The relationship between pigmentations of pupae and insecticide resistance of adults of *Bactrocera dorsalis* (Hendel)

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ABSTRACT

The oriental fruit fly *Bactrocera dorsalis* (Hendel) is a worldwide pest leading to crop production. The management of *B. dorsalis* is becoming more and more difficult because of the emerging insecticide resistance. Searching for new and effective methods in managing this pest has become an urgent task. Here, we explored the relationship between pigmentation and insecticide resistance through conducting studies on 5 strains of *B. dorsalis* (CK, CZ, CB, LC and LX) with

various traits of pigmentation and insecticide resistance. Strain CK was a wild strain with brown pupae coloration while strain CZ was a *beta*-cypermethrin-resistant strain with pupae coloration was brown. Strain CB was a pigmentation mutation strain with white pupae coloration. Strain LC was the female of pigmentation sex genetic strain whose pupae coloration was white. Strain LX was the male of pigmentation sex genetic strain whose pupae coloration was brown. Efficacy tests were employed to evaluate the resistance performance of the 5 trains, transcriptome sequencing and gene quantifications were used to explore the potential genes in both pigmentation and detoxification pathway. The brief of results were as follow:

Results of efficacy tests of the 5 strains with *beta*-cypermethrin, abamectin and trichlorfon respectively showed that resistances to these three insecticide of strains LX and LC were very low. The corrective mortalities of strains LX and LC in 128 ug/ml of abamectin were 49.48±3.01%, 59.06±1.89% respectively while that of strain CK was 11.80%±1.37. The corrective mortality of strain CZ were lowest in all concentrations level of all test reagent. The resistance of strain CK was similar to CB. Transcriptomic analysis showed that 24 genes and 165 genes were associated with pigmentation and detoxification. Gene expression pattern of strain CK was similar to strain CB while these two strains were different to strains LC and LX, and the gene expression pattern of strain CZ was more similar to strains LC and LX. Results of gene quantifications in *B. dorsalis* pupae showed that the relative expressions of *yellow* gene *MK529913*, *DDC* gene *MK515141*, *laccase2* gene *MK515143* and *PO* gene *MK515140* in brown pupae (CK, CZ, LX) were significantly higher than those in white pupae (CB, LC) while the relative expressions of *AANAT* gene *MK515144* in white pupae were significantly higher than those in yellow pupae. In summary, analyses of gene quantifications and efficacy tests indicated that high expressions of P450 gene, *AANAT* gene might contribute to resistances of adults of *B. dorsalis* to *beta*-cypermethrin and abamectin; high expression of *yellow* gene might contribute to resistances of adults which emergenced from brown pupae to *beta*-cypermethrin and abamectin; high expressions of *yellow* genes, *laccase2* gene, *PO* gene and *DDC* gene might contribute to the resistance of *B. dorsalis* to trichlorfon.

Subjects: Agricultural Science, Ecology, Entomology.

Keywords: *Bactrocera dorsalis*, Pigmentation, Insecticide, Detoxification, Dopachrome conversion, Arylalkylamine-N-acetyltransferase, Dopa decarboxylase, Phenoloxidase, Cytochrome P450.

INTRODUCTION

The oriental fruit fly *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) is a polyhagous pest. It is recognized as a worldwide pest causing severe damage to agriculture because of its aggressive invasiveness (Clarke *et al.*, 2005). This pest feeds on a great variety of economic crops such as *Mangifera indica* L., *Psidium guajava* L., *Averrhoa carambola* L. and so on (Ye, 2001; Xu *et al.*, 2005; Chen *et al.*, 2012; Lin *et al.*, 2014; Yuan *et al.*, 2015). Females of *B. dorsalis* can lay eggs into fruits. Larvae can cause decay and fruit dropping by feeding in fruits after incubation (Lin *et al.*, 2014). Investigations showed that this pest was known to cause devastating losses in fruit commodities, especially in Asia-Pacific and Africa regions (Ye 2001; Verghese *et al.*, 2004; Clarke *et al.*, 2005; Meyer *et al.*, 2009; Li *et al.*, 2011; Khamis *et al.* 2012).

Today, chemical control has been widely accepted to be the most effective method in managing *B. dorsalis*. Some insecticides were proved to be effective in the management of this pest, such as *beta*-cypermethrin, abamectin and trichlorfon (Lin *et al.*, 2014). *Beta*-cypermethrin was a kind of Pyrethroids insecticides. Pyrethroids insecticides could effect on voltage-dependent sodium channel of insects, and could obstruct the conduction of excitement in nerve, and poison insects as result (Narahashi, 1996; Smith *et al.*, 1998; Vais *et al.*, 2000; Soderlund *et al.*, 2002; Wu *et al.*, 2004b). Meanwhile, pyrethroids insecticides could also effect on nervous system including Na^{+}-K^{+}-ATPase and Ca^{2+}-Mg^{2+}-APTase (Ning *et al.*, 1998). Abamectin was a kind of Avermectins (AVMs) insecticides. Gamma aminobu-tyric acid (GABA) was a neurotransmitter which could regulate the activity of motor nervous system. GABA could be released out from cell membrane when nerve impulses reach nerve endings. AVMs could effect on the CL^{-} channel which were controlled by specific glutamate gate or be sensitive to GABA. Insects were caused

to be paralyzed or dead while the conduction of excitation signals was inhibited (Shoop *et al.*, 1995; Mckellar *et al.*, 1996). Trichlorfon was a kind of organophosphorus insecticides. Organophosphorus insecticides could inhibit the activity of acetylcholinesterase(*AcE*) and lead voltage-dependent sodium channel to remain open. Insects were caused into convulsion or to be dead as the result of excessive accumulation of Na^+ (Villatte *et al.*, 2002; Jiang, 2009).

Meanwhile, many researchers had conducted extensive studies on *B. dorsalis* and made it clear in understanding the responding of *B. dorsalis* to insecticides (Shen *et al.*, 2011; Hu, 2012; Huang, 2016). Some enzymes and genes related to detoxification were found to play important roles in resistance of *B. dorsalis* mainly including Cytochrome P450 (P450) (Nelson, 2009; Rene, 2014), Glutathione *S*-transferases (GST) (Board *et al.*, 1997; Sheehan *et al.*, 2001; Tu *et al.*, 2005; Hu, 2012), *AcE* and Carboxylesterases (*CarE*) (Shen *et al.*, 2011).

B. dorsalis is an insect belonging to Diptera. Enzymes and genes related to detoxification were studied intensively in other insects which belong to Diptera either, such as *Bactrocera oleae* (Vontas *et al.*, 2002), *Drosophila melanogaster* (Ranson *et al.*, 2002; Terhzaz *et al.*, 2015) and *Culex quinquefasciatus*(Arensburger *et al.*, 2010). The enzymes and genes mentioned above were studied intensively either in insects belonging to Lepidoptera or Orthoptera, such as *Bombyx mori*(Yu *et al.*, 2008), *Spodoptera litura*(Wang *et al.*, 2017) and *Locusta migratoria* (Guo *et al.*, 2016). Researches on these insects proved that enzymes and genes related to detoxification could contribute to the adaptation of insects to environment.

The mechanism of pigmentation of insects had been well studied. Pigmentations of insects were studied mainly about Aposematic coloration (Poulton, 1890; Finkbeiner *et al.*, 2014), mimicry (Bates, 1861), cryptic coloration (Bond *et al.*, 2002; Llaurens *et al.*, 2014), courtship (Katayama *et al.*, 2014), thermoregulation and so on (Umbers *et al.*, 2013).

As studies progresses, melanin had been found to play an important role in the adaptation of insects to environment. Studies of the pigmentation of insects were mainly about melanization. Regulation and function of the malanization in insects were studied mainly about the immune responses to microorganisms infections, defense to parasitic enemies and the healing response to

wounds. (1) In immune responses of insects to microorganisms, melanin capsule was an important role which could be syntheticed by insects after the infection of bacteria. Melanization which was intermediated by 3,4-dihydrozyphenylalanine (DOPA) could exhibit antimicrobial activity in the blood of *Sarcophaga peregrine* (Leem *et al.*, 1996). Meanwhile, melanization could be activated by pattern recognition receptors (PRRs) as the response of *Tenebrio molitor* to the infection of Gram-positive bacteria (Park *et al.*, 2007). Microoranisms could be sequestered at wound sites by melanin (Lemaitre *et al.*, 2007). Dopa decarboxylase (DDC) was recognized as an enzyme that could convert DOPA to dopamine. Dopamine could be converted in to melanin. The expression levels of DDC and tyrosine hydroxylase (*TH*) of *Drosophila* could up regulate after the infection of a mixture of *Escherichia coli* and *Micrococcus luteus* (Gregorio *et al.*, 2001). (2) In defendence to parasitic enemies, phenoloxidase(*PO*) was recognized as an important enzyme of insects with tyrosinase-like activity that could catalyze the oxidation of monophenols to diphenols and quinines. The *PO* genes *PPO3* were studied to be significantly upregulated after *Drosophila* larvae were infected by parasitoid wasps (Lanot *et al.*, 2001; Wertheim *et al.*, 2005). Reports showed that the encapsulation reaction against the parasitic wasp *Leptopilina boulardi* were reduced severely while the DDC activity of *Drosophila* larvae was reduced (Nappi *et al.*, 1991; Rizki *et al.*, 1990). Melanization were inhibited as the result of the over expression of serpin Spn27A in the hemolymph of *D. melanogaster*. The ability of encapsulating and destroying *L.boulardi* were reduced as result (Nappi *et al.*, 2004). Some reports showed that parasites were killed after initiation of the melanization reaction in *Anopheles gambiae* and indicated that melanization seemed to be important for *A. gambiae* defending to parasites (Blandin *et al.*, 2004; Volz *et al.*, 2006). (3) In the healing response to wounds of insects, some melanizations seemed to represent wound healing responses. For example, melanization could be activated in a few hours at a larval epidermal wound (Galko *et al.*, 2004; Bidla *et al.*, 2007). Melanotic capsules could be formed by hemocytes where a basement membrane was disrupted (Rizki *et al.*, 1984).

Mean while, the synthesis mechanism of pigmentation such as melanin had been studied well.

