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# Cytochrome P450-mediated detoxification involves in phosphine resistance mechanism in the red flour beetle of *Tribolium castaneum*

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**ABSTRACT**

Cytochrome P450-mediated detoxification is one of the most important mechanisms involved in insecticide resistance. High levels of inheritable resistance to phosphine in *Tribolium castaneum* have been detected. There is a paucity of reports on the molecular mechanism, especially on the role of P450-mediated detoxification, in phosphine resistance of stored product insect pests. A high-level phosphine resistant strain of *T. castaneum* was screened (resistance factor = 724). PBO synergistic ratios of resistant strains were low (1.77-3.81x). But P450 reductase and cytochrome b5 contents in resistant strains were significantly higher than that of a susceptible strain at 10d, 20d larval and adult (3-4 d of age) stages ($P<0.05$). The concentrations of P450 reductase and cytochrome b5 were positively correlated with $LC_{50}$. P450 associated genes CYP4Q4 and CYP4Q7 were overexpressed in the midgut of resistance strains, while CYP similar gene was overexpressed in the fat body of resistant strains ($P<0.01$).Oxidative metabolism could be involved in phosphine resistance of *T. castaneum* as high levels of P450 reductase and b5 were detected in resistant strains compared to a susceptible strain. Furthermore, qPCR revealed that P450 associated genes, CYP4Q7, CYP4Q4 and CYP, were up-regulated in the phosphine resistant strains. The study provides the first direct evidence that cytochrome P450 monooxgenases and the over-expression of its related genes are one of metabolic mechanism responsible for phosphine resistance in *T.castaneum.*

**Key words**: Phosphine resistance, *Tribolium castaneum,* P450 Monooxygenases, CYP4Q4, CYP4Q7, CYP similar gene.

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## INTRODUCTION

Phosphine fumigation is a popular practice against stored product pests for a variety of commodities in large and small storage facilities in the past four decades(Wang et al., 2006). It became a much more important fumigant as the international community reached an agreement to phase out the use of methyl bromide by 2015 (UNEP 1995). Because of its popularity and improper practices such as less perfect air-tight conditions resulting in more frequent applications, insect pests have developed genetic resistance to phosphine over the past few decades (Daglish 2004; Pimentel et al., 2008; Benhalima et al., 2004). The highest resistance level reported is over 1000-fold and greatly threatens the future of phosphine as a fumigant(Pimentel et al., 2010; Campbell et al., 2010).

Red flour beetle, *Tribolium castaneum* (Coleoptera: Tanebrionidae) is a major cosmopolitan pest of stored agricultural products worldwide causing damage to flour, cereal, beans, and nuts, etc(Weston and Rattlingourd. 2000). The management of red flour beetle heavily depends on synthetic insecticides and the insect has been reported to resistant to all five classes of insecticides and fumigants used against it in many geographical areas(Athie

and Mills 2005). The development of high-level resistance in many stored product insects now threatens its continued use. With complete genome sequencing and its ability to develop resistance rapidly, *T. castaneum* has become an ideal model organism for research on insecticide resistance(Collins 1990; Zhu et al., 2010; Jeremy et a., 2009).

*T. castaneum* and the lesser grain borer, *Rhyzopertha dominica* has confirmed that strong resistance is primarily conferred by two major genes, rph1 and rph2(Jagadeesan et al.,2012; Collinset al., 2002; ). Dihydrolipoamide dehydrogenase (dld), a gene encoding a core metabolic enzyme, has been identified as rph2(Schlipalius et al., 2012)in both *R. dominica* and *T. castaneum* and a free soil living nematode *Caenorhabditis elegans*. A single amino acid mutation in DLD in populations of *T. castaneum* and *R. dominica* with strong resistance was identified as P45S in *T. castaneum* and P49S in *R. dominica*, both collected from northern Oklahoma, USA(Chen et al., 2015). Two loci, tc_rph1 on chromosome 8 and tc_rph2 on chromosome 9 of *T. castaneum* were identified by regional SNP frequency and they are together responsible for high level resistance against phosphine. Next-generation sequencing technology were carried out to identify the candidate genes for phosphine resistance of *T. castaneum* and found that there are two genes(TC006822 and TC006823) are actually the first and last exons of the dihydrolipoamide dehydrogenase (dld) (tc-rph2) gene. In addition, there were two genes (TC006231 and TC006232) that have strong homologies to genes of known cytochrome b5 related function and one gene (Tc06829) has strong homology to cytochrome P450(Jagadeesan et al.,2013). The possible role of cytochromes P450 in the toxicity or detoxification of phosphine had been studied(Schlipalius et al., 2008; Pratt. 2003) . However, the results of different treatment had no consistent conclusion and therefore it is appears to be unlikely that the microsomal mixed function oxidases(MFO) play any significantly role in the toxic action of phosphine in insects(Nisa et al., 2011).

