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Molecular cloning and expression of glutathione *S*-transferases involved in propargite resistance of the carmine spider mite, *Tetranychus cinnabarinus* (Boisduval)

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ABSTRACT

The carmine spider mite (CSM) *Tetranychus cinnabarinus* has become a serious pest in China and has developed resistance to acaricide propargite as it is used to control mites worldwide including *T. cinnabarinus*. In this study, a resistant colony of *T. cinnabarinus*, PRR34 (37.78-fold resistant ratio), was established after 34 generations of propargite selection, and cross-resistance patterns of 7 other acaricides were determined in comparison with a susceptible strain (SS). The contribution of detoxification enzymes to propargite tolerance were investigated using biological, biochemical and molecular approaches. Enzyme inhibitor synergist tests suggested glutathione *S*-transferases (GST) involvement in propargite-resistance of PRR34, and GST activity against 1-chloro-2,4-dinitrobenzene (CDNB) was correlated with the development of resistance. Eight novel GST genes (*TcGSTd1, TcGSTd2, TcGSTm1, TcGSTm2, TcGSTm3, TcGSTm4* and *TcGSTm5*) were cloned, and phylogenetic analysis showed that the eight GST genes were most closely related to GST family delta and mu from *Tetranychus urticae*. Quantitative RT-PCR revealed that the expression level of GSTs in PPR34 strain increased in larvae, nymphs and adults, while decreased in eggs compared with that of SS. Collectively, these results support a role of GSTs in mediating resistance to propargite in the PRR34 strain. *TcGSTd1,TcGSTd2* and *TcGSTm2* genes might play significant roles in propargite resistance of CSM, especially at adult stage. This is the first attempt to define specific genes involved in GST mediated propargite resistance of *T. cinnabarinus* at the transcriptional level.

1. Introduction

The carmine spider mite (CSM), *Tetranychus cinnabarinus* (Boisduval) (Acarina: Tetranychidae), is a polyphagous species with nearly 100 host plant species, and is one of the most serious pests in many cropping systems worldwide including various vegetables and fruits, cotton, and ornamentals [1,2]. The control of CSM is still very much dependant on acaricides/insecticides. A number of compounds with different chemical structures and modes of action have been used. Moreover, CSM has a strong ability to develop resistance to chemicals rapidly due to its short life cycle, high fecundity, inbreeding, and parthenogenesis reproduction [3].

Propargite has demonstrated significant acaricidal effects against mites in ornamentals and orchards [4]. Since introduction almost 50 years ago, propargite has been widely used in China and in other regions of the world. Consequently, propargite resistance has been reported in a number of studies [4–6] .

Understanding insecticide resistance mechanism is important for sustainable pest management. Similar to insects, enhanced enzymatic detoxification (quantitative or qualitative) and target site insensitivity were major causes of acaricide resistance development in Acari. Molecular analysis allows identification of specific changes at genomic level in target sites and/or in characteristics of detoxification enzymes in resistant populations [7]. The major detoxification enzymes that metabolize acaricides before they reach at their target sites are carboxylesterase (COEs), cytochrome P450 monooxygenases (P450s) and glutathione *S*-transferases (GSTs). Metabolic resistance mechanisms associated

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with enhanced activities of P450s, GSTs and COEs were reported in mites, such as abamectin and fenpropathrin-selected CMS, bifenthrin and k-cyhalothrin-selected CMS and *Tetranychus evansi* [2,8]. Several studies have implicated GST enzyme family conferring resistance to multiple classes of insecticides [9–11].

In present study, systemic efforts with a multi-level approach (biological, biochemical and molecular levels) were made to understand propargite resistance mechanisms in *T. cinnabarinus*. First, we focused on generating a propargite-resistant strain of *T. cinnabarinus* through laboratory selections. Then, cross-resistance to other acaricides and the effects of three enzyme inhibitory synergists [piperonyl butoxide (PBO), triphenyl phosphate (TPP) and diethyl maleate (DEM)] on the resistance were investigated. Sequences of cDNA of GSTs were cloned from the SS stain and phylogenetic analysis was performed. Finally, comparative analyses of mRNA expression of the identified GST genes in susceptible (SS) and propargite-resistant (PRR34) strains were performed for mites of different life-stages. The results not only provide valuable guidance for resistance management strategy of mites, but also provide insight information for mite resistance specifically to propargite and to mitochondrial ATP synthase inhibitor acaricides in general.

2. Experimental methods

2.1. Acaricides and synergists

Seven acaricides (technical grade) belong to 5 classes of mode of action according to IRAC [12] were tested in this study. They were dichlorvos (80% purity), phoxim (92%) and methomyl (80%) (Kunming Pesticide Factory, Kunming, China) of IRAC MoA group 1 – acetylcholinesterase (AchE) inhibitors; propargite (90%) and diafenthiuron (95%) (Qingdao Pesticide Factor, Qingdao, China) of IRAC MoA group 12 – inhibitors of mitochondrial ATP synthase; pyridaben (96%) (Qingdao Pesticide Factor, Qingdao, China) of IRAC MoA group 21 – mitochondrial complex I electron transport inhibitors; avermectins (95.5%) (Wangfeng Pesticide Factory, Beijing, China) of IRAC MoA group 6 – chloride channel activators; and chlorfenapyr (98.8%) (Yunnan Chemical Research Institute) of IRAC MoA group 13 – uncouplers of oxidative phosphorylation via disruption of the proton gradient.