The formation of melanin was related to contents of substances including phenylalanine, tyrosine, *DOPA*, N-acetyl-dopamine (*NADA*), *N-beta*-alanyl-dopamine (*NBAD*) (Wittkopp *et al.*, 2003; Sekine *et al.*, 2011) and so on. These substances were related to some enzymes including phenylalanine hydroxylase (*PAH*) (Kwok *et al.*, 1985; Zhang, 2004; Infanger *et al.*, 2004), *TH* (Futahashi *et al.*, 2008), *DDC*(Fang *et al.*, 2000; Chen *et al.*, 2002; Paskewitz *et al.*, 2008; Giegling *et al.*, 2008; Bertoldi *et al.*, 2009; Ferguson *et al.*, 2011; Hodges *et al.*, 2012), *PO* (Sachdev *et al.*, 2017; Zhang *et al.*, 2018a), Phenoloxidase(laccase2) (Yatsu *et al.*, 2009; Futahashi *et al.*, 2010), *NBAD* synthetase (*ebony*) (Hovemann *et al.*, 1998; Wittkopp *et al.*, 2002; Wittkopp *et al.*, 2003; Takahashi *et al.*, 2008; Takahashi *et a*l., 2011; Ferguson *et al.*, 2011), *NBAD* hydrolase (*tan*) (Futahashi *et al.*, 2010; Wagner *et al.*, 2010; Du *et al.*, 2018), dopachrome conversion enzyme family (*yellow*) (Wittkopp *et al.*, 2003; Futahashi *et al.*, 2008; Ferguson *et al.*, 2011; Futahashi *et al.*, 2012), aspartate decarboxylase (*ADC*) (Arakane *et al.*, 2009; Dai *et al.*, 2015) and arylalkylamine-N-acetyltransferase (*AANAT*) (Hintermann *et al.*, 1996; Tsugehara *et al.*, 2007; Zhan *et al.*, 2010; Zhang *et al.*, 2018b). These enzymes and genes were mostly studied on the growing developments of insects (Bai *et al.*, 2014; Sachdev *et al.*, 2017; Zhang *et al.*, 2018b). Meanwhile, they were studied on defense responses of other organisms (Johnson *et al.*, 2003; Ma *et al.*, 2005; Luna *et al.*, 2011; Geomela *et al.*, 2012; Hodges *et al.*, 2012; Coates., 2018; Coaglio *et al.*, 2018).

These enzymes and genes involved in pigmentation were studied in *B. dorsalis* as well (Wang, 2016; Wang *et al.*, 2017). Some genetic laws of *B. dorsalis* had been reported (Zacharopoulou *et al.*, 2013). Methods of enzyme inhibitions were used in studies of these enzymes and genes usually. *PO* and *TH* were found to contribute to growth, development and immune defense of *B. dorsalis* (Bai *et al.*, 2014; Chen *et al.*, 2017; Hou *et al.*, 2017; Sachdev *et al.*, 2017). Pigments were recognized as being distributed in tissues of epidermis and intestines (Nijhout, 1991), and could be performed in cuticle (Barbakadze *et al.*, 2006) while the cuticle was performed by secretion secreted in epidermal cells and contributed to the environmental adaptability of insects (Tang, 2009; Li, 2012; Fedorka *et al.*, 2013). Here, whether pigmentation enzymes and genes

could reduce the accession of efficacy into insect body is still unknown. Meanwhile, the management of *B. dorsalis* is becoming more and more difficult with the emergence of insecticide resistance (Zhang *et al.*, 2007; Jin *et al.*, 2010; Jin *et al.*, 2014; Lin *et al.*, 2014). There is still a lack in studies of enzymes and genes associated with pigmentations of *B. dorsalis*, especially in studies of those associated with pigmentation of *B. dorsalis* pupae. For these reasons, we conducted efficacy tests of *B. dorsalis* adults, transcriptome sequencing of 5 trains of *B. dorsalis* pupae and gene quantifications and tried to obtain systematical knowledge about enzymes or genes which both associated with pigmentations of *B. dorsalis* pupae and associated with resistance of *B. dorsalis* further.

MATERIALS AND METHODS

Efficacy tests of *B. dorsalis* adults

Insects. There were 5 strains of *B. dorsalis* including strain CK, CZ, CB, LC and LX (Fig. 1). **(1)** Strain CK: the wild strain of *B. dorsalis* collected from field. Strain CK had been cultured for more than 60 generations in laboratory. The pigmentation of pupa of this strain was brown. **(2)** Strain CZ: the resistant strains for *beta*-cypermethrin. Strain CZ had been selected and cultured for more than 30 generations in laboratory and the resistance multiple (the ratio of LC50 of the stain CZ to stain CK) was 98.57. The pigmentation of pupa of this strain was brown. **(3)** Strain CB: the whitening mutation from strain CK. Strain CB had been selected and cultured for more than 9 generations in laboratory. The pigmentation of pupa of this strain was white. **(4)** Strain LC: the female of pigmentation sex genetic strain of *B. dorsalis* from Professor Kostas Bourtzis who was working in Austrian International Atomic Energy Agency Department of Pest Control. The pigmentation of pupa of this strain was white. **(5)** Strain LX: the male of pigmentation sex genetic strain of *B. dorsalis* mentioned above. The pigmentation of pupa of this strain was brown. *B. dorsalis* was cultured in our laboratory at 28℃ under a photoperiod of 14 h light /10 h dark (Lin *et al.*, 2014). Adults of *B. dorsalis* were reared with artificial diets including sugar and yeast in a ratio of 1:1. Larvae of *B. dorsalis* were reared with artificial diets including paper, banana, corn flour, suger, yeast, water, hydrochloric acid and sodium benzoate in appropriate proportion

in the laboratory. Adults samples used in efficacy tests were collected 3 days after emergence. These adults were normally feared before efficacy tests.

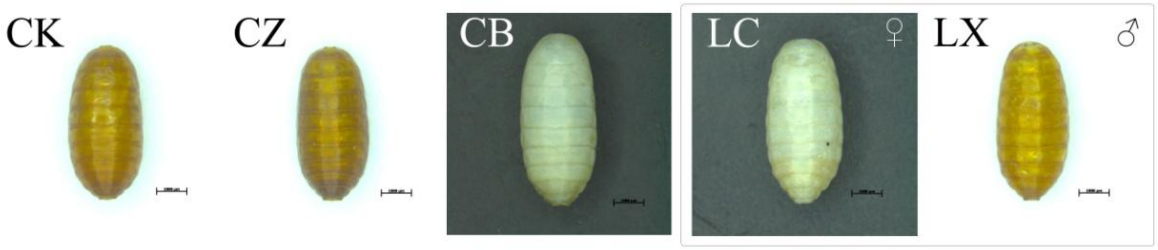

Figure 1 5 strains of *B. dorsalis*.

Equipments and materials. 250ml triangle bottles, cotton, rubber strips, pipettes, wooden frames, acetone, *beta*-cypermethrin, trichlorfon and abamectin. *Beta*-cypermethrin, abamectin and trichlorfon were used in the efficacy tests respectively. These 3 pharmacies were diluted to 8mg/L, 16mg/L, 32mg/L, 64mg/L and 128mg/L with acetone respectively. The group studied only with acetone was regarded as control group and was used as blank control in the calculation of corrected mortality.

Methods. Membrane method was used in the study (Lin *et al.*, 2014). 30 adults were applied to one sample and 15 samples were applied to each strain in the efficacy tests with either *Beta*-cypermethrin, abamectin or trichlorfon. Adults were reared without anything during the study time. Numbers of adult deaths were recored every 12 hours. The data used in this paper was only the data of adult deaths in 24 hours. The corrected mortality was computed based on the number of adult deaths. The differences between strains were analyzed by SAS. Figures were constructed by software R (RStudio).

Transcriptome sequencings of pupae of 5 *B. dorsalis* strains

Insects. Samples used in transcriptome sequencings were pupae of 5 *B. dorsalis* strains mentioned above (Fig. 1).

Laboratory equipments. Centrifuge 580/5804 high speed refrigerated centrifuge (Eppendorf, Germany), Biorad GelDoc XR Gel Imager (Bio-Rad, USA), Nano-drop UV spectrophotometer (Nanodrop, USA), DYY7C horizontal electrophoresis instrument (Beijing Liuyi Instrument Factory, China), Quantitative PCR instrument Agilent MX3500p (USA), Mastercycler Pro

gradient PCR instrument (Eppendorf, Germany), Minbeadbeaater-16 grinding bead homogenizer (Biospec, USA), Pipettes (Eppendorf, Germany).

Consumptive materials. RNA extraction reagent Trizol Reagent (Beijing Tiangen Biotechnology Co., Ltd., China), RNA-free 1.5 mL centrifuge tubes (Anexy, USA), RNase-free 2 mL centrifuge tubes (Anexy, USA), RNase-free 200 uL PCR tubes (Anexy, USA), An RNase-free pipette (Anexy, USA), Chloroform, isopropanol, absolute ethanol and so on.

Methods. In samples extractions of each strain, 2 pupae pupated 2 days old were used in the total RNA extraction of one sample. For preventing experimental bias caused by too little sample, three samples were set for each strain. **(1)** RNA extraction: pupae were extracted in Trizol reagent. The experiment was carried out on super clean bench, consumptive materials were treated for preventing RNase contaminations. **(2)** transcriptome sequencing: transcriptome sequencings of all samples were performed on Illumina HiSeq/MiSeq (Illumina HiSeqTM2000). Clean reads were filtered from raw reads and further spliced by Trinity (Grabherr *et al.*, 2011). The longest transcript of each gene was regarded as unigene. **(3)** Annotations of gene functions: assembled unigenes were annotated with software NCBI blast 2.2.28 in 7 databases including NR, Nt, Pfam, KOG/COG, Swiss-prot, KEGG and GO. The threshold of e-value used in the gene function annotations in databases including NR, Nt and Swiss-prot was 1e-5, and the threshold of e-value used in database KOG/COG was 1e-3. The e-value used in software HUMMER 3.0 and in database Pfam was 0.01. KAAS KEGG Automatic Annotation Server was used in database KEGG. BlastGOv2.5 and self-written scripts were used in database GO. The similarity comparison between sequences of native species and relative species was obtained in sequences comparations and annotations in NR. **(4)** CDS prediction: The ORF coding frame information was extracted after the blast of Unigenes in databases NR and Swissprot. ORFs of the Unigenes without annotations in NR and Swissprot were predicted by software extscan (3.0.3). **(5)** Differential expression analysis of unigenes: the transcriptome obtained by splicing trinity was used as reference sequence (ref). Clean reads of all samples were blasted to ref by software RSEM. **(6)** Screening of differentially expressed genes between each two strains: the read counts

were obtained in the analysis of gene expression levels. Difference analyses were performed by DEGseq after standardized processing of read counts by TMM. For controlling the occurrence of false positive rates, we set the screening criteria in the definition of differential genes as qualue<0.005 and $|\log_2(\text{foldchange})|>1$. Genes with $\log_2(\text{foldchange})>0$ were defined as up-regulated and genes with $\log_2(\text{foldchange})<0$ were defined as down-regulated. Differentially expressed genes were further enriched by GO enrichment (Young *et al.*, 2010) and KEGG enrichment (Kanehisa M *et al.* 2008).