Cytochrome P450 monooxygenases were confirmed be a major metabolic factor responsible for pyrethroid and other organophosphates insecticide resistance(Schenkman & Jansson. 2003). Up-regulated P450 super-family gene (s) expression has been proved to be closely associated with resistance to pyrethroid insecticides in many insects, e.g. over expression of CYP6D123 and CYP6A3624 in resistant strains of houseflies(Zhu et al., 2008) and CYP6F1 in *Drosophila melanogaster* (Daborn et al., 2007). Studies had showed that gene CYP4Q4 (AY337337) and CYP4Q7 (AF254755) were related to pyrethroid resistance in *T. castaneum*(Assie et al., 2007). And CYP similar gene has be indicated in National Center for Biotechnology Information (NCBI) to be a cytochrome P450 related gene. We speculate that the toxicity of phosphine would be key to understanding the toxicity of phosphine and cytochrome P450-mediated detoxification may involve in phosphine resistance mechanism. Using three genes CYP4Q4, CYP4Q7 and CYP similar gene as model.The purpose of this study was to warranty if cytochrome P450-mediated detoxification involve a potential phosphine resistance mechanism in *T. castaneum*

Cytochrome P450 enzyme function and completion of catalytic cycle requires the delivery of two electrons: NADPH: cytochrome P450 reductase (EC 1.6.2.4; hereafter “P450 reductase”) (Feyereisen. 2005) and cytochrome b5 (hereafter b5) (Schenkman. & Jansson 1998). A number of studies have suggested that the interactions of P450 reductase and b5 in P450 reaction played an important role in cytochrome P450-mediated detoxification insecticide resistance(Murataliev et al.2008 )

The objectives of this study were: (1) artificially screened for high-level phosphine resistant strain of *T. castaneum*; (2) determined the resistant levels of three available resistant strains and the synergistic effect of piperonyl butoxide (PBO); (3) quantified the content of P450 reductase and cytochrome $b_5$ of susceptible and resistant strains at 4 developmental stages (10 d and 20 d old larva, pupa and adult); (4) cloned and expressed three P450 associated genes. The information shade light on understanding the molecular mechanism of phosphine resistance in *T. castaneum* and in insects in general.

## MATERIALS AND METHODS

2.1. Insects

The strains of *T. castaneum* used in this study included a phosphine-susceptible strain (AK), obtained from Department of Primary Industries and Fisheries, Queensland, Australia, two phosphine resistant strains, SQ($F_0$) and SQ($F_2$), collected in a wheat warehouse in Shandong Qihe National Storage Facility (Qihe, Shandong, China 37.26°N, 116.17° E), and a high-level resistant strain, SQ($F_4$), obtained by laboratory screening from SQ(F2) in this study. All strains were reared on organic wheat flour containing 5% yeast under 30ºC, 70% RH and 12 L: 12D.

2.2. Chemicals

Phosphine gas (*ca*. 86% pure) was produced using aluminum phosphide (0.6 g pellets, Jiangsu Shuanglin Chemical Industry Company) and acidified water (sulfuric acid 5%)[31]. Other chemicals used including piperonyl butoxide (PBO) (94%, Sigma®, USA) and Coomassie brilliant blue G-250 (Alfa Aesar®). Disodium hydrogen phosphate ($Na_2HPO_4$, >99%), sodium dihydrogen phosphate ($NaH_2PO_4$, >99%) and sodium hyposulpdit ($Na_2S_2O_4$, > 90%) were purchased from Tianjin Kemio Chemistry Ltd. (Tianjin, China). BSA (albumin bovine serum) and DTT (1,4-dithiothreitol, > 97 %) were purchased from Sangon Biotech Co. Ltd. (Shanghai, China). CO (>99.9%) was from Shanghai Shengkai Gas Ltd. (Shanghai, China).

**2.3. Fumigation bioassay of phosphine resistance and PBO synergism**

Phosphine resistance levels in adults were determined using FAO recommended discriminating concentrations and method[17] under 30 ±1ºC and RH 70 ±5%. Fumigation process was performed using air-tight desiccators. Within each desiccator there were 3 fumigation cages (replications) each contained 50 healthy unsexed adults (14 d old). The insects were acclimated in the desiccators for 12 h before phosphine gas was injected with air-tight syringes to create the desired concentrations. Uniform distribution of phosphine within a desiccator was achieved by mixing with a magnetic mixer for 2-3 min. After 24 h of fumigation, the insects were transferred from fumigation cages to petri dishes (diameter 11cm) with organic wheat flour as a food source and kept under 32±1ºC and RH 70±5%. Mortalities were checked 14d after fumigation. The insects were considered dead if no movement of any body parts was observed after touched by a fine brush. There were 5 phosphine concentrations and a blank control. The mortality was assessed each 12h after phosphine treatments and was less than 6% in control. The lethal concentrations ($LC_{50}$ and $LC_{90}$) using SAS Proc Probit Program.