Three enzyme inhibitor synergists tested were P450 inhibitor piperonyl butoxide (PBO, 90%), GST inhibitor diethyl maleate (DEM, 97%) (Sigma–Aldrich, Dorset, UK), and COE inhibitor triphenyl phosphate (TPP, 100%) (Kelong Chemical Company, Chengdu, China).

2.2. Mite strains and sample preparation

A susceptible strain (SS) of *T. cinnabarinus* was established from a field collection in Chongqing of China in 2000, and maintained in the laboratory with no insecticide/acaricide exposure. This susceptible strain was selected with propargite consecutively for 34 generations under laboratory conditions to generate a propargite-resistant strain (PRR34; see below for more information). All rearing of *T. cinnabarinus* were performed on cowpea plants (*Vigna unguiculata*) under 25 ± 1 °C, 60–80% relative humidity (RH) and a 14L:10D photoperiod.

To prepare mites of different stages for genetic analysis, cowpea leaves were cut into 4 cm diameter discs and each was placed on a 5 cm layer of water-saturated cotton in petri dishes (9 cm diameter). A total of 90 such petri dishes were prepared, and each was infested with 30 egglaying female adults for 24 h before being removed. Eggs on 30 of the leaf discs were brushed into three centrifuge tubes and stored at −80 °C for RNA extraction. Eggs on the rest of 60 leaf discs were untouched and allowed to develop in the petri dishes for collection of larvae, nymphs, and female adults. Fresh leaf discs were supplied as needed [13].

2.2.1. Resistance selection

Propargite resistant strain of *T. cinnabarinus* was selected according to He et al. [3]. Briefly, propargite was sprayed on cowpea plants infested with mites at concentrations that killed about 85% of the populations. At 48 h after spraying, mortality was recorded and survival individuals were transferred to fresh cowpea plants and allowed to stay for 48 h for oviposition. When the new generation developed to adults, a dose–response bioassay was performed to monitor susceptibility changes (LC_{50}) and to determine the proper concentration for next selection run. Probit analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, USA) for LC_{50} calculations. Resistance ratio (RR) = LC_{50} value of resistance-selected strain/LC_{50} value of SS strain.

2.3. Bioassay

2.3.1. Acaricide and synergist bioassay

A slide-dip method recommended for spider mites by Busvine (1980) [14] was used. Acaricides were first dissolved in acetone (10000 mg/L), and then diluted with double-distilled water (ddH_2O) to desirable concentrations. Female adults (25–30 individuals) were fixed on a slide to dip into the acaricide solutions for 5 s. After dipping, the slides were kept under laboratory conditions described above. Mortality was recorded after 24 h. A mite was considered dead if no movement of any appendages after stimulation by a fine brush. Each population was tested with six concentrations of each acaricide (pre-determined by preliminary tests), plus a ddH_2O only treatment as control with three replications.

Synergist bioassays were performed similarly, except that the mites were treated with maximum sublethal doses of PBO (200 mg/L), TPP (1000 mg/L) and DEM (1000 mg/L) 60 min prior to acaricide dipping [15]. Synergistic ratios (SR) and synergistic resistance ratios (SRR) were calculated using the following formula: SR = LC_{50} for propargite alone/LC_{50} for propargite with synergist; SRR = LC_{50} of PRR34 strain/LC_{50} of SS strain.

2.3.2. Enzyme sample preparation and activity assays

Two hundred female adult mites of each strain were homogenized in 1000 μL of ice-cold sodium phosphate buffer (0.04 M, pH 6.5). The homogenate was centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was transferred into a clean micro-centrifuge tube and was used as enzyme source. There were at least three biological replications for each strain.

GST activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma–Aldrich) as a substrate in a 96-well microplates [16,17]. The total reaction volume in each microplate well was 300 μL, consisting of 100 μL of the enzyme solution, and 100 μL CDNB (containing 2% (v/v) ethanol), giving final concentrations of 0.6 mM CDNB. The control wells contained a mixture of CDNB without the enzyme solutions. The absorbance of 340 nm was measured (xMark Microplate Reader, BIO-RAD, USA) at 15 s interval during a 5-min reaction period at 37 °C. Changes in absorbance per minute were converted into nmol CDNB conjugated/min/mg protein based on the extinction coefficient of the resulting 2, 4-dinitrophenyl-glutathione (ε_{340nm} = 9.6 mM^{-1} cm^{-1}). Total protein amount of the enzyme source was determined according to the Bradford method with BSA as the standard [18]. Data were analyzed by one-way ANOVA followed by *t*-test for mean comparison using SPSS16.0.

