developed high resistance to some commonly used insecticides (Kady & Devine, 2003; Roditakis

et al., 2005; Ahmad, 2007). Typically, enhanced metabolic detoxification among insects may contribute to insecticide resistance (Low et al., 2007), the metabolic detoxification system in insects consists of three major groups of enzymes. The phase I detoxification enzymes, acting on a broad range of substrates directly to reduce their toxicity, are represented by cytochrome P450. The phase II enzymes, including GST, UDP-glucuronosyltransferases (UGTs), and COE, facilitate the excretion of hydrophobic toxic compounds by improving their hydrophilicity. Many studied indicated that increased levels of detoxification gene expression are known to result in increased levels of detoxification enzymes and detoxification enzymes activities that are responsible for insecticide resistance (Liu & Scott, 1998; Liu et al., 2011; Schule, 2011; Gong et al., 2013; Zhang et al., 2015).

The biochemical characteristics and gene expression profiles of detoxification enzymes in the TH-R and TH-S strain were compared in the present study, respectively, as enhanced metabolic detoxification often contributes to insecticide resistance among insects (Li et al., 2007). The activity of cytochrome P450 towards *P*-NA was significantly greater in the TH-R strain than in the TH-S strain in our study. This is somewhat consistent with that the major mechanism in all samples investigated so far has appeared to be enhanced detoxification by cytochrome P450 (Rauch & Nauen, 2003). Furthermore, the cytochrome P450 system has been previously reported to be involved in the resistance to many insecticides in many insects, including abamectin resistance in *Leptinotarsa decemlineata* (Argentine et al., 1992), *Tetranychus urticae* (Stumpf & Nauen, 2002) and *B. tabaci* (Wang & Wu, 2007), permethrin resistance in *Culex quinquefasciatus* (Liu et al., 2011; Gong et al., 2013), and imidacloprid resistance in *Nilaparvata lugens* (Bass et al., 2011). In particular, the resistance of *B. tabaci* to imidacloprid was strongly related to enhanced detoxification by cytochrome P450 (Nauen et al., 2002; Feng et al., 2010), and cytochrome P450 activity was significantly greater in the resistant strains than in the corresponding susceptible strains (Rauch & Nauen, 2003). These are consistent with our results that cytochrome P450 may be the major mechanism in all detoxification enzymes investigated in *B. tabaci*.

In addition to comparing enzyme activities in the TH-R strain and the TH-S strain, the expression profiles of 7 cytochrome P450 genes were also compared. Expression levels for *CYP6CX1*, *CYP6CX4*, *CYP6DW3*, *CYP6DZ6*, and *CYP9F* were significantly higher in the TH-R strain than in the TH-S strain. In addition, though the other two cytochrome P450 genes (*CYP6CM1* and *CYP6A*) were not overexpressed in the TH-R strain, they could be significantly induced in the TH-R strain or the TH-S strain, respectively (data not shown). In previous studies, Rauch and Nauen (2003) and Cahill et al. (1996) reported that enhanced metabolic detoxification resulting from increased expression of cytochrome P450 is a major factor resulting in pest tolerance to neonicotinoid insecticides. Cytochrome P450s metabolize endogenous compounds, and the CYP6 family has been implicated in insecticide resistance more than any other cytochrome P450 families (Pavek & Dvorak, 2008; Scott, 2008; Puinean et al., 2011). For example, *CYP6CM1* and *CYP9F* have also been reported that linked with imidacloprid resistance in whitefly (Karunker et al., 2008; Qiu et al., 2009), *CYP6CX1* exhibited significantly higher mRNA in imidacloprid and thiamethoxam resistance in whitefly (Zhuang et al., 2011; Yang et al., 2013). These findings are somewhat consistent with that *CYP6CM1*, *CYP6CX1*, and *CYP9F* may be not only involved in imidacloprid and thiamethoxam resistance, but also involved in diafenthiuron resistance in whitefly. In the present study, expression levels of *CYP6DW3*, *CYP6DZ7*, and *CYP6CX5* were significantly greater in *B. tabaci* Q than in B, suggesting that the enzymes encoded by these genes may contribute to the different sensitivities to insecticides (Guo et al., 2014). In our study, the overexpression of *CYP6DW3*, *CYP6DZ6*, and *CYP6CX4* in the TH-R strain was also observed, suggesting that multiple cytochrome P450s were involved in insecticide resistance. At the same time, the target-site modifications and enhanced detoxification for imidacloprid resistance have been identified. Studies have indicated that a target-site mutation, Y151S, within the nAChR N1 α1 and N1 α3 subunits, which was involved in imidacloprid resistance in the laboratory-selected brown planthopper (Liu et al., 2005).

In addition to cytochrome P450, GST and COE could also be involved in the detoxification of allelochemicals (Karunker et al., 2008). Enhanced expression of GST has been shown to be a mechanism of resistance to DDT and organophosphates, and has also been implicated in resistance to pyrethroids in some insects (Huang et al., 1998; Ranson et al., 2001; Vontas et al., 2001). GST activity and single *GST* expression were significantly higher in the TH-R strain than in the TH-S strain in the present study. The involvement of this GST in the resistance of the TH-R strain to diafenthiuron clearly warrants further investigation, including functional validation of the ability of this enzyme to metabolize diafenthiuron and/or its primary metabolites.

Organophosphate resistance in the B-biotype of *B. tabaci* is associated with a point mutation in an ace1-type acetylcholinesterase and overexpression of COEs (Alon et al., 2008). *COE1* displayed a higher level of expression in thiamethoxam resistance in B-biotype *B. tabaci* by Yang et al. (2014). COE activity was

significantly higher in the TH-R strain than in the TH-S strain in our present study. Although *COE1* was not difference between the TH-R strain and the TH-S strain, significant induction by diafenthiuron was observed. Further evidence was showed that the activities of COE of the resistant strain increased significantly (Rauch & Nauen, 2003). Alon et al. (2008) also showed that *COE1* and another *COE* displayed a higher level of expression, whereas, only a two-fold increase in expression of *COE2* in the resistant strain compared to the susceptible strain. This was consistent with some of our results and those of previous studies. These findings strongly suggest that COE and cytochrome P450 were responsible for diafenthiuron resistance.

5. Conclusions

We have shown that selection of the TH-R strain with diafenthiuron over many generations has induced significant changes in activities and gene expression of detoxification enzymes, indicating that enhanced detoxification by cytochrome P450, COE and Glutathione GST are mainly responsible for this resistance. The target insensitivity or inheritance patterns of diafenthiuron resistance in *B. tabaci* will also require additional research in order to confirm the resistance mechanism and to establish more efficient management strategies for this pest.

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