The PBO synergism was evaluated by topically applying 0.2 µg/insect of PBO (in acetone) 1 h before subjected to the fumigation resistance bioassay. The synergistic factors (SF) were calculated as the ratio of $LC_{50}$ of treatments with insecticide alone to the $LC_{50}$of treatment with the synergist and insecticide. An analysis of variance (ANOVA) was carried out on the biochemical data and comparisons were made between the means using the PLSD Fisher test using the Statview software program.

2.4. Resistance strain screening

Phosphine resistance was artificially selected from SQ($F_2$) strain. One thousand unsexed adults (45-52 d old) were fumigated at the $LC_{90}$ concentration for 24 h in the same manner as described above. After aeration, the adults were reared under the conditions described above for 14 d. The surviving adults were transferred into a new culture bottle and allowed to stay for 7 d to oviposition. After the adults of the $F_1$ generation reached the age of 45-52 d, the fumigation was carried out again. A high-level resistant strain SQ($F_4$) was obtained after screening for 7 generations as determined by the resistant bioassay described above.

2.5. Detection P450 reductase and cytochrome $b_5$ contents

Microsomal fractions of 10 d, 20 d old larvae, 3-4 d old pupae and 4-7 d old adults of different strains were prepared according to Masaphy (1996)[32]. Briefly, 120 individuals of each developmental stages were pooled, the surface was washed with 0.9% sodium chloride solution. After frozen at -20 ºC for 1h the insects were homogenized in ice-cold phosphate buffer (0.1M, pH 7.6) containing 1mM EDTA, 0.1mM DTT, 1mM PTU, 1mM PMSF and10% glycerol. The homogenates were filtered with four layers of medical gauze and the filtrated fluids were centrifuged at 4°C and 10000g for 20min. The supernatants were filtered again by two layers of medical gauze and the filtrated fluids were centrifuged at 4°C and 105000 g for 60 min in a Hitachi CP100MX ultra-speed refrigerated centrifuge to pellet the microsomal fraction. The total protein content of the pellets (extracts) was assayed by Bradford method[33] using a standard curve generated from bovine serum albumin.

P450 reductase and Cytochrome $b_5$ in the microsomal fractions were quantified in triplets using reduced carbon monoxide (CO) difference spectroscopy (DU733 Beckman, U.S.A.)[34]. The extracts were diluted to around 1 mg/mL suspension. Three mg of solid sodium dithionite was added to 10 ml of the suspension and reacted for 5 min. The resulted solution was divided into two cuvettes, one as sample and the other as reference. Using the reference cuvette as a blank, the sample cuvette was scanned between 400 to 500 nm at 60 nm/min speed to achieve a stable base line. Then, CO gas (99.9% purity) was lightly bubbled into the sample cuvette for 1 min and scanned again. The maximum and minimum absorbance was recorded at around 450 nm and 490 nm, respectively. P450 content was calculated as nmol.$mg^{-1}$ microsomal protein using the extinction coefficient of 91 $mM^{-1}cm^{-1}$ and the absorbance difference between the maximum and the minimum.Cytochrome $b_5$ was quantified before P450 reductase in the

similar way without CO bubbled into the samples . The content of $b_5$ was determined as nmol.mg$^{-1}$ microsomal protein using the extinction coefficient of 185 mM$^{-1}$ cm$^{-1}$ and the absorbance difference between 420 and 409 nm.

Enzyme activity of different strains was analyzed with one-way ANOVA followed by Turkey's HSD test at $P<0.05$ for mean comparisons.