2.4. Cloning of GST genes from the CSM susceptible strain

Total RNA was extracted from homogenate of *T. cinnabarinus* female adults of the susceptible strain using RNeasy® plus Micro Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instruction. Genomic DNA was removed by passing through a gDNA elimination column supplied by the kit. A reverse transcription was carried out using PrimeScript® 1st Strand cDNA Synthesis Kit (Takara Biotechnology Dalian Co., Ltd., Dalian, China) and the synthesized cDNA was stored at −20 °C. Based on the available genome sequence of *Tetranychus urticae* (http://bioinformatics.psb.ugent.be/webtools/bogas/overview/Teur), gene-specific primers were designed to amplify the complete GSTs open reading frame in overlapping fragments (Table S1). PCRs were carried out in a C1000™ Thermal Cycler (Bio-Rad, Laboratories, Inc., USA). The total volume of PCR was 25 μL that contained 15.25 μL ddH_2O, 2.5 μL 10× PCR buffer (Mg^{2+} free), 2.0 μL Mg^{2+}, 2.0 μL dNTP Mixture (2.5 mM), 1 μL cDNA as a template, 1 μL each primer (10 μM), and 0.25 μL rTaq™ polymerase (Takara, Tokyo, Japan). The program was pre-denaturation for 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 62–65 °C, 60 s at 72 °C and a final extension of 3 min at 72 °C. PCR products were analyzed on a 1% agarose gel and the band of interest was purified using Gel Extraction Mini Kit (Watson Viotechnologies, Inc., Shanghai, China). Purified cDNA fragments were cloned into the pMD-19T easy vector and transfected into *Escherichia coli* JM109 cells (Takara). Several recombinant clones were identified by PCR amplification and the products were subjected to direct sequencing (Invitrogen Life Technologies, Shanghai, China).

DNA sequences were edited with DNAMAN 5.2.2 (Lynnon BioSoft). The deduced amino acid sequences for GST genes were trimmed and aligned using Clustal X. The molecular weight of the proteins was calculated by ExPASy Proteomics Server (http://cn.exasy.org/tools/pi_tool.html). Homologous GST cDNAs and protein sequences were searched by BLASTX and BLASTP in the NCBI databases (http://www.nibi.nlm.nih.gov). Phylogenetic tree was constructed with MEGA 3.0 [19] using the Maximum Likelihood method.

2.5. Quantitative real-time PCR (RT-qPCR)

The expression level of mRNA was measured for different life stages of the two mite strains by normalizing against a stable reference gene, RPS18, evaluated by Sun et al. [20]. Specific primer pairs of GST genes were designed using Primer 3.0 (http://frodo.wi.mit.edu/). The primers for the genes of interest and the reference gene (RPS18) are listed in Table S2. Mite samples, RNA isolation and DNase treatment were performed as described above. The qPCR reactions were performed on a StepOne Plus Real-Time PCR system (Applied Biosystems, USA) using the Maxima SYBR green qPCR master mix with ROX solution (Fermentas Life Sciences, USA) according to the manufacturer's instructions. The efficiency of PCR amplification for gene-specific primers was analyzed using one cDNA sample with five serial dilutions of three technical replications. The reaction consisted of 2 μL cDNA, 10 μL SYBR® Premix Ex Taq ™ II (Perfect Real Time) (Takara) and 0.2 mM each of forward and reverse gene specific primers. The optimized thermal program was 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s. A fluorescence reading was used to determine the extension of amplification at the end of each cycle. Quantification of the transcript level or relative number of gene copies was conducted according to the $2^{-\Delta Ct}$ methodology [21]. The mRNA expression in egg, larva, nymph, and female adult was evaluated for the SS and PRR34 strains, respectively. The mRNA expression level of eggs served as the calibrator with mRAN expression ratio of 1.0. Each RT-qPCR experiment consisted of three independent biological repeats with three technical replicates. Differences in expression levels among different developmental stages and between different strains were analyzed by one-way analysis of variance (ANOVA), followed by *t*- test using SPSS16.0. The level of significance was set at $P < 0.05$.

3. Results

3.1. Resistance strain selection

The propargite selection increased LC_{50} gradually. At 26th generation (PPR26), the LC_{50} increased from 24.160 mg/L to 260.92 mg/L with resistance ratio of 10.80-fold (moderate resistance). After continuous exposure to propargite for 34 generations, the LC_{50} increased to 912.750 mg/L with RR of 37.78-fold (Table 1). This population (PRR34) was used as the resistance strain in this study. This result confirmed that continuous acaricide pressure can induce a certain level of *T. cinnabarinus* resistance to propargite.

3.2. Cross-resistance profile

Cross-resistance profile of the PRR26 and PRR34 strains was characterized (Table 2). Except for phoxim, PPR26 with 10.80-fold resistance to propargite showed no significant cross-resistance to the 6 tested acaricides. However, PRR34 showed various degrees of cross-resistance to all the 7 acaricides tested. The highest level of cross-resistance occurred to phoxim (71.08-fold), followed by pyridaben (32.27-fold) and chlorfenapyr (15.65-fold). The RR of methomyl, diafenthiuron, dichlorvos, and abamectin were 5.58, 3.60, 2.43 and 1.71-fold, respectively. Interestingly, methomyl and abamectin exhibited a negative cross-resistance pattern. With increasing resistance to propargite from PPR26 to PPR34, the resistance ratio of methomyl increased from 0.61-fold to 5.58-fold, but the resistance ratio of abamectin decreased from 2.29-fold to 1.71-fold. Diafenthiuron has the same mode of action as propargite, but the RR of PRR34 was only 3.6-fold. These results suggest that phoxim, pyridaben and chlorfenapyr should not be used as alternatives to manage propargite resistant mite populations, while abamectin, dichlorvos, diafenthiuron and methomyl may be the better options.

3.3. Synergism

The synergistic effect of DEM, PBO and TPP on PPR34 strain was 2.58, 1.47 and 1.06-fold, respectively. The synergistic data indicated the 95% LC_{50} CI for propargite and propargite plus DEM on

Table 1
Susceptibility *T. cinnabarinus* to propargite during laboratory selection.