Indentifications of genes in different gene families

Methods used in identifications of genes were as follow: (1) According to the database annotations in transcriptome sequencings of 5 trains, genes associated to detoxifications or pigmentations were identified into different gene families; (2) Genes were performed by blastx in NCBI; (3) Genes were identified in phylogenetic trees based on sequence characteristics of gene families which had been reported. Phylogenetic trees were built in MEGA4 (Tamura *et al.*, 2007). Gene families which had been reported were as follow:

Genes of P450 in transcriptomes were searched for and identified based on studies reported. There were several species in these studies about P450 genes including: *B. dorsalis* (Huang, 2016), *D. melanogaster* (Terhzaz *et al.*, 2015), *A. gambiae* (David *et al.*, 2005; Muller *et al.*, 2008), *Anopheles stephensi* (Vontas *et al.*, 2007), *Aedes aegypti* (Strode *et al.*, 2008), *Culex quinquefasciatus* (Yang *et al.*, 2011; Reid *et al.*, 2012), *Culex pipiens quinquefasciatus* (Yan *et al.*, 2012; Yan *et al.*, 2012), *Acyrthosiphon pisum* (Ramsey *et al.*, 2010), *Myzus persicae* (Ramsey *et al.*, 2010) and *Mamestra brassicae* (Coisne *et al.*, 2010).Protein sequences and alignments of P450 genes were showed in supplemental file 1.

Genes of GST in transcriptomes were searched for and identified based on studies reported (Hemingway *et al.*, 2000). Many species were used in identifications of GST genes and as follow: *B. dorsalis*(Hu, 2012), *D. melanogaster* (Ranson *et al.*, 2002), *Musca domestica* (Fournier *et al.*, 1992), *B. mori* (Yu *et al.*, 2008), honeybee (Claudianos *et al.*, 2006), *Nasonia vitripennis* (Oakeshott *et al.*, 2010), *L.migratoria* (Qin *et al.*, 2011), *A. pisum* (Ramsey *et al.*, 2010), *M.*

persicae (Ramsey *et al.*, 2010), *Plutella xylostella* (Sonoda *et al.*, 2006), *Spodoptera litura* (Deng *et al.*, 2009), *Manduca sexta* (Snyder *et al.*, 1995), *A. gambiae* (Ranson *et al.*, 2001; Ding *et al.*, 2003; Lumjuan *et al.*, 2005; David *et al.*, 2005), *Anopheles arabiensis* (Muller *et al.*, 2008), *A. stephensi* (Vontas *et al.*, 2007), *A. aegypti* (Lumjuan *et al.*, 2007; Strode *et al.*, 2008), *C. pipiens quinquefasciatus* (Reddy *et al.*, 2011; Yan *et al.*, 2012) and *Grapholita molesta* (Li *et al.*, 2018).Protein sequences and alignments of GST genes were showed in supplemental file 2.

Genes of *AcE* in transcriptomes were searched for and identified based on studies reported (Fournier *et al.*, 1992; Baxter *et al.*, 2002; Gao *et al.*, 2002). Many species were used in identifications of *AcE* genes and as follow: *B. dorsalis* (Shen *et al.*, 2011), *D. melanogaster* (Shi, 2003), *M. domestica* (Kozaki *et al.*, 2001; Walsh *et al.*, 2001; Shi, 2003), *B. oleae* (Vontas *et al.*, 2002), *Culex*. *tritaeniorhynchus* (Nabeshima *et al.*, 2004), *Aphis. gossypii* (Andrews *et al.*, 2004; Toda *et al.*, 2004; Javed *et al.*, 2003), *M. persicae* (Nabeshima *et al.*, 2003; Javed *et al.*, 2003), *Nephotettix cincticeps* (Tomita *et al.*, 2000), *Lucilia cuprina* (Chen *et al.*, 2001), *Bemisia tabaci* (Javed *et al.*, 2003), *Trialeurodes vaporariorum* (Javed *et al.*, 2003), *Tetranychus urticae* (Anazawa *et al.*, 2003) and *Ditylenchus destructor* (Ding *et al.*, 2008). Protein sequences and alignments of *AcE* genes were showed in supplemental file 3.

Genes of *CarE* in transcriptomes were searched for and identified based on studies reported. Species were used in identifications of *CarE* genes and as follow: *B. dorsalis* (Shen *et al.*, 2011; Shen *et al.*, 2014), human(Kroetz *et al.*, 1993), *D. melanogaster* (Robin *et al.*, 1996; Ranson *et al.*, 2002), *L. cuprina* (Newcomb *et al.*, 1997a), *Anisopteromalus calandrae* (Zhu *et al.*, 1999), *Apis mellifera* (Claudianos *et al.*, 2006), *C. quinquefasciatus* (Vaughan *et al.*, 1995; Gong, 2015), *C. pipiens quinquefasciatus* (Yan *et al.*, 2012), *A. gambiae* (David *et al.*, 2005), *Nilaparvata lugens* (Small *et al.*, 2000), *M. persicae* (Field *et al.*, 1993), *A. gossypii* (Li *et al.*, 2002), *S. litura* (Huang *et al.*, 2010) and *Helicoverpa armigera* (Wang *et al.*, 2008). Protein sequences and alignments of *CarE* genes were showed in supplemental file 4.

Enzymes and genes associated to pigmentations of insects were searched and identificated based on studies reported. There were 9 families of enzymes or genes as follow: Dopachrome

converting enzyme family DEC(*yellow*) (Wittkopp *et al.*, 2003; Futahashi *et al.*, 2008; Ferguson *et al.*, 2011; Futahashi *et al.*, 2012), N-acetyltransferase(*AANAT*) (Hintermann *et al.*, 1996; Tsugehara *et al.*, 2007; Zhang *et al.*, 2018b), Phenoloxidase(*laccase2*) (Futahashi *et al.*, 2010; Yatsu *et al.*, 2009; Futahashi *et al.*, 2010), NBAD synthetase (*ebony*) (Hovemann *et al.*, 1998; Richardt *et al.*, 2002; Wittkopp *et al.*, 2002; Wittkopp *et al.*, 2003; Futahashi *et al.*, 2008; Pool *et al.*, 2010; Takahashi *et al.*, 2011; Ferguson *et al.*, 2011; Zhang *et al.*, 2018b), NBAD hydrolase (*tan*) (Futahashi *et al.*, 2010; Ferguson *et al.*, 2011; Du *et al.*, 2018), Phenoloxidase(*PO*) (Zhang *et al.*, 1997; Luna *et al.*, 2011; Sachdev *et al.*, 2017; Zhang *et al.*, 2018a), Aspartate decarboxylase (*ADC*)(Arakane *et al.*, 2009; Dai *et al.*, 2015), DOPA decarboxylase (*DDC\AADC*) (Fang *et al.*, 2000; Hawi *et al.*, 2001; Chen *et al.*, 2002; Ma *et al.*, 2005; Yu *et al.*, 2006; Giegling *et al.*, 2008; Paskewitz *et al.*, 2008; Bertoldi *et al.*, 2009; Ferguson *et al.*, 2011; Geomela *et al.*, 2012), Phenylalanine hydroxylase(*PAH*) (Kwok *et al.*, 1985; Johnson *et al.*, 2003; Infanger *et al.*, 2004; Zhang *et al.*, 2004; Wang *et al.*, 2013), and Tyrosine hydroxylase (*TH*) (Futahashi *et al.*, 2008). Protein sequences and alignments of *yellow* genes, *AANAT* genes, *laccase2* genes, *ebony* genes, *tan* genes, *PO* genes, *ADC* genes, *DDC* genes, *PAH* genes and *TH* genes were showed in supplemental files 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 respectively.

Analysis of relationships of 5 strains in gene ontology (GO)

Based on the indentifications of genes associated to pigmentations and detoxifications above, we obtained biological processes, cell components and molecular functions which these genes participated in. We conducted analyses in veen figures of GO IDs of these biological processes, cell components and molecular functions, and obtained figures 3A, 3B and 3C.

Analysis of relationships of 5 strains in genes expressions

Heat maps were constructed based on all transcriptomes of 5 strains. On the two strains in 5 strains were combined to be a differential comparison group. And 10 differential comparison groups were obtained including CK vs CZ, CK vs CB, CK vs LC, CK vs LX, CZ vs CB, CZ vs LC, CZ vs LX, CB vs LC, CB vs LX and LC vs LX. We set the screening criteria in the definition of differential genes as qvalue<0.005 and $|\log_2(\text{foldchange})|>1$. FPKM values obtained

in transcriptome sequencings of 5 strains were used for basic data in the heap map Figure 4A. FPKM values of genes associated to detoxifications (P450, GST, *AcE* and *CarE*) were used for basic data in the heap map Figure 4B. FPKM values of genes associated to pigmentations (*PAH*, *DDC*, *PO*, *laccase2*, *ebony*, *tan*, *yellow*, *ADC* and *AANAT*) were used for basic data in the heap map Figure 4C. FPKM values of genes which were up-regulated or down-regulated in comparisons mentioned above and both associated to detoxifications and pigmentations were used for basic data in the heap map Figure 4D. FPKM values used in these heat maps were changed with logarithm by R language (RStudio) and were normalized by column parameters. Pearson correlation was used in the column clusters of heat maps.

Analysis of expressions of genes in *B. dorsalis* pupae

Six strains of *B. dorsalis* were performed in the PCR amplifications of 9 target genes. Strain CB_Z was a new strain compared with 5 strains mentioned above. And the strain CB_Z was a strain selected for 10 generations base on strain CB. Pupae samples of 6 strains were collected 2 days after pupations. Three pupae were used in each one sample. For preventing experimental bias caused by too little sample, three biological replicates were set for each train.

Genes. There were 9 target genes up-regulated or down-regulated in the 10 differential comparison groups mentioned above including 4 genes of *yellow* (*MK529913*, *MK529914*, *MK529912* and *MK529915*), 1gene of *laccase2* (*MK515143*), 1 gene of *AANAT* (*MK515144*), 1 gene of *PO* (*MK515140*), 1 gene of *DDC* (*MK515141*) and 1 gene of P450 (*MK515142*). Gene *a-tub* was used as reference gene (Shen *et al.*, 2010; Shen *et al.*, 2013).