**2.6 mRNA expression of CYP4Q7, CYP4Q4, and CYP similar genes**

Midgut and fat body tissues were dissected on ice from 15-20 larvae (20 d old) of one susceptible (AK) and two resistant strains, SQ($F_0$) and SQ($F_4$). Three replicate samples were prepared from each strain. Total RNA extraction, RT-PCR and SYBR real-time quantitative PCR (qPCR) were conducted following the procedures described in Li et al. (2009)[22] with some modifications. Total RNA was extracted using TRIZOL reagent (Invitrogen Co., San Diego, CA, USA). First-strand cDNA was synthesized from total RNA using Prime Script$^{TM}$ RT-PCR reverse transcriptase with oligo $dT_{18}$ primer (TARAKA, Japan) under programmed reaction conditions of 5 min at 65°C, 30 min at 42°C, and 5 min at 95°C. The primer sequences for PCR amplification were given in Table 1. Primers specific to 18sRNA (internal control gene) and genes encoding CYP4Q7, CYPQ4 and CYP similar genes were designed based on the sequences in *Tribolium* genome (GenBank: FM877879, AF254755 and FJ209361) using Beacon Designer 7.0 (Premier Biosoft International, Palo Alto, CA, USA). qPCR was performed in a total reaction volume of 25 µl containing 12.5 µl of 1×SYBR GREEN PCR mix (TaKaRa Code: RR041A), 1µl 1:10 diluted cDNA templates, 1µl sets of each primer and 9.5µl Rnase free dd water according to the manufacturer's instructions. Reaction conditions were as follows: 94°C for 30 s and 40 cycles of 95°C for 5 s and X°C for 15 s, and 72°C for 10 s. X°C was 49.5°C, 52.1°C, 57.3°C and 58.6°C, respectively, for the genes encoding 18sRNA, CYP4Q7, CYPQ4 and CYP similar genes. Amplification and detection of SYBR-Green were performed with the MyiQ (Bio-Rad, Hercules, CA, USA). Data were collected as CT (PCR cycle number where fluorescence is detected above threshold and decreases linearly with increasing input target quantity) using MyiQ optical system software version 1.0 (Bio-Rad). Each replicate was run on PCR plates in triplicate and the data were pooled. The CT of each sample was used to calculate ΔCT values (target gene CT subtracted from 18sRNA gene CT). Each primer pair was tested with a logarithmic dilution of a cDNA mixture to generate a linear standard curve, which was used to determine primer pair efficiency. The relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method [35]. Data from PCR runs were analyzed in the MyiQ optical system software version 1.0 (Bio-Rad). The expression quantity was calculated using the SigmaPlot version 9.0 software (Systat software Inc. USA).

Gene expression data was analyzed with one-way ANOVA followed by Tukey's HSD test at $P<0.05$ for mean comparisons.

**Table1 Details of primers used for real time PCR**

| Target Gene | Primers | Sequences (5'–3') | Tm value (°C) | Product length (bp) | GenBank accession number |
|---|---|---|---|---|---|
| 18sRNA | FR | AAACGGCTACCACATCCAA | 49.3 | 396 | FM877879 |
| | RR | TGTTCAAAGTAAACGTGCCG | | | |
| CYP similar gene | FR | CATCCGCAAACACAACAAAC | 58.8 | 283 | FJ209361 |
| | RR | CGACTGGTCGCTACACTTCA | | | |
| CYP4Q4 | FR | TGATCCTGATCGGTTCCTTC | 57.5 | 249 | AF251548 |
| | RR | GCACATCTGGGGACAAACTT | | | |
| CYP4Q7 | FR | AGGACTGCGAGCTGGTTTTA | 52.6 | 300 | AF254755 |
| | RR | CCATTGCTGTCTCTGCGATA | | | |

## RESULTS

3.1. Resistance levels and synergism of PBO of different strains

Phosphine $LC_{50}$ with or without PBO of the susceptible strain AK, resistant strains SQ($F_0$), SQ($F_2$) and SQ($F_4$) was reported with Table 2. Based on these $LC_{50}$s the resistance factor of SQ($F_0$) , SQ($F_2$) and SQ($F_4$) and PBO synergism ratios were calculated (Table 2). The highest resistance factor was 725 of SQ($F_4$) . The dramatic difference in $LC_{50}$ among different strains indicated that phosphine resistance could be selected and the populations of different strains were genetically stable.

When PBO, an oxidase inhibitor, was applied, the synergistic ratios were 1.77-, 2.81- and 3.81-fold for SQ($F_0$), SQ($F_2$) and SQ($F_4$) strains, respectively (Table 2). The low synergistic effect of PBO implied that oxidative metabolism could be involved in phosphine resistance but probably be less important compared to other potential

resistance mechanisms.