Strains	χ^2	Slope (SE)	LC_{50} (95% CI[a]) (mg/L)	RR[b]
SS	5.65	2.60 (0.27)	24.16 (20.11–28.53)	1.00
PRR1	5.06	1.80 (0.40)	32.85 (11.10–53.19)	1.36
PRR5	11.85	2.24 (0.51)	62.16 (50.44–74.28)	2.57
PRR9	28.55	1.81 (0.20)	51.12 (36.06–71.18)	2.12
PRR12	15.60	2.47 (0.22)	153.21 (131.21–179.90)	6.34
PRR19	11.77	1.54 (0.20)	209.86 (159.27–281.24)	8.69
PRR24	15.28	1.93 (0.36)	208.92 (157.81–281.28)	8.65
PRR25	22.84	1.78 (0.21)	234.38 (149.30–368.18)	9.70
PRR26	22.18	0.84 (0.11)	260.92 (179.89–406.70)	10.80
PRR30	25.92	1.78 (0.16)	300.28 (234.97–393.17)	12.43
PRR31	17.13	1.40 (0.12)	280.48 (234.22–341.66)	11.61
PRR33	21.43	1.21 (0.34)	470.67 (320.75–516.08)	19.48
PRR34	23.89	1.04 (0.18)	912.75 (740.97–10079.74)	37.78

[a] Confidence interval.
[b] Resistance ratio (RR) = LC_{50} of resistance selected strain/LC_{50} of the SS strain.

Table 2
Resistance of PRR26 and PRR34 strain of *T. cinnabarinus* to 7 acaricides.

Acaricide	Strain	N[a]	χ^2	Slope (SE)	LC_{50} (95% CI[b]) (mg/L)	RR[c]
Diafenthiuron	SS	420	26.5	2.35 (0.24)	16.46 (10.41–23.48)	1.00
	PRR26	420	15.18	3.24 (0.28)	40.99 (32.75–51.08)	2.49
	PRR34	420	15.66	2.48 (0.20)	59.26 (42.81–86.66)	3.6
Dichlorvos	SS	420	1.67	3.20 (0.34)	93.68 (79.79–108.68)	1.00
	PRR26	420	5.09	4.02 (0.59)	142.63 (119.73–163.82)	1.52
	PRR34	420	19.53	2.51 (0.21)	227.60 (142.81–374.57)	2.43
Phoxim	SS	425	5.52	2.15 (0.23)	49.28 (9.55–59.67)	1.00
	PRR26	420	13.75	2.70 (0.41)	1469.23 (465.89–2517.81)	29.81
	PRR34	420	17.53	1.51 (0.18)	3502.66 (1847.84–16671.24)	71.08
Methomyl	SS	418	5.31	2.92 (0.31)	87.32 (73.29–102.18)	1.00
	PRR26	420	2.78	2.16 (0.20)	53.63 (44.77–63.61)	0.61
	PRR34	420	14.5	1.32 (0.16)	487.29 (377.53–657.25)	5.58
Pyridaben	SS	432	4.62	1.96 (0.19)	3.14 (2.54–3.80)	1.00
	PRR26	420	25.87	2.17 (0.24)	5.33 (1.94–15.27)	1.70
	PRR34	420	18.23	0.70 (0.16)	101.33 (35.46–1433.01)	32.27
Abamectin	SS	435	5.20	1.76 (0.26)	0.01 (0.01–0.02)	1.00
	PRR26	420	1.67	3.37 (0.39)	0.03 (0.03–0.04)	2.29
	PRR34	420	7.41	1.30 (0.15)	0.02 (0.02–0.03)	1.71
Chlorfenapyr	SS	442	4.13	2.49 (0.33)	0.86 (0.66–1.04)	1.00
	PRR26	420	11.98	3.31 (0.29)	4.69 (3.31–6.73)	5.46
	PRR34	420	21.45	1.25 (0.15)	13.46 (8.87–25.27)	15.65

[a] Total number of mites used in the experiments.
[b] Confidence interval.
[c] Resistance ratio (RR) = LC_{50} of resistance selected strain/LC_{50} of the SS strain.

PRR34 strain were 740.97–10079.74 and 258.04–561.50 respectively. The non-overlapping 95% CI at the LC_{50} level suggests that DEM significantly increased the susceptibility of PRR34 strain to propargite, showing the strongest synergistic effect. PBO also showed some level of synergistic effect, though it was not statistically significant. On the other hand, none of the synergists showed a significant synergistic effect on the SS strain (Table 3). These results indicated that P450-dependent monoxygenase- and GSTs-mediated metabolism may associate with the resistance and cross-resistance of the PRR34 strain. With DEM and PBO, the RR was decreased from 37.78-fold to 13.33- and 19.20-fold, respectively, which suggested that the metabolic detoxification by GSTs and P450s contributed about 65% and 49% of resistance to propargite in CSM, respectively. GST mediated metabolism may play a major role in propargite resistance in the PRR34 strain.

3.4. GST activity

Results of specific activity of GSTs with CDNB as the substrate in PRR26 and PRR34 were shown in Table 4. As RR increased from PRR26 and PRR34, the GST activity increased. The relative ratio of GST specific activity in adult mites of PRR26 was 1.22-fold compared to SS strain (SS = 10.54 ± 0.87 nmol min^{-1} μg^{-1}, PRR26 = 12.90 ± 3.13 nmol min^{-1} μg^{-1}, $P < 0.05$), while the activity was 2.72-fold in PRR34 strain compared to the SS stains (PRR34 = 28.67 ± 5.171 nmol min^{-1} μg^{-1}, $P < 0.05$). Such correlation between resistance level and GST activity strongly suggested that increased GST activity might be associated with propargite tolerance in *T. cinnabarinus*. This result agreed with the synergistic effect of DEM on the PRR34 strain that also suggested a role of GSTs in the propargite resistance.