Primers. Primers of Genes *MK529913*, *MK529914*, *MK529912*, *MK529915*, *MK515143*, *MK515144*, *MK515140*, *MK515141*, *MK515142* and *a-tub* were as follows respectively: TGCTCAGTGTTCTCCACGTC (forward), GAAGAATCCCCAGAGCCCAC (reverse); AGGGGCACCTAAATCAGCAG (forward), TAGACTCGCGTATTGCAGGC (reverse); CGATCGTCGCGGAATGAGTA (forward), AACTGACAAAGTGTTGCGCC (reverse); CAAACAACCGATTGCCCCAG (forward), CACTTCGCAATTTGGCTGCT (reverse); GTTACTGCCTTGGGACGTGA (forward), GCAGAATTAAGCCCATGCCG (reverse);

ACCCTCGCCATCTGCTAAAC (forward), AGTACGCTCAGTCAGGCAAC (reverse); TCTCGCGTGTACCGCTATTC (forward), CGCCAGTAGCCGAGATCTTT (reverse); CGCCCAATTCGCAATAAGCA (forward), TCGTTATTGGCGCAGGACTT (reverse); GCAGAAAGATTGTGGCCTGC (forward), AATGCGCTCTACACAACCGA (reverse); CGCATTCATGGTTGATAACG (forward), GGGCACCAAGTTAGTCTGGA (reverse). PCR amplifications were performed in CFX-96_CFX Connect, beginning with a 15 min incubation at 95℃ followed by 40 cycles of 95℃ for 10 seconds, 55℃ for 20 seconds and 72℃ for 25 seconds. Melting curve analyses were performed to confirm the amplification specificity in these PCR amplifications.

Equipments and materials. Real-time PCR instrument (CFX-96_CFX Connect, Bio-Rad, USA), Total RNA extraction kit of micro-sample (centrifugal column, DP420), RNA-free 1.5mL centrifuge tubes, RNase-free 200uL PCR tubes, RNase-free gun heads and RNA-free PCR eight-tubes from Beijing Tiangen Biotechnology Co., Ltd., China. Other instruments were consistent to the instruments used in the extractions of transcriptomes samples.

Mehods. RNA samples of the 6 stains pupae pupated 3 days old were extracted respectively. The quality of these RNA samples were detected combining the following two methods: (1) the concentrations and OD260/280 values of these RNA samples were detected by Nucleic acid protein detector; (2) these RNA samples were observed by electrophoresis and gel imaging. RNA samples of the 6 stains obtained above were reversed transcription into cDNA respectively. The concentrations of these cDNA samples were detected by Nucleic acid protein detector. Expressions of target genes in these cDNA samples obtained above were quantified Real-time PCR instrument.

Analysis of expressions of genes in *B. dorsalis* adults

The Adult samples of 6 strains were collected 5 days after emergences. Four adults were used in each one sample. For preventing experimental bias caused by too little sample, three biological replicates were set for each train. Primers of 9 target genes, instruments, methods of RNA extractions and detections, methods of RNA reversions and methods of Real-time PCR were

consistent to those used in the determination of relative expressions of 9 target genes in *B. dorsalis* pupae.

RESULTS

Efficacy tests of *B. dorsalis* adults

There were 5 strains in efficacy tests with *beta*-cypermethrin including strains CK, CZ, CB, LC and LX (Fig. 2A).

Strain CZ showed high resistance in the five concentrations to *beta*-cypermethrin. Corrected mortalities of strain CZ in 4 concentrations 16 mg/L, 32 mg/L, 64 mg/L and 128 mg/L were 0.07%±0.06, 0.04%±0.12, 0.06%±0.24 and 1.06%±0.59 respectively, significantly lower than other 4 strains. The resistance of strain CB ranked second in resistances of 5 strains. Corrected mortalities of strain CB in 4 concentrations 16 mg/L, 32 mg/L, 64 mg/L and 128 mg/L were 2.19%±0.60, 6.21%±0.56, 14.24%±1.70 and 22.48%±2.11 respectively, significantly higher than strain CZ and significantly lower than strains CK, LC and LX. Corrected mortalities of strain CK in 5 concentrations were 8.02%±1.20, 13.78%±1.94, 15.52%±2.06, 21.28%±2.04 and 34.71%±3.22 respectively. The resistance of strain LX was similar to strain LC. Compared with strains CK, CZ and CB, the resistance of strain LX was indicated to be lower in concentrations 16 mg/L, 64 mg/L and 128 mg/L. Corrected mortalities of strain LX in these 3 concentrations were 16.25%±1.81, 34.83%±2.35 and 55.84%±2.38 respectively. Strain LC showed low resistances either in concentrations 32 mg/L, 64 mg/L or 128 mg/L. Corrected mortalities in these 3 concentrations were 26.75%±1.92, 37.95%±3.75 and 47.81%±2.17 respectively.

In general, resistances of the 5 strains in the efficacy tests to *beta*-cypermethrin were roughly as: CZ > CB > CK > LC ≈ LX (high to low).

There were 5 strains in the efficacy test with abamectin including strain CK, CZ, CB, LC and LX (Fig. 2B).

Strain CZ showed high resistances in the five concentrations to abamectin. Corrected

mortalities of strain CZ in 4 concentrations 16 mg/L, 32 mg/L, 64 mg/L and 128 mg/L were 0, 0.56%±0.28, 3.74%±0.73 and 2.49%±0.50 respectively, significantly lower than other 4 strains. Resistance of strain CB ranked second in resistances of 5 strains. Corrected mortalities of strain CB in 3 concentrations 32 mg/L, 64 mg/L and 128 mg/L were 1.76%±0.90, 7.52%±2.05 and 7.00%±2.36 respectively, significantly higher than strain CZ and significantly lower than strains CK, LC and LX. Corrected mortalities of strain CK in 4 concentrations 16 mg/L, 32 mg/L, 64 mg/L and 128 mg/L were 6.70%±1.20, 9.64%±1.26, 10.29%±1.42 and 11.80%±1.37 respectively. The resistance of strain LX was similar to strain LC. Compared with strains CK, CZ and CB, the resistance of strain LX was indicated to be lower in 5 concentrations 8 mg/L, 16 mg/L, 32 mg/L, 64 mg/L and 128 mg/L. Corrected mortalities of strain LX in these 5 concentrations were 8.93%±1.33, 12.16%±1.97, 24.96%±3.95, 37.37%±4.66 and 49.48%±3.01 respectively, significantly higher than strains CK, CZ and CB. Strain LC showed low resistances either in concentrations 32 mg/L, 64 mg/L or 128 mg/L. Corrected mortalities in these 3 concentrations were 12.31%±1.97, 24.28%±1.90 and 59.06%±1.89 respectively.

In general, resistances of the 5 strains in the efficacy test with abamectin were roughly as: CZ > CB > CK > LC ≈ LX (high to low).

There were 5 strains in the efficacy test with trichlorfon including strain CK, CZ, CB, LC and LX (Fig. 2C).

Corrected mortalities of strains CK, LC and LX in concentrations 64 mg/L and 128 mg/L reached high levels. Strain CZ showed high resistances in concentrations 64 mg/L and 128 mg/L while strain LC took second place of it. Strain CZ showed high resistance in the five concentrations of trichlorfon. Corrected mortalities of strain CZ in 4 concentrations 16 mg/L, 32 mg/L, 64 mg/L and 128 mg/L were 1.21%±0.24, 4.93%±0.46, 64.77%±2.88 and 97.49%±0.48 respectively, significantly lower than other 4 strains. The resistance of strain LC ranked second in resistances of 5 strains. Corrected mortalities of strain LC in 3 concentrations 16 mg/L, 32 mg/L and 64 mg/L were 14.66%±5.17, 65.36%±8.87 and 87.26%±5.02 respectively, significantly lower than strain CB and LX. Resistances of strain CB were lowest among 5 strains. Corrected

mortalities of strain CB in 4 concentrations 16 mg/L, 32 mg/L, 64 mg/L and 128 mg/L were 63.19%±6.74, 97.53%±1.34, 100% and 100% respectively, significantly higher than strains CZ and LC. The resistance of strain LX was similar to strain LC. Corrected mortalities of strain LX in 5 concentrations 8mg/L, 16 mg/L, 32 mg/L, 64 mg/L and 128 mg/L were 10.45%±3.99, 33.77%±8.77, 89.33%±3.76, 99.63%±0.19 and 100% respectively. Corrected mortalities of strain CK in 5 concentrations 8mg/L, 16 mg/L, 32 mg/L, 64 mg/L and 128 mg/L were 6.51%±1.47, 38.25%±3.92, 74.77%±6.55, 98.71%±0.66 and 100% respectively.

In general, resistances of the 5 strains in the efficacy test with trichlorfon were roughly as: CZ > LC > CK > LX ≈ CB (high to low).

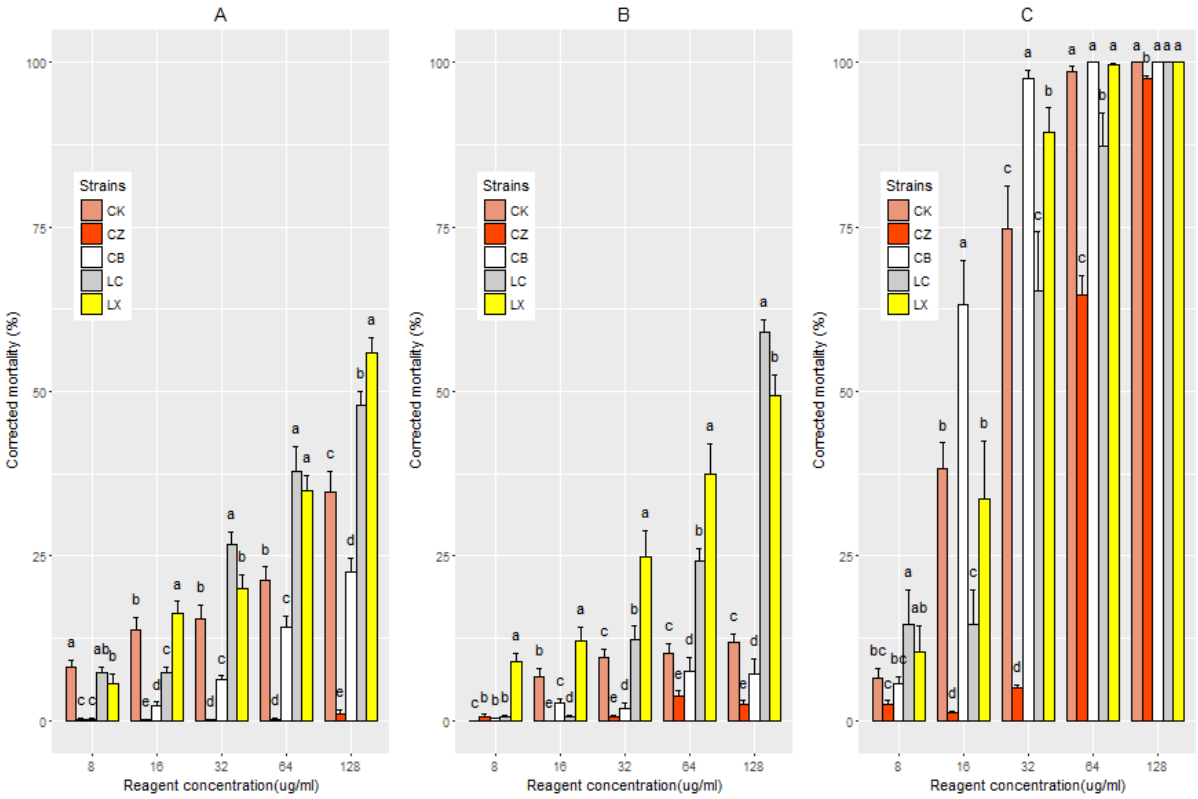

Figure 2 Respondings of *B. dorsalis* adults to *beta*-cypermethrin, abamectin and trichlorfon.