**Table2 Phosphine ($PH_3$) toxicity of *T.castaneum* susceptible and resistant strains and synergistic effect of PBO**

| Strains | Test | $LC_{50}$ | 95% confidence interval | Slope(SE) | RR[a] | SR[b] |
|---|---|---|---|---|---|---|
| AK | | 0.0061 | 0.0055—0.0072 | 11.52（±0.61） | 1.00 | |
| SQ($F_0$) | $PH_3$ | 0.243 | 0.20—0.32 | 8.13（±0.26） | 39.84 | |
| | $PH_3$ +PBO | 0.137 | 0.12—0.13 | 8.02（±0.53） | 22.45 | 1.77 |
| SQ($F_2$) | $PH_3$ | 2.780 | 2.11—5.51 | 2.87（±0.38） | 455.74 | |
| | PH +PBO | 0.989 | 0.94—0.10 | 2.51（±0.21） | 162.13 | 2.81 |
| SQ($F_4$) | $PH_3$ | 4.42 | 2.78—9.40 | 1.38（±0.42） | 724.59 | |
| | PH +PBO | 1.16 | 1.53—1.24 | 1.48（±0.30） | 190.16 | 3.81 |

[a] *RR (resistance ratio) = $LC_{50}$ of resistance strain / $LC_{50}$ of susceptible strain AK*
[b] *SR (synergism ratio) = $LC_{50}$ of PH alone/$LC_{50}$ of PH+PBO*

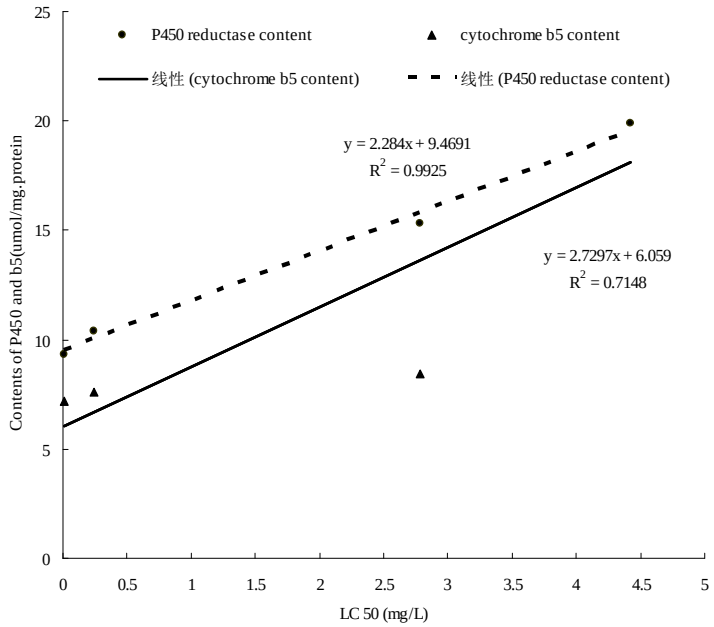


**Fig.1 Positive correlations between phosphine $LC_{50}$ and the concentrations of P450 and cytochrome $b_5$**

3.2. P450 reductase and cytochrome b5 concentrations

Both P450 reductase and cytochrome b5 concentrations were significantly different among strains at 10 d and 20 d larval and adult stages, but no statistic difference was detected at pupal stage (Table 3). The concentrations of P450 and b5 increased as phosphine resistance increased. The high-level resistant strain SQ($F_4$) had the highest P450 and b5 concentrations at all four tested developmental stages. Adult $LC_{50}$ and contents of P450 reductase was higher correlated ($R^2$ = 0.9925, p=0.002), but the relationship between adult $LC_{50}$ and contents of cytochrome $b_5$ was not very strong ($R^2$ = 0.7148, p=0.00346) (Fig.1).

3.3. Expression profiles of CYP4Q4, CYP4Q7 and CYP similar gene of different strains

According to the primer designs, the specific brands of P450 related genes (CYP4Q4 249bp, CYP4Q7 300bp, CYP similar gene 283bp and the inference gene 18sRNA 396bp) in midgut and fat body tissues of the susceptible strain AK, the low-level resistant strain SQ($F_0$) and the high-level resistant strain SQ($F_4$) were cloned by qPCR (Fig.2). The result indicated that the primers used for each gene were specific and suitable to use for qPCR. The melting curves were automatically generated by fluorescence quantitative PCR. The main peaks occurred at 83.5°C, 83.5°C, 83.5°C and 90.5°C for CYP4Q7, CYP4Q4, CYP similar gene and 18sRNA, respectively, which indicated that there was specific amplification with no primer dimer contamination in the PCR products (Fig.3).