3.5. cDNA cloning and phylogenetic analysis

Eight GST cDNA sequences were cloned and submitted to GenBank (accession numbers: 557131867, 557131869, 557131871, 557131881, 557131875, 557131877, 557131879 and 557131889) with names of *TcGSTd1*, *TcGSTd2*, *TcGSTm1*, *TcGSTm2*, *TcGSTm3*, *TcGSTm4*, *TcGSTm5* and *TcGSTz1*, respectively. The identified GST cDNA coding sequences were 651–684 bp long with deduced sequences of 216–242 amino acids (AA), giving an estimated molecular weights of 24.51–27.91 kDa and predicted iso-electric points of 4.74–8.42 (Table S1). Comparison of sequences identified by the NCBI BLASTP search revealed that these translated cDNA sequences displayed a moderate level of similarity with GSTs of

Table 3
Synergistic effects of DEM, TPP and PBO on propargite susceptibility of PRR34 and SS strains of *T. cinnabarinus*.

Strain	Treatment (synergist)	N[a]	χ^2	Slope (SE)	LC_{50} (95% CI[b]) (mg/L)	SRR[c]	SR[d]
PRR34	Propargite	380	23.89	1.04(0.18)	912.75(740.97–10079.74)	37.78	1.00
	+DEM	647	46.39	1.98(0.17)	353.32(258.04–561.50)	13.33	2.58
	+TPP	665	6.96	1.76(0.24)	861.84(620.81–1454.75)	34.57	1.06
	+PBO	697	3.81	1.02(0.13)	620.36(420.07–1110.24)	19.20	1.47
SS	Propargite	416	5.65	2.60(0.27)	24.16 (20.11–28.53)	1.00	1.00
	+DEM	420	20.23	2.97(0.25)	26.51(14.18–60.17)	1.00	0.91
	+TPP	420	38.87	3.02(0.26)	24.93(19.66–32.18)	1.00	0.97
	+PBO	420	46.03	5.56(0.60)	32.31(25.71–41.24)	1.00	0.75

[a] Total number of mites used in the experiments.
[b] Confidence interval.
[c] Synergistic resistance ratio = LC_{50} PRR34/LC_{50} SS strain.
[d] Synergistic ratio = LC_{50} of propargite alone/LC_{50} of propargite with the synergist.

Table 4
Glutathione *S*-transferase activities of female adults in different strains of *T. cinnabarinus*.

Strains	Protein (μg mL^{-1})	Activity (nmol min^{-1})	Specific activity (nmol min^{-1} μg^{-1})	RR[a]
SS	3.26 ± 0.22	33.91 ± 0.01	10.54 ± 0.87	1.00
PRR26	1.07 ± 0.55	12.10 ± 2.93	12.90 ± 3.13	1.22
PRR34	2.56 ± 0.77	50.14 ± 0.01	28.66 ± 5.17*	2.72

* Indicating significant difference compared to SS strain with ANOVA followed by *t*-test ($P < 0.05$).
[a] Relative ratio (RR) = Specific activity value of resistance selected strain/specific activity value of the SS strain.

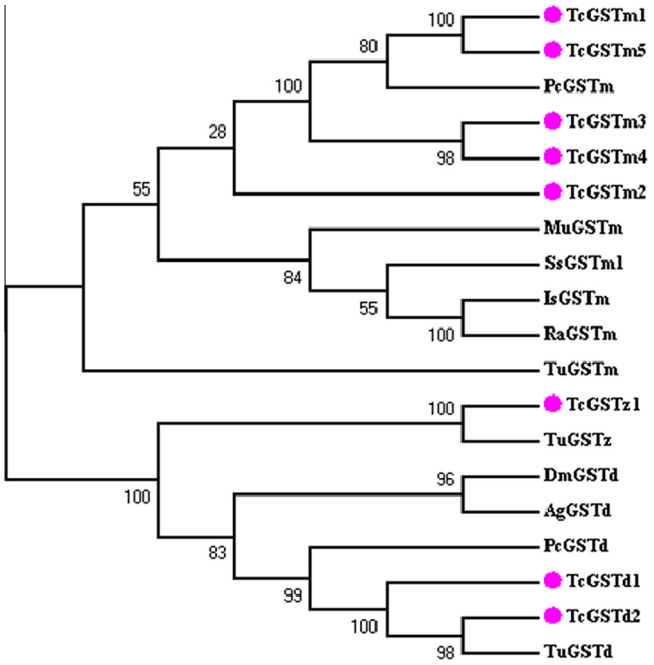

Fig. 1. Cluser analysis of GST genes cloned from SS strain of *T. cinnabarinus* Ag: *Anopheles gambiae* (AgGSTd, AAM53609.1). Dm: *Drosophila melanogaster* (DmGSTd, NP_524916.1). Pc: *Panonychus citri* (PcGSTd, AFD36886.1; GSTm, AFY08505.1). Tu: *Tetranychus urticae* (TuGSTd, tetur26g01510; TuGSTm, tetur05g05290; TuGSTz, tetur07g02560). Is: *Ixodes scapularis* (IsGSTm, XP_002401749.1). Ra: *Rhipicephalus annulatus* (RaGSTm, ABR24785.1). Ss: *Sarcoptes scabiei* (SsGSTm, AF462190.1). Mu: *Mus musculus* (MuGSTm, NP_034488.1).

insect species, such as *Pediculus humanus corporis* (60%), *Acyrthosiphon pisum* (60%) and *Tribolium castaneum* (59%).