(A) Corrected mortalities of 5 strains in *beta*-cypermethrin. (B) Corrected mortalities of 5 strains in abamectin. (C) Corrected mortalities of 5 strains in trichlorfon.

Notes: X-axis standed for strains of *B. dorsalis*. Y-axis standed for corrected mortalities of genes. Length of column standed for the average of corrected mortalitie. Line on columns standed for standard error. The difference of letters

on columns standed for significant by DMRT (a=0.05) analysis of variance.

Transcriptome sequencings of pupae of 5 *B. dorsalis* strains

The pupae pupated for 2 days old of 5 strains (CK, CZ, CB, LC and LX) were detected in illumine high-throughput sequencing. Raw reads of these 5 strains were 3.16G, 2.56G, 3.35G, 3.35G and 3.15G respectively. Numbers of clean reads of these 5 strains were 25271645, 20441155, 26767170, 28109880 and 26822233 respectively. The Q20 and Q30 were up to 97.03% and 93.97% respectively. By sequence assembly, the mean length, N50 and N90 of these transcripts were 916bp, 1906bp and 314bp. The mean length, N50 and N90 of these unigenes were 729bp, 1379bp and 265bp respectively. 136941 transcripts and 97809 unigenes were annotated in all databases.

Indentifications of genes in different gene families

After indentifications of the gene sequences, we obtained 165 genes related to detoxification enzymes including 127 genes of family Cytochrome P450 (P450), 33 genes of family Glutathione S-transferases (GST), 1 gene of Acetylcholinesterase (*AcE*) and 4 genes of carboxylesterases (*CarE*). We obtained 24 genes related to pigmentation enzymes including 1 gene of phenylalanine hydroxylase (*PAH*), 2 genes of Tyrosine hydroxylase (*TH*), 2 genes of DOPA decarboxylase (*DDC*), 2 genes of phenoloxidase (*PO*), 2 genes of *laccase2*, 1 gene of *ebony*, 1 gene of *tan* (NBAD hydrolase), 10 genes of *yellow* (dopachrome converting enzyme, *DEC*), 2 genes of aspartate decarboxylase (*ADC*) and 1 gene of N-acetyltransferase (*AANAT*).

Analysis of relationships of 5 strains in gene ontology (GO)

There were 66 and 37 biological processes associated with detoxifications and pigmentations respectively (Fig. 3A). There were 8 points in the intersection including 0006281(DNA repair), 0006355(regulation of transcription, DNA-templated), 0006813(potassium ion transport), 0007165(signal transduction), 0008033(tRNA processing), 0008152(metabolic process), 0009405(pathogenesis) and 0055114(oxidation-reduction process).

There were 23 and 11 cell components associated with detoxifications and pigmentations respectively (Fig. 3B). There were 8 points in the intersection including 0005576(extracellular

region), 0005622(intracellular), 0005634(nucleus), 0005643(nuclear pore), 0005667(transcription factor complex), 0005737(cytoplasm), 0016020(membrane) and 0016021(integral component of membrane).

There were 62 and 32 molecular functions associated with detoxifications and pigmentations respectively (Fig. 3C). There were 14 points in the intersection including 0003677(DNA binding), 0003700(sequence-specific DNA binding transcription factor activity), 0003824(catalytic activity), 0005488(binding), 0005506(iron ion binding), 0005515(protein binding), 0005524(ATP binding), 0008168(methyltransferase activity), 0008270(zinc ion binding), 0016491(oxidoreductase activity), 0016597(amino acid binding), 0016705(oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen), 0016740(transferase activity) and 0050660(flavin adenine dinucleotide binding).

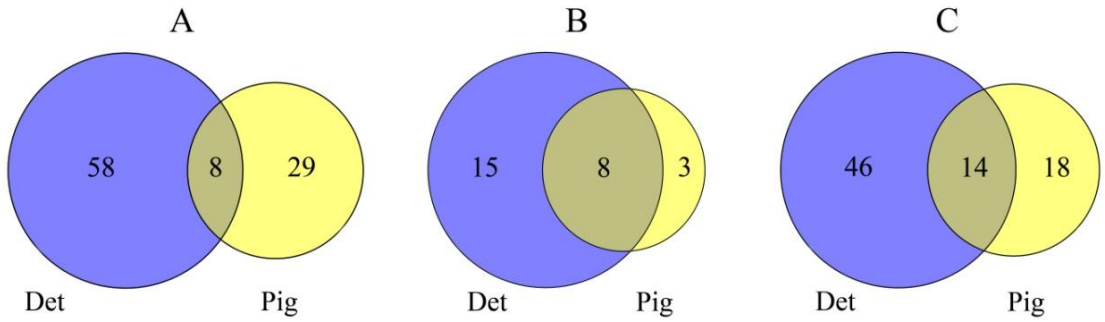

Figure 3 The relationship of 5 strains in biological process, cell component and molecular function. (A) Numbers of biological process associated with detoxifications and pigmentations, (B) Numbers of cell component associated with detoxifications and pigmentations, (C) Numbers of molecular function associated with detoxifications and pigmentations.

Note: "Det" and "Pig" in the figure were abbreviations of Detoxification and Pigmentation respectively.

Analysis of relationships of 5 strains in genes expressions

Five strains of *B. dorsalis* were divided into 2 groups approximately. Strain CK and strain CB are divided into a same group. Strain LC, LX and CZ were divided to a same group. Gene expression of strain CK was similar to strain CB in either all genes, 165 genes associated with detoxification enzymes or in 24 genes associated with pigmentations while the gene expression of strain LC was

similar to strain LX (Figs. 4A, 4B, 4C). 116 genes up-regulated or down-regulated in 10 differential comparison groups were as follow: 18 genes associated with pigmentations, 74 P450 genes, 21 GST genes and 3 *CarE* gene. The expression pattern of these 116 genes also showed that expressions of these 116 genes in strain CK were similar to strain CB while expressions of these 116 genes in strain LC were similar to strain LX (Fig. 4D).

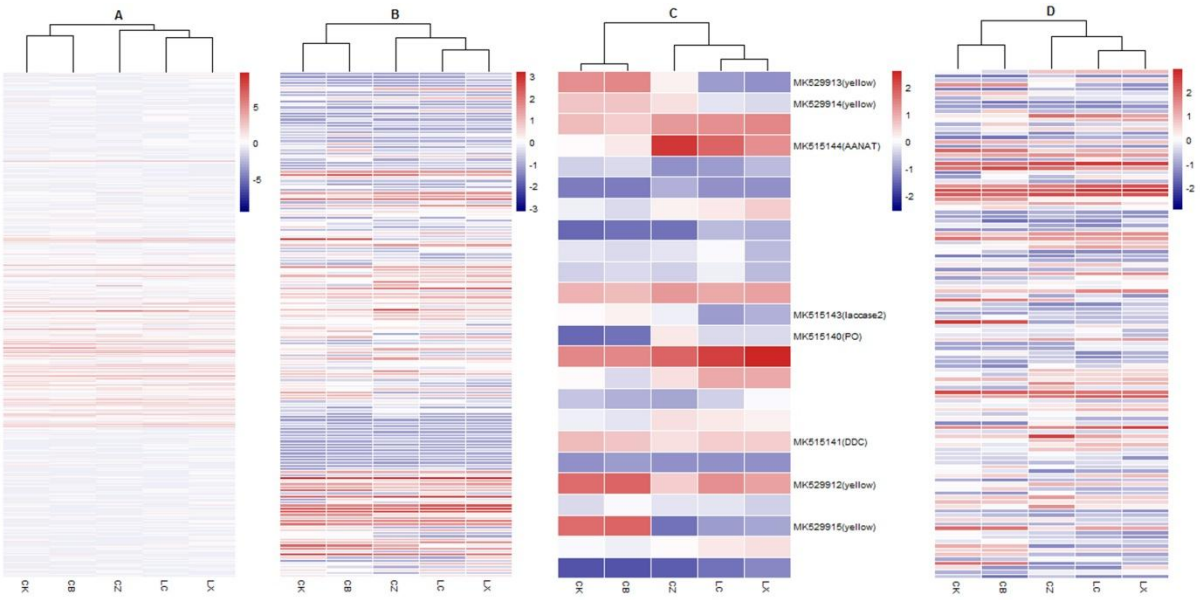

Figure 4 Relationships of gene expressions of 5 strains. (A) All genes of 5 strains, (B) 165 genes associated with detoxification enzymes of 5 strains, (C) 24 genes associated with pigmentations of 5 strains, (D) 116 genes up-regulated or down-regulated in 10 differential comparison groups of 5 strains.

Notes: The row name was the family name of gene. The number in the expansion was the accession number of gene in GenBank. Genes associated with pigmentations and marked with accession numbers were subjected to real-time PCR amplifications.

Analysis of expressions of genes in *B. dorsalis* pupae

Expressions of 9 target genes in 6 strains were quantified in the PCR amplifications respectively. Strain CB_Z was a strain selected for 10 generations base on strain CB. Expressions of 9 target genes in strain CB_Z were used for observations. Genes expressions of strains CK were regarded as references with relative expressions for 1.00.