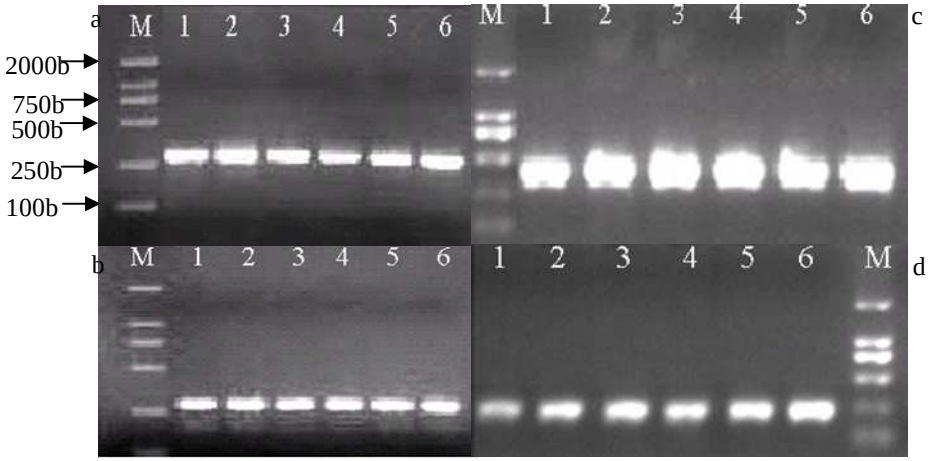


**Fig 2. Electrophoresis image of qPCR products of target genes CYP4 Q7 (300bp) (a), CYP similar gene (283bp) (b), internal reference gene 18sRNA (396bp) (c), and CYP4Q4 (249bp) (d). M=marker, 2000bp; 1, 2, 3, 4, 5, and 6 represents AK midgut, AK fat body, SQ($F_0$) midgut, SQ($F_0$) fat body, SQ($F_4$) midgut and SQ($F_4$) fat body, respectively**

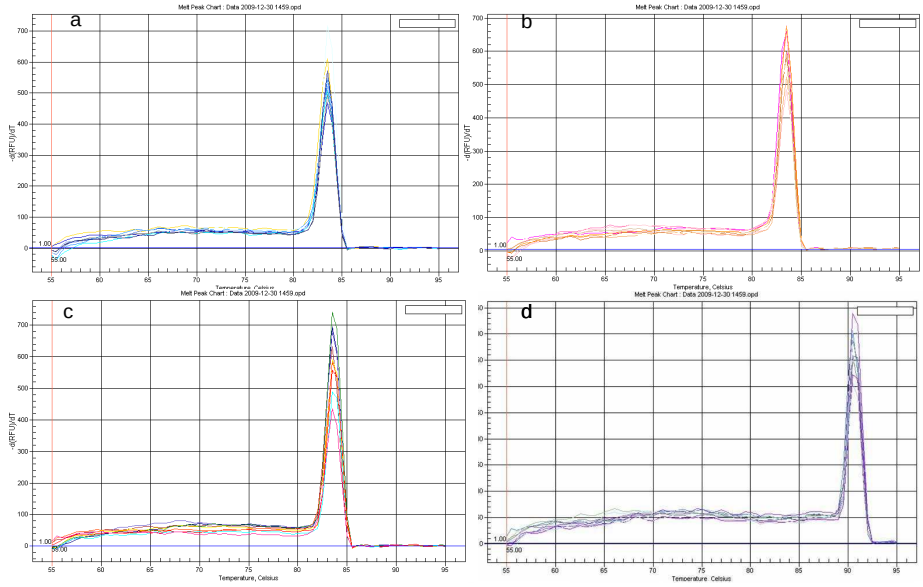


**Fig 3. Melting curves of the qPCR of CYP4Q7 (a), CYP4Q4 (b), CYP similar gene (c) and 18sRNA (d) genes showing specific product for each primer. Specifically, the main melting peak of CYP4Q7, CYP4Q4, CYP similar gene and 18sRNA was at 83.5°C, 83.5°C, 83.5°C and 90.5°C, respectively**