In addition to the 8 GST sequences identified in this study, 8 GST sequences from other species obtained by GenBank search and 3 GST proteins of *T. urticae* from Bioinformatics & Systems Biology (http://bioinformatics.psb.ugent.be/webtools/bogas/overview/Teur) were included in the phylogenetic analysis (Fig. 1). A tree was generated with minimum evolution (ME) analysis using MEGA 3.0 under the Poisson correction model of amino acid substitution [19]. The analysis revealed that *TcGSTz1* originated from the same evolutionary root as *TuGSTz* from *T. urticae* with 100% bootstrap, and *TcGSTd2* was most closely related to *TuGSTd* from *T. urticae* to form a single clade with the bootstrap value of 98. Evolutionary relatedness and similar physiological functions might exist between *TuGSTm1* and *TuGSTm5*, and *TuGSTm3* and *TuGSTm4*. *TuGSTm1*, *TuGSTm5*, *TuGSTm3* and *TuGSTm4* had a close relationship with *PcGSTm1* from *Panonychus citri*.

3.6. Expression profile of GST genes of different strains and different life stages

When compared between PRR34 and SS strains, *TcGSTd1* gene were significantly down regulated in eggs (0.23-fold) but significantly up regulated in larvae (5.89-fold), nymphs (13.35-fold) and adults (16.95-fold) ($P < 0.05$). *TcGSTd2* gene showed significantly higher expression in nymphs (7.90-fold) and adults (65.99-fold) but down-regulated in eggs (0.31-fold) ($P < 0.05$). There were no significant difference at mRNA levels in egg, larval, nymph and adult stages for *TcGSTm1*, *TcGSTm3*, *TcGSTm4* and *TcGSTm5* between the two strains, while *TcGSTm2* transcript had a significant higher expression in the adult stage (22.51-fold) ($P < 0.05$). The expression of *TcGSTz1* in PRR34 was the highest in nymphs (8.36-fold), followed by adults, larvae and eggs (0.69-, 1.54- and 2.08- fold, respectively) relative to SS strain (Fig. 2). The eight GST genes in delta, mu and zeta family were detectable in all the life stages of *T. cinnabaranus*, with distinct patterns (Fig. 3). *TcGSTd1* and *TcGSTd2* express patterns between life stages were distinctively different between SS and PRR34. In SS, express levels were similar among different life stages, while in PRR34, the express levels were significantly higher in larvae, nymphs and adults than in eggs. Except for *TcGSTm3* and *TcGSTsm5*, the expression levels relative to the egg stage of other mu family genes were higher in the resistant strain PRR34 than susceptible strain (SS). In SS, the highest transcription levels of the other genes appeared in larval stage, with 4.31-, 3.51- and 7.26-fold relative to eggs, respectively. While in PRR34, the highest transcription levels of *TcGSTm1*, *TcGSTm2*, and *TcGSTm4* delayed and appeared in nymph stage, with 11.00-, 57.80- and 21.60-fold relative to eggs, respectively. For *TcGSTz1*, there were no significant differences among the four life-stage in SS, while significantly higher transcripts at nymph stage (13.78-fold relative to eggs) in PRR34 were observed.

4. Discussion

Glutathione *S*-transferases belong to a diverse family that are major detoxification enzymes protecting from oxidative damage [22]. In insects, elevated GST activity has been associated with resistance to all the major classes of insecticides [23], including organophosphates, pyrethroids, carbamates, and chlorinated hydrocarbons such as DDT [9,24,25]. Although resistance research in Acari has not been kept in pace with that in Insecta, a number of studies on resistance mechanisms of mites has been conducted previously [7]. A total of 32 GST genes were detected in the *T. urticae* genome, which is similar to that in insects [26]. Increased transcription of *GSTmu 1*, *delta 1* and *delta 3* were observed in a permethrin-resistant *Sarcoptes scabiei* var.*hominis* strain [27]. Elevated levels of GST activity were determined to be associated with spider mite resistance to acaricides, such as abamectin resistance of *T. urticae* [28] and fenpropathrin resistance of *T. cinnabaranus* [3]. In the current study, the involvement of GSTs in propargite resistance of *T. cinnabaranus* was demonstrated at multiple levels. First, synergist test showed that the synergism effect of DEM was significantly higher compared to PBO and PTT for the propargite resistance strain (PRR34) suggesting that GSTs may play an important role in the resistance. Direct measurement of GST activity also suggested that GSTs played an important role in the propargite resistance as GST activity was positively correlated with the level of resistance. Finally, comparative expression levels of GST genes