Six strains were analyzed based on 8 comparison groups in 4 parts: two comparison groups CK vs CZ and CB vs CB_Z were conducted to reveal the relationships between resistant strains and non-resistant strains; one comparison group CK vs LX was conducted to reveal the relationships between brown-pupae strains; one comparison group CB vs LC was conducted to reveal the relationships between white-pupae strains; four comparison groups CK vs CB, CK vs LC, LX vs CB and LX vs LC were conducted to reveal the relationships between brown-pupae strains and white-pupae strains.

In the revelation of the relationship between brown pupae and white pupae, results showed in groups LX vs CB and LX vs LC were as follow: relative expressions of *yellow* gene *MK529913*, *DDC* gene *MK515141* and *laccase2* gene *MK515143* of pupae with brown pigmentation (strain LX) were significantly higher than those in pupae with white pigmentation (strains CB, LC) respectively. Relative expressions of *yellow* gene *MK529913* in strains LX, CB and LC were 6.88±0.22, 0.98±0.28 and 2.03±0.80 respectively (Fig. 5A). Relative expressions of *DDC* gene *MK515141* in strains LX, CB and LC were 2.96±0.42, 0.83±0.13 and 0.44±0.23 respectively (Fig. 5H). Relative expressions of *laccase2* gene *MK515143* in strains LX, CB and LC were 16.40±4.89, 1.35±0.42 and 2.13±1.27 respectively (Fig. 5E). Group LX vs CB showed that the relative expression of *PO* gene *MK515140* in strain LX was 3.08±1.06 which was significantly higher than 0.08±0.01 in strain CB (Fig. 5G). Group CB vs LC was conducted for reveling relationships between white pupae. It showed that relative expressions of nine target genes including *yellow* gene *MK529913* (Fig. 5A), *DDC* gene *MK515141* (Fig. 5H), *laccase2* gene *MK515143* (Fig. 5E) and *PO* gene *MK515140* (Fig. 5G) in strain CB and LC were not significantly higher or lower than each other respectively.

These results above indicated that *yellow* gene *MK529913*, *DDC* gene *MK515141*, *laccase2* gene *MK515143* and *PO* gene *MK515140* played important roles in the pupae pigmentation of *B. dorsalis*.

The relative expression of *AANAT* gene *MK515144* in strain CK was significantly lower than 2.06±0.54 in strain LX (Fig. 5F). Combined with that the pigmentation of pupae of strain LX was

lighter than strain CK, we estimated that *AANAT* gene *MK515144* might contribute to the color-less pigmentation in pupae of *B. dorsalis*.

There was no significant difference of P450 gene *MK515142* between each two strains in 8 comparison groups mentioned above (Fig. 5I).

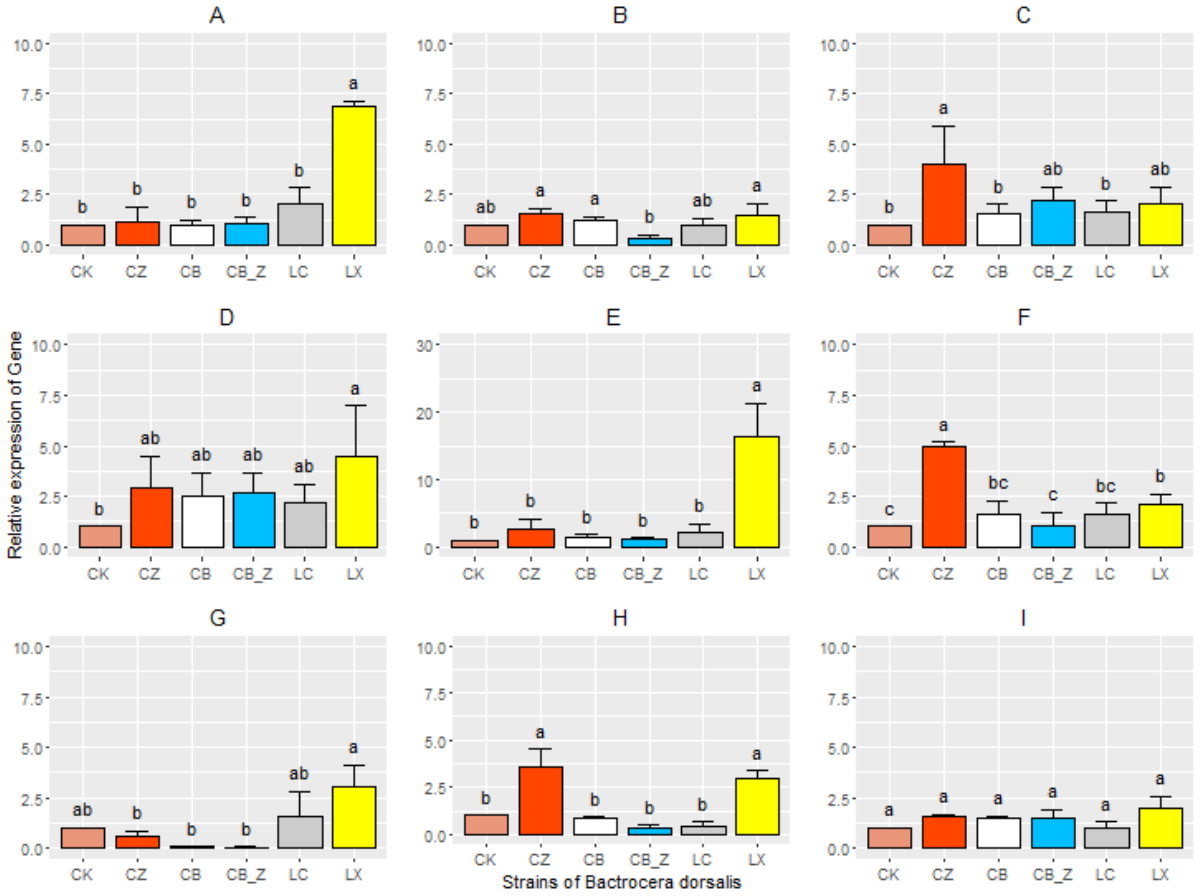

Figure 5 Expresions of 9 genes in pupae of 6 strains of *B. dorsalis*.

(A) Expresion of gene *MK529913*(*yellow*). (B) Expresion of gene *MK529914*(*yellow*). (C) Expresion of gene *MK529912*(*yellow*). (D) Expresion of gene *MK529915*(*yellow*). (E) Expresion of gene *MK515143*(*laccase2*). (F) Expresion of gene *MK515144*(*AANAT*). (G) Expresion of gene *MK515140*(*PO*). (H) Expresion of gene *MK515141*(*DDC*). (I) Expresion of gene *MK515142*(P450).

Notes: X-axis standed for strains of *B. dorsalis*. Y-axis standed for relative expression of genes. Length of column standed for the average of relative expression of gene. Line on column standed for standard error. The difference of letters on columns standed for significant by DMRT (a=0.05) analysis of variance.

Analysis of expressions of genes in *B. dorsalis* adults

We combined results of efficacy tests of adults mentioned above with expressions of 9 target genes in adults. Results of efficacy tests showed that resistances of 5 strains in the efficacy test of *beta*-cypermethrin and abamectin were roughly as CZ > CB > CK > LC ≈ LX (high to low) in general. Resistances of 5 strains in the efficacy test of trichlorfon were roughly as CZ > LC > CK > LX ≈ CB (high to low).

In groups CK vs CZ and CB vs CB_Z, we known that P450 gene *MK515142* showed a relative expression 0.19 ±0.03 in strain CZ significantly lower than 1 in strain CK and showed a relative expression 0.07 ±0.00 in strain CB_Z significantly lower than 0.51 ±0.06 in strain CB (Fig. 6I). These results could not indicated that the high expression of P450 gene *MK515142* could contributed to resistances of *B. dorsalis* to *beta*-cypermethrin and abamectin.

In the other hand, from groups CK vs LX, CB vs LC, CK vs LC and LX vs CB, we known that P450 gene *MK515142* showed a significantly higher relative expression in strain CK than 0.16 ±0.04 in strain LX (Fig. 6I). The relative expression of this gene in strain CK was 0.51 ±0.06 significantly higher than 0.11 ±0.02 in strain LC (Fig. 6I). The relative expression of this gene in strain LX was 0.16 ±0.04 significantly higher than 0.51 ±0.06 in strain CB (Fig. 6I). These results indicated that the high expressions of P450 gene *MK515142* might contribute to resistances of *B. dorsalis* to *beta*-cypermethrin and abamectin.

Efficacy tests of adults with trichlorfon showed that resistances of 5 strains were roughly as CZ > LC > CK > LX ≈ CB (high to low). The relative expression of P450 gene *MK515142* in strain CK was higher than 0.16 ±0.04 in strain LX (Fig. 6I). These results indicated that the high expression of P450 gene *MK515142* might contribute to resistances of *B. dorsalis* to trichlorfon.

Gene expressions patterns of *yellow* gene *MK529912* were similar to gene expressions patterns of *yellow* gene *MK529914*. Relative expressions of these two genes in strain CB were 0.81 ±0.06, 1.97 ±0.53 respectively, significantly lower than 2.50 ±1.01, 6.14 ±1.33 in strain LC respectively (Figs. 6C, 6B). These results indicated that expressions of genes could be significantly different between white-pupae strains (CB and LC) while these expressions could contribute to

pigmentations of *B.dorsalis* pupae.

Group CK vs LC showed that relative expressions of *yellow* genes *MK529912* and *MK529914* in strain CK were significantly lower than 2.50±1.01 and 6.14±1.33 respectively (Figs. 6C, 6B). In the other hand, group LX vs LC showed that expressions of these two genes were 0.57±0.10 and 1.52±0.48 respectively, significantly lower than expressions of these two genes in strain LC (Figs. 6C, 6B). These results indicated that expressions of *yellow* genes *MK529912* and *MK529914* in white-pupae strains could be significantly higher than what in brown-pupae strains although these two genes were reported to play roles in melanization promotings.

Based on these 3 groups (CB vs LC, CK vs LC and LX vs LC), we could not estimated that high expressions of *yellow* genes *MK529912* and *MK529914* could contributed to resistances of *B. dorsalis* to *beta*-cypermethrin and abamectin. Instead of this result, these 3 groups proved that high expressions of *yellow* genes *MK529912* and *MK529914* could contribute to the resistance of *B. dorsalis* to trichlorfon.