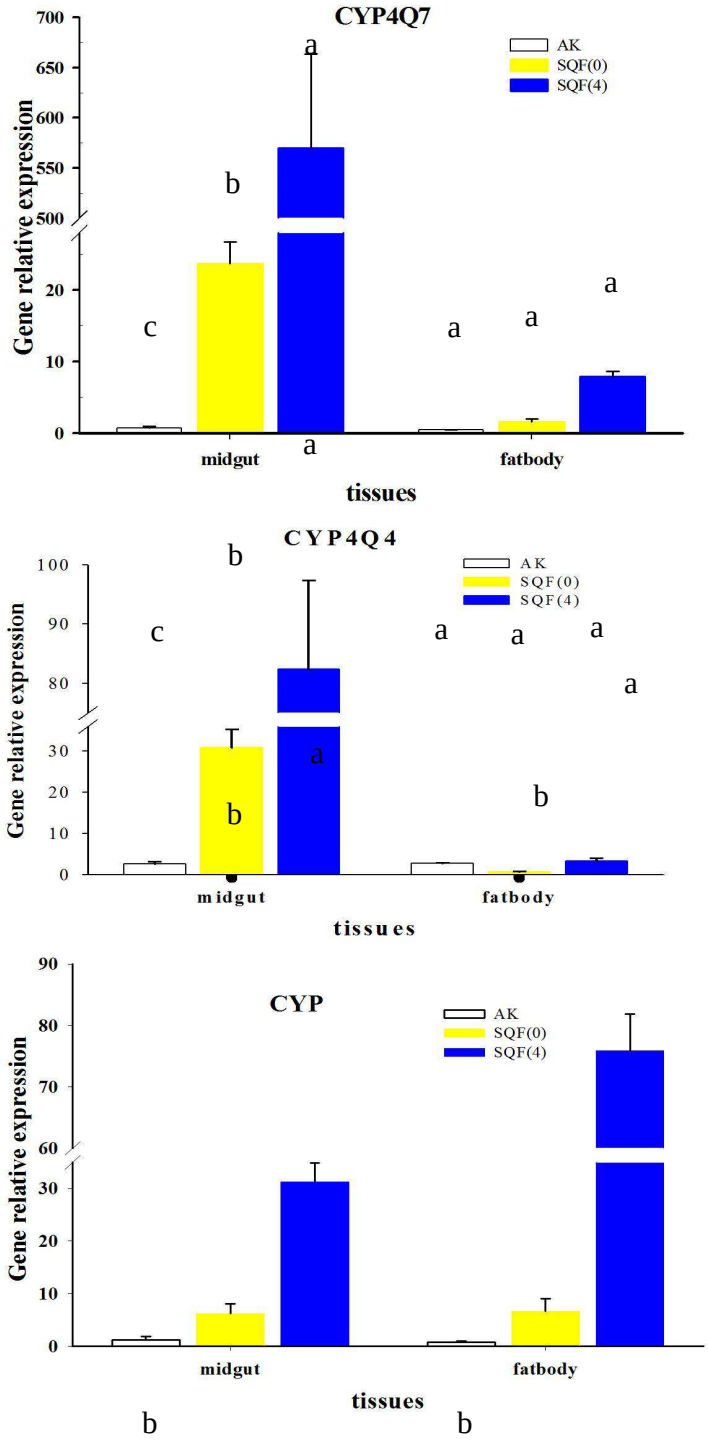


**Fig. 4. CYP4Q4, CYP4Q7 and CYP gene relative transcriptional expression in midgut and fat body tissues of susceptible stain AK and two resistance strains SQF(0) and SQF(4) of *T.castaneum* (20 d old larvae) using qPCR with 18sRNA as the normalizer. Gene relative expression was calculated based on 20 ng of total RNA. Bars with different letters were significantly different within each tissue type (Tukey's HSD Test, P< 0.05)**

The quantitative difference in the relative transcript level of the three P450 related genes were varied in different tissues and among different strains (Fig.4). For midgut tissue, the relative transcription level of CYP4Q7, CYP4Q4 and CYP similar genes were significantly different among strains. The expression increased dramatically as

phosphine resistance increased. This is especially true for CYP4Q7 and CYP4Q4 as significant difference was found for all pair-wise comparisons. CYP similar gene expression was not significantly different between susceptible (AK) and to low-level resistant strain [SQ($F_0$)]. The relative transcription level of CYP4Q7 and CYP4Q4 in fat body was not significantly different among strains, but CYP similar gene expression was significantly higher in SQ($F_4$) strain than that of AK and SQ($F_0$) strains. The results indicated that the over-expressions of CYP4Q7, CYP4Q4 and CYP similar genes were associated with phosphine resistance in *T. castaneum*, and specifically that the over-expression of CYP4Q7 and CYP4Q4 gene in midgut and CYP similar gene in fat body were more important than others.

## DISCUSSION

P450s are well known detoxification enzymes and have been proven, through conventional and/or modern molecular techniques, as a major resistance mechanism of many insect species to various insecticides (Yang et al., 2006). In this study, we successfully selected a high-level (724-fold) phosphine resistant *T. castaneum* strain and demonstrated that resistant strains of different levels had proportionally higher P450 reductase and cytochrome $b_5$ concentrations. Furthermore, using qPCR technology, three P450s related genes were showed to be up-regulated in resistant strains. These results strongly suggest that cytochrome P450 complex is involved in phosphine resistance, just like resistance to other classes of insecticides. However, our in vivo PBO synergist evaluation seems to suggest otherwise as the synergistic ratios of the resistant strains were very low (<4 fold). The possible role of cytochromes P450 in the toxicity or detoxification of phosphine has been studied previously. *T. castaneum*, *S. granarius* and the house fly *Musca domestica* L. treated with insecticide synergists PBO and SKF525A [2-(diethylamino) ethyl 2,2-diphenylvalerate], that are known to inhibit microsomal oxidase enzymes, produced only minor increases in the toxicity of phosphine and any significant role of microsomal oxidases in the detoxification of phosphine was discounted (Zhu et al., 2010;Shorey. 1961; Markussen & Kristensen. 2010).