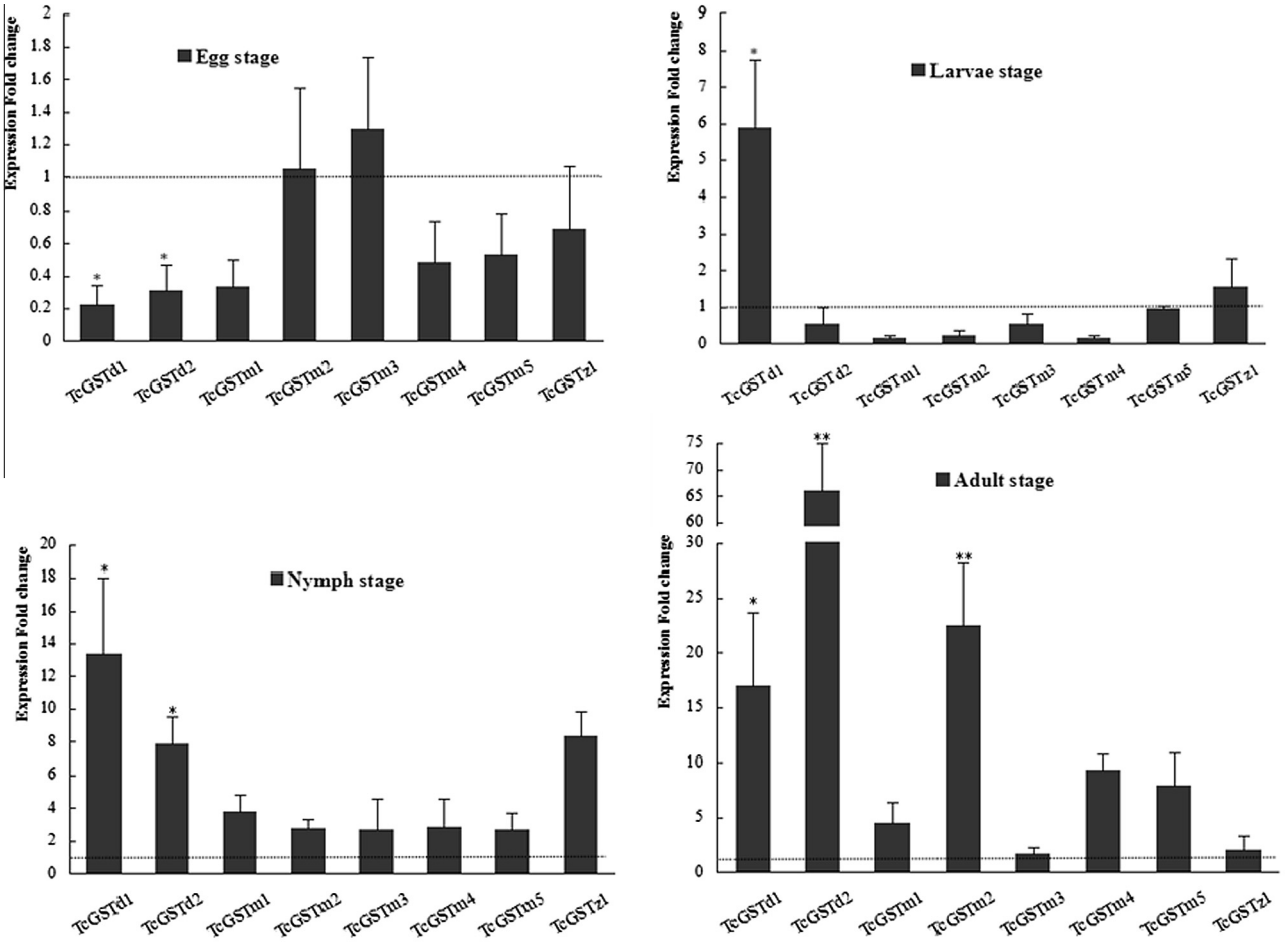

Fig. 2. Comparison of GSTs mRNA expression level in the two strains of *T. cinnabarinus*. *Y*-axis is the ratio of gene expression level between the resistant strain PPR34 and the susceptible strain. Error bars indicate standard error of the mean. Statistically significant differences were evaluated with ANOVA followed by *t*- test relative to the susceptible strain ($P < 0.05$ indicated by * and $P < 0.01$ as **).