Groups CK vs LX, LX vs CB and LX vs LC showed that the relative expression of *yellow* gene *MK529913* in strain LX was 4.88±1.99, significantly higher than 1.00 in strain CK, 0.43±0.21 in strain CB and 0.94±0.31 in strain LC respectively (Fig. 6A). It could not be indicated that high expressions of this gene could contribute to resistance of *B. dorsalis* adults to *beta*-cypermethrin and abamectin. However, it could be indicated that high expression of this gene could both contribute to pigmentations of pupae and contribute to resistances of adults which emergenced from brown pupae to *beta*-cypermethrin and abamectin.

Groups CK vs CZ and CB vs LC showed that the relative expression of *yellow* gene *MK529915* in strain resistance strain CZ was 0.38±0.08 significantly lower than 1.00 in strain CK. The relative expression of this gene in strain CB was 0.29±0.06 significantly lower than 0.95±0.31 in strain LC (Fig. 6D). These results indicated that high expressions of *yellow* gene *MK529915* might reduce resistances of adults to *beta*-cypermethrin and abamectin. But in the other hand, groups CK vs LX and LX vs LC showed that the relative expression in strain LX was 0.32±0.06 significantly lower than 1.00 in strain CK and 0.95±0.31 in strain LC (Fig. 6D). It

indicated that high expressions of this gene might contribute to resistances of adults to *beta*-cypermethrin and abamectin. For results above, it could not be improved that high expressions of *yellow* gene *MK529915* could contribute to resistances of adults to *beta*-cypermethrin and abamectin. But these results showed that *yellow* gene *MK529915* might contribute to resistances of adults to trichlorfon.

Results showed in groups CK vs CZ and CB vs CB_Z were as follow: the relative expression of *AANAT* gene *MK515144* in strain CZ was 0.32±0.22 significantly lower than 1.00 in strain CK; the relative expression of this gene in strain CB_Z was 0.27±0.19 significantly lower than 1.29±0.13 in strain CB (Fig. 6F). We could not estimate that whether high expressions of *AANAT* gene *MK515144* could contribute to resistances of adults to *beta*-cypermethrin and abamectin from these results.

In the other hand, groups CK vs LX, CB vs LC, CK vs LC and LX vs CB showed that the relative expression of *AANAT* gene *MK515144* in strain CK was significantly higher than 0.05±0.03 in strain LX; the relative expression of this gene in strain CB was 1.29±0.13 significantly higher than 0.16±0.12 in strain LC; the relative expression of this gene in strain CK was significantly higher than 0.16±0.12 in strain LC; the relative expression of this gene in strain LX was significantly lower than the relative expression of this gene in strain CB (Fig. 6F). All of these results indicated that high expressions of *AANAT* gene *MK515144* might contribute to resistances of adults to *beta*-cypermethrin and abamectin.

Group CK vs LC showed that the relative expression of *DDC* gene *MK515141* in strain CK was significantly lower than 3.82±1.43 in strain LC (Fig. 6H). It indicated that there could be a significantly higher expression of this gene in adults whose pupae coloration were white, and either indicated that high expressions of *DDC* gene *MK515141* might reduce resistances of adults to *beta*-cypermethrin and abamectin while mitht contribute to resistances of adults to trichlorfon.

Group LX vs CB showed that the relative expression of *laccase2* gene *MK515143* in strain LX was 2.73±1.03 significantly higher than 0.45±0.20 in strain CB (Fig. 6E). It indicated that this gene played an important role in the pigmentation of pupae of *B. dorsalis*, and either indicated

that there could be a significantly higher expression of this gene in adults whose pupae coloration were brown. At the same time, the relative expression of *laccase2* gene *MK515143* in strain CK was significantly lower than 2.73±1.03 in strain LX, the relative expression of this gene in strain CB was significantly lower than 2.03±0.89 in strain LC (Fig. 6E). All of these results indicated that high expressions of *laccase2* gene *MK515143* might reduce resistances of adults to *beta*-cypermethrin and abamectin and either indicated that high expressions of this gene might contribute to resistances of adults to trichlorfon.

Group CK vs LC showed that the relative expression of *PO* gene *MK515140* in CK was significantly lower than 14.97±5.29 in strain LC (Fig. 6G). Group LX vs CB showed that the relative expression of this gene in strain LX was 15.87±1.83 significantly higher than 5.17±2.16 in strain CB (Fig. 6G). These results indicated that there could be a significantly higher expression either in adults whose pupae colorations were white or in adults whose pupae colorations were brown. However, all of these results consistently indicated that high expressions of *PO* gene *MK515140* might reduce resistances of adults to *beta*-cypermethrin and abamectin, and either indicated that high expressions of this gene might contribute to resistances of adults to trichlorfon.

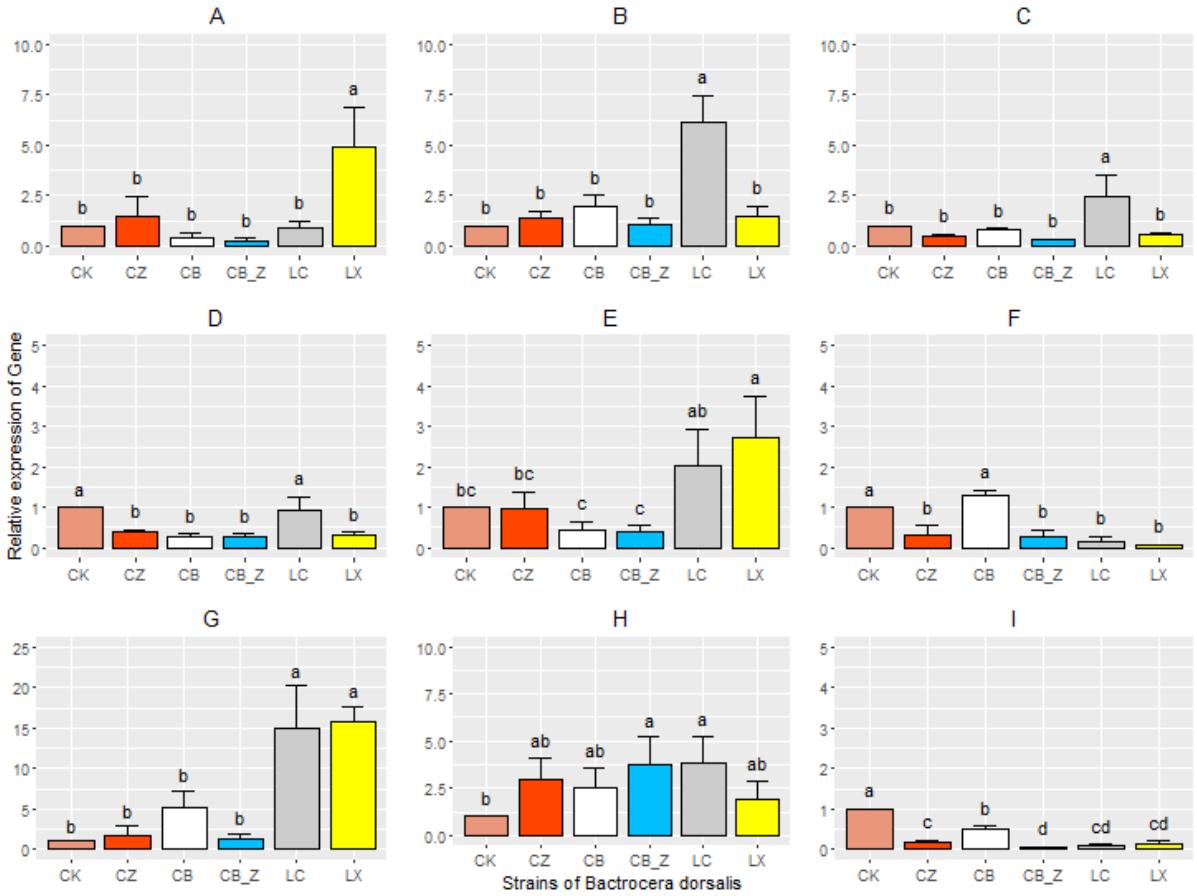

Figure 6 Expresions of 9 genes in adults of 6 strains of *B. dorsalis*.

(A) Expresion of gene *MK529913*(*yellow*). (B) Expresion of gene *MK529914*(*yellow*). (C) Expresion of gene *MK529912*(*yellow*). (D) Expresion of gene *MK529915*(*yellow*). (E) Expresion of gene *MK515143*(*laccase2*). (F) Expresion of gene *MK515144*(*AANAT*). (G) Expresion of gene *MK515140*(*PO*). (H) Expresion of gene *MK515141*(*DDC*). (I) Expresion of gene *MK515142*(P450).

Notes: X-axis standed for strains of *B. dorsalis*. Y-axis standed for relative expression of genes. Length of column standed for the average of relative expression of gene. Line on column standed for standard error. The difference of letters on columns standed for significant by DMRT(a=0.05) analysis of variance.

DISCUSSION

In this study, the relationship between pigmentations of pupae and resistances of adults in 5 strains of *B. dorsalis* was studied based on two basic logics: **a.** It was feasible to take

comparisons between pigmentations of *B. dorsalis* pupae and resistance of *B. dorsalis* adults; **b.** High expressions of genes could effectively contribute to actions of enzymes related to pigmentations and resistance (Salam *et al.*, 1981; Hernandez *et al.*, 2002; Gao *et al.*, 2002; David *et al.*, 2005; Lumjuan *et al.*, 2005; Strode *et al.*, 2008; Deng *et al.*, 2009; Lumjuan *et al.*, 2011; Qin *et al.*, 2011; Reid *et al.*, 2012; Rene *et al.*, 2014).

In all studies in this study, we tried to confirm the following four conjectures: **a.** 4 *yellow* genes (*MK529913*, *MK529914*, *MK529912* and *MK529915*), *laccase2* gene *MK515143*, *PO* gene *MK515140* and *DDC* gene *MK515141* were the main enzymes or the basic enzymes which could contribute to pigmentations of insects. As results, expressions of these genes in brown pupae should be consistently higher than expressions in white pupae. **b.** Gene *AANAT* (*MK515144*) could promote color-less in insects. As the result, expressions of it in white pupae should be higher than expressions in brown pupae. **c.** Resistances of *B. dorsalis* adults emergenced from brown pupae should be consistently higher or lower than resistances of *B. dorsalis* adults emergenced from white pupae. **d.** We could find some genes that are both significantly associated with pigmentations of *B. dorsalis* pupae and significantly associated with resistances of *B. dorsalis* adults. In other words, we hoped we could find some genes which could meet the following conditions: a. Expressions of these genes were high; b. Expressions of these genes in high-resistance *B. dorsalis* could significantly higher or lower than expressions in low-resistant *B. dorsalis*.