The metabolic mechanisms of insecticide resistance are complicated phenomena(Rajak, & Hewlett. 1971) Although PBO is considered as a specific P450 inhibitor and is widely used as an indication of P450 conferred resistance(Tozzi,1998), the metabolic pathways specifically blocked by it still are not fully understood and its effect could be species-dependent due to the complexity of P450 systems. For example, PBO was showed to act as an esterase inhibitor in pyrethroid resistant strains of *Helicoverpa armigera*(Young et al., 2005). Additionally, studies have shown that PBO synergistic ratios ranged from 10 to 450-fold for different insecticide resistant strains, for example，10.3-fold for *Trichoplusia ni* against Sevin(Wang et al., 2004),462 -fold for YGF strain of *Helicoverpa armigera* against fenvalerate(Castells & Berenbaum. 2008), and 84.4 – and 74.67- fold for *Aedes aegypti*, *Anopheles culicifacies* against deltamethrin respectively(Yang et al.,2004); suggesting that the synergistic efficacy could heavily depend on the involved insecticides and insect species. The low PBO synergistic ratio of this study could not be an artifact as similar result was reported previously(Gunning 2006). In this respect, the seemingly contradicted result of high P450s content but low PBO synergism in current study provides a strong hint that phosphine resistance could be very different from other classes of insecticides.

The involvement of P450 in insecticide resistance could also be indicated by high enzyme contents of resistant strains. In current study, in addition to reporting higher levels of P450 reductase and b5 contents in phosphine resistant *T. castaneum* strains, we further demonstrated overexpression of three known P450 related genes in *T. castaneum* phosphine resistant strains. P450s are a sophisticated group with numerous isoenzymes and there is ample evidence that insecticide resistances are commonly conferred by several P450s working together. For example, CYP6G1 and CYP12D1 overexpression in DDT-resistant strains of *Drosophila melanogaster*(Festucci-Buselli 2005). Four P450 genes (CYP4D4V2, CYP4G2, CYP6A38, and CYP6A36) are overexpressed in a permethrin resistant housefly strain(Markussen,& Kristensen, 2010; Cariño et al.,1992). Two overexpressed P450s genes, CYP6P4 and CYP6P9, are associated with pyrethroid resistance in *A. funestus* strains(Wondji et al., 2009). Yang et al(2004)reported that constitutive overexpression of multiple cytochrome P450 genes (CYP9A12, CYP9A14 and CYP6B7) was associated with pyrethroid resistance in *Helicoverpa. armigera* in Asia. Based on the dual enzyme content analysis and gene expression results of current study we could hypothesize that multiple P450s are involved in conferring phosphine resistance in *T. castanetum*. Of cause, to definitely prove this hypothesis, studies such as RNAi suppression and/or transgenic expression are needed.

The midgut, Malphighian tubules and fat body are the tissues recognized as the major sites of P450-mediated detoxification in insects(Feyereisen, 2005). For example, gene CYP12A4 is expressed specifically in midgut and

Malpighian tubules in a lufenuron resistance strain of *D. melanogaster*. Eight *D. melanogaster* P450s were expressed in the midgut, Malpighian tubules and fat body, and some of them conferred resistance to multiple insecticides(Bogwitz et al., 2005). In *T. castaneum*, P450 gene CYP6BQ9 is expressed mainly in the brain of a deltamethrin resistant strain[21]. DNA microarray analysis in *Drosophia* have confirmed that P450 transcripts are enriched in midgut, Malphighian tubules(Le et al., 2003). We examined the expression of three P450 genes (CYP4Q4, CYP4Q7 and CYP ) in midgut and abdominal fat body tissues. All 3 genes were overexpressed in midgut of the high-level resistant strain while only CYP gene was overexpressed in fat body. Insecticide resistance associated tissue-specific P450s expression could provide information about the mode of action, movement, distribution and metabolize of insecticides after entering insects. For example, for neurotoxins that many insecticides are, central nerve system such as the brain could be a prime site for resistance related overexpressions(Zhu et al., 2010). As phosphine is not a neurotoxin, it should be interesting to know P450s expression in brains of resistant strains. Thereby, systemically investigating P450 expression in various tissues of phosphine resistant strains will be one of future studies.

In conclusion, the current study successfully screened a high level phosphine resistant strain of *T. castaneum* and demonstrated that high P450 content levels in resistant strains with three P450 associated genes up-regulated. The results provide a strong hint that P450 enzyme complex is involved in phosphine resistance of *T. castaneum*.

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