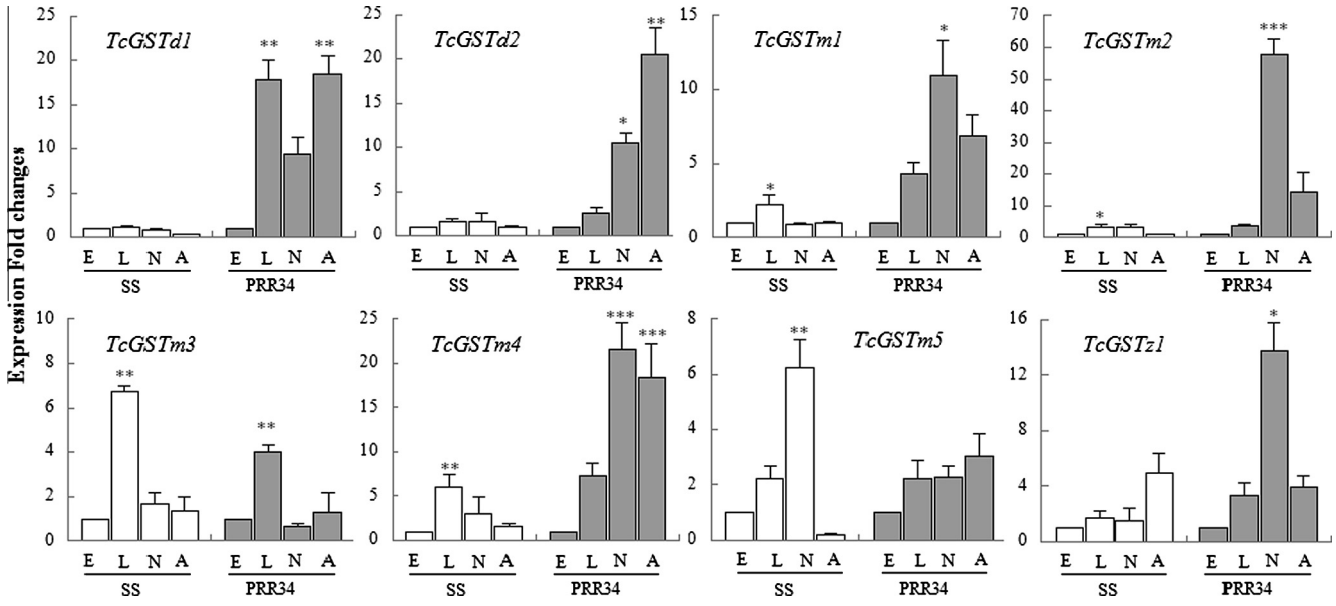

Fig. 3. Comparison of GST mRNA expression level in different life-stages of the two strains of *T. cinnabarinus*. E = egg, L = larva, N = nymph and A = adult. *Y*-axis is the ratio of expression level relative to the egg stage of susceptible strain (SS) or propargite-resistant strain (PRR 34), respectively. Error bars indicate standard error of the mean. Statistically significant differences were evaluated with ANOVA followed by *t*- test between egg and other three life-stages ($P < 0.05$ indicated by * and $P < 0.01$ as **).

between resistant and susceptible strains showed that three GST genes (*TcGSTd1, TcGSTd2* and *TcGSTm2*) were significantly up regulated in larvae, nymphs and adults of PRR34, and once again suggested GST involvement in the resistance. More direct approaches, such as RANi and in vivo expression are needed for confirmation, and will be persuaded in future. Nevertheless, our study provides foundational insights for future research on molecular mechanisms of GST detoxification-mediated acaricide resistance in *T. cinnabaranus*.

Additionally the current study conducted laboratory selection of resistance strain and cross resistance profile characterization. The results showed that selection with propargite for 34 generations resulted in a resistance ratio of 37.78-fold (Table 1). In *T. urticae*, laboratory selections with fenpyroximate generated 64.43-fold resistance after 14 generations [15], and selections with abamectin generated 13.30-fold resistance after only 5 generations [29]. It seems that *T. cinnabarinus* resistance develops relatively slower. This is supported by previous report where 8.7-fold resistance after selection of 42 generations by abamectin, and 28.7-fold after 20 generations of selection by fenpropathrin [3]. Such difference suggests that *T. cinnabarinus* may inherently have lower resistant gene frequency compared to other mite species [30].

Resistance management strategies are dependent very much on cross-resistance profile, i.e. whether a population that is resistant to one particular pesticide also is resistant to other acaricides. The current study characterized the propargite cross-resistance profile of PRR26 and PRR34 strains. The relative order of the resistance developed in the PRR34 strain was phoxim > pyridaben > chlorfenapyr > methomyl > diafenthiuron > dichlorvos > abamectin. The ranking suggested that the propargite-resistant PRR34 strain exhibited cross-resistance to phoxim, pyridaben and chlorfenapyr that belong to different chemical classes. This may suggest multiple mechanisms involved in the cross-resistance of the PRR34 strain of CSM. Keena and Granett reported that propargite resistance is inherited intermediately and probably involves more than one gene [31]. In the PRR34 strain of CSM, enhanced detoxification by increased activity of GSTs was observed which may at least partially explain the cross-resistance spectrum. The GSTs are major phase II detoxification enzymes that play a role in catalyzing the conjugation of electrophilic substrates to glutathione (GSH) [22]. GSTs are characterized by two domains, a N-terminal domain of 80 amino acid sites and a C-terminal domain of 120 sites. The N-terminal domain contains a site which interacts with a glutathione molecule. The C-terminal domain contains a site which interacts with the substrate [23]. GSTs can be responsible for resistance to a range of insecticides by conjugating reduced GSH to insecticide molecular or its toxic primary metabolic products [32]. The GSTs are important in phase I metabolism of organophosphorus insecticides (OP) and play a significant role in OP resistance [33]. Propargite, pyridaben and chlorfenapyr belong to three different modes of action group but all affect energy metabolism. Chlorfenapyr is a novel pyrrole compound and a prodrug activated by oxidative removal of a *N*-ethoxymethyl group [34]; its *N*-dealkylated metabolite is a potent uncoupler of mitochondrial oxidative phosphorylation [35]. Pyridaben belongs to mitochondrial complex I electron transport inhibitors (METIs) which contain heterocyclic rings with two nitrogen atoms associated with long hydrophobic tail structures and at least one tertiary butylgroup. Hydroxylation of this tertiary butylgroup could be a common mechanism of oxidative detoxification [36]. GSTs metabolize various electrophilic xenobiotics, including nitro compounds, organophosphates and oxides mentioned above. The high GST activity in the PRR34 strain may explain the cross-resistance among those three acaricides. Merely selecting acaricides with different MoAs in a spray program is not enough and studies alike the current one are very much in need. The current study suggested that diafenthiuron, methomyl, dichlorvos and abamectin were effective substitutes/rotation partners for managing propargite resistance of CSM.

5. Conclusion

Despite resistance reports, propargite is still the main choice for control spider mite. Therefore, understanding resistance mechanism is paramount for resistance management of propargite. This study is the first report on GST gene involvement in propargite resistance of *T. cinnabaranus*. The results provide new insights for exploratory molecular studies of resistant mechanism in CSM, which will be beneficial to spider mite resistance management in the field.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pestbp.2014.07.004.

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