Most of these four conjectures had been confirmed in our studies. Results were showed in conclusions. Among these results, the more important results were as follow: *yellow* gene *MK529913*, *DDC* gene *MK515141*, *laccase2* gene *MK515143* and *PO* gene *MK515140* were proved to contribute to pigmentations of *B. dorsalis* pupae; *AANAT* gene *MK515144* was proved to contribute to color-less of *B. dorsalis* pupae; high expressions of P450 gene *MK515142*, *AANAT* gene *MK515144* were indicated to contribute to resistances of *B. dorsalis* adults to *beta*-cypermethrin and abamectin; high expression of *yellow* gene *MK529913* might contribute to resistances of adults which emergenced from brown pupae to *beta*-cypermethrin and abamectin;

high expressions of *yellow* genes (*MK529912*, *MK529914*, *MK529915*) and *laccase2* gene *MK515143*, *PO* gene *MK515140* and *DDC* gene *MK515141* were indicated to contribute to trichlorfon. All of these results were consistent to what showed in many reports as: *yellow* gene, *DDC* gene, *laccase2* gene and *PO* gene were genes which could promote pigmentations of insects (Wittkopp *et al.*, 2003; Ferguson *et al.*, 2011; Futahashi *et al.*, 2008; Futahashi *et al.*, 2010; Yatsu *et al.*, 2009; Ferguson *et al.*, 2011; Futahashi *et al.*, 2012) , *AANAT* gene *MK515144* was the gene which could promote color-less of insect (Tsugehara *et al.*, 2007; Zhan *et al.*, 2010; Zhang *et al.*, 2018b), P450 genes were genes which could contribute to resistances of insects (Arensburger *et al.*, 2010; Rene, 2014; Pang *et al.*, 2014; Peng *et al.*, 2016; Terhzaz *et al.*, 2015; Xi *et al.*, 2015).

In addition, we found some interesting phenomena as follow:

(1) Strain CZ had been selected with *beta*-cypermethrin from strain CK for a long time. The pattern of genes expressions of strain CZ was different from the pattern of genes expressions of Strain CK. The pattern of genes expressions of strain CZ was similar to patterns of genes expressions of Stains LC and LX which were the artificial mutant strains named GSS strains (Ji *et al.*, 2007; Isasawin *et al.*, 2012).

Gender separation techniques had been studied well in *Ceratitis capitata* (Augustinos *et al.*, 2017), *Anopheline mosquitoes*(Federiac *et al.*, 2018) and other insects (Lees *et al.*, 2015; Zhang *et al.*, 2015b; Bourtzis *et al.*, 2016; Zhang *et al.*, 2016). Gene *Yob*, gene *rdl* and other genes had been proved to be important for Gender separations (Cyrille *et al.*, 2018; Elzbieta *et al.*, 2018). Gender separations could effect on classical genetic, molecular, mechanical, behavioral, developmental and symbiont-based approaches of insects (Federiac *et al.*, 2018).

Therefore, we estimated that expressions of abundant genes of *B. dorsalis* might change because of artificial mutants. As of it, why genes expressions patterns of strains LC and LX were different to other 2 strains (CK, CB) could be explained. We estimated that genes expressions of *B. dorsalis* could not change much because of natural mutants. As of it, why genes expressions patterns of strain CB was similar to strain CK and deeply different to strains LC and LX could be

explained. These conjectures had not been proved and should be further verified.

(2) Relative expressions of P450 gene *MK515142* of *B. dorsalis* pupae did not showed significantly difference between each two strains. Mean while, relative expressions of this of *B. dorsalis* adults showed significantly difference between each 2 strains.

Here, we estimated that some genes which were associated to pigmentations or detoxifications of *B. dorsalis* could show differences in adults and contribute to environmental adaptabilities of *B. dorsalis* adults. As of it, developments of insects should be an important factor to be considered in searches of genes associated to resistance.

(3) Groups CK vs CZ and CB vs CB_Z showed that the relative expression of P450 gene *MK515142* in strain CZ was significantly lower than the relative expression in strain CK, the relative expression of this gene in strain CB_Z was significantly lower than the relative expression in strain CB while strains CZ and CB_Z were resistant strains. But in the other hand, this gene were proved to contribute to resistances of adults to *beta*-cypermethrin and abamectin in groups CK vs LX, CB vs LC, CK vs LC and LX vs CB. Here, we estimated that resistance of *B. dorsalis* should be determined by a combination of factors instead of a few genes associated to detoxifications (Gao *et al.*, 2013; Huang *et al.*, 2013; Huang *et al.*, 2016).

In general, it was revealed that there were many differences between strains of *B. dorsalis* in both genes associated to pupae pigmentations of *B. dorsalis* and resistance of adults. Therefore, the direction of our studies was right to studying relationships between pigmentations of *B. dorsalis* pupae and resistance of *B. dorsalis* adults. Desirable results were obtained in our studies also.

Here, we hoped we could contribute to managements of *B. dorsalis* by further studies which were about relationships between pupae pigmentations and resistance of *B. dorsalis*.

CONCLUSIONS

This paper improved our understanding of the relationship between pigmentations of pupae and insecticide resistance of adults of *B. dorsalis*. Efficacy tests, transcriptome sequencings of pupae

and gene quantifications showed that some genes could both contribute to pigmentations of pupae and insecticide resistance of *B. dorsalis* adults.

We combined results of efficacy tests of adults mentioned above with expressions of 9 target genes in adults. Results of efficacy tests showed that resistances of 5 strains in the efficacy tests of *beta*-cypermethrin and abamectin were roughly as CZ > CB > CK > LC ≈ LX (high to low) in general (Figs. 2A, 2B). Resistances of 5 strains in the efficacy tests of trichlorfon were roughly as CZ > LC > CK > LX ≈ CB (high to low) (Fig. 2C).

We conducted analysis for expressions of 5 strains (CK, CZ, CB, LC and LX) obtained in the transcriptome sequencings. We found that genes associated with pigmentations and genes associated with detoxifications could both play roles in 8 biological processes, 8 cell components and 14 molecular functions (Figs. 3A, 3B and 3C).

Based on heat maps analysis of genes expressions with Pearson correlation methods, we found that the pattern of genes expressions of strain CK (wild strain) was similar to strain CB (pigmentation mutation) while these two strains were far away from strains LC and LX, and the gene expression of strain CZ was more similar to strains LC and LX (Figs. 4A, 4B, 4C and 4D).

Analysis of relative expressions of *B. dorsalis* pupae showed that *yellow* gene *MK529913*, *DDC* gene *MK515141*, *laccase2* gene *MK515143* and *PO* gene *MK515140* played important roles in the pupae pigmentation of *B. dorsalis* (Figs. 5A, 5E, 5G and 5H). We estimated that *AANAT* gene *MK515144* might contribute to the color-less pigmentation in pupae of *B. dorsalis* (Fig. 5F). There was no significant difference of P450 gene *MK515142* between each two strains in 8 comparison groups mentioned above (Fig. 5I).

Analysis of relative expressions of *B. dorsalis* adults showed that relative expressions of P450 gene *MK515142* in strains CK and CB were significantly higher than those in resistance-strains CZ and CB_Z respectively. It could not be indicated that whether the high expressions of this gene could contribute to resistance of *B. dorsalis* adults. However, comparisons between other strains proved that the high expressions of this gene could contribute to resistances of *B. dorsalis* adults to *beta*-cypermethrin and abamectin(Fig. 6I).

The high expression of *yellow* gene *MK529913* could both contribute to pigmentations of pupae and contribute to resistance of adults which emergenced from brown pupae to *beta*-cypermethrin and abamectin(Fig. 6A). High expressions of *yellow* genes *MK529912*, *MK529914* and *MK529915* might contribute to the resistance of *B. dorsalis* to trichlorfon. However, we could not estimated that whether high expressions of these 3 genes could contribute to resistances of *B. dorsalis* to *beta*-cypermethrin and abamectin (Figs. 6C, 6B and 6D).

The high expression of *AANAT* gene *MK515144* might contribute to resistances of adults to *beta*-cypermethrin and abamectin (Fig. 6F). High expressions of *laccase2* gene *MK515143*, *PO* gene *MK515140* and *DDC* gene *MK515141* might reduce resistances of adults to *beta*-cypermethrin and abamectin, while they might contribute to resistance of adults to trichlorfon.

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Competing Interests

All authors declare that they have no competing interests. All authors declare their employment with any non-academic affiliations in the Competing Interest statement. Gu-Qian Wang is employed by Jiangxi Yang's Fruit CO., Ltd.

Author Contributions

- Yu-Peng Chen conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and tables, authored or reviewed drafts of paper, approved the final draft.

- Gu-Qian Wang, Ting Yu, Xiao-Yan Peng, Tao Li, Zhou Gao, Xue Bai, Chun-Yan Zheng designed the experiments.
- Guang-Wen Liang approved the final draft.
- Yong-Yue Lu conceived and designed the experiments authored or reviewed drafts of the paper, approved the final draft.

Supplemental Information

Protein sequences and alignments were provided in Supplemental files.

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Figure 1 5 strains of *B. dorsalis*.

Figure 2 Respondings of *B. dorsalis* adults to *beta*-cypermethrin, abamectin and trichlorfon.

Figure 3 The relationship of 5 strains in biological process, cell component and molecular function.

Figure 4 Relationships of gene expressions of 5 strains.

Figure 5 Expresions of 9 genes in pupae of 6 strains of *B. dorsalis*.

Figure 6 Expresions of 9 genes in adults of 6 strains of *B. dorsalis*.

Supplemental file 1 The protein sequences and alignments of 127 P450 genes.

Supplemental file 2 The protein sequences and alignments of 33 GST genes.

Supplemental file 3 The protein sequences and alignments of 1 AcE gene.

Supplemental file 4 The protein sequences and alignments of 4 CarE genes.

Supplemental file 5 The protein sequences and alignments of 10 yellow genes.

Supplemental file 6 The protein sequences and alignments of 1 AANAT gene.

Supplemental file 7 The protein sequences and alignments of 2 laccase2 genes.

Supplemental file 8 The protein sequences and alignments of 1 ebony gene.

Supplemental file 9 The protein sequences and alignments of 1 tan gene.

Supplemental file 10 The protein sequences and alignments of 2 PO genes.

Supplemental file 11 The protein sequences and alignments of 2 ADC genes.

Supplemental file 12 The protein sequences and alignments of 2 DDC genes.

Supplemental file 13 The protein sequences and alignments of 1 PAH gene.

Supplemental file 14 The protein sequences and alignments of 1 TH gene.

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