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Differential analysis of the cytochrome p450 acaricide-resistance genes in *Panonychus citri* (Trombidiformes: Tetranychidae) strains

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Abstract

The citrus red mite, *Panonychus citri* (McGregor) (Trombidiformes: Tetranychidae), a highly destructive pest in citrus groves around the world, has developed resistance to many registered acaricides. Hexythiazox is a selective miticide that has been widely used to control citrus mites in a variety of crops. Forty-six cytochrome P450 mono-oxygenase genes related to general pesticide resistance in other insect species were obtained from the transcriptomes of the hexythiazox-resistant (RR) and hexythiazox-susceptible (SS) strains of *P. citri* and divided into 4 clans, 15 families and 24 subfamilies. Sequence analyses of each CYP resulted in detection of 3 mutationsin the *CYP307A1* gene (841-A to C, 1395-T to C, 1491-T to C) that differed between the 2 strains. Only the change at an amino acid position (278-lysine to glutamine) resulted in a sense mutation. One SNP site was also detected in *CYP381A2* (40-A to T) causing a sense amino acid mutation (14-threonine to serine). Seven of these P450s belonged to the CYP2 clan, CYP3 clan and CYP4 clan based on digital gene expression (DGE) library sequencing with a $|\log_2 \text{ratio}|$ value greater than 2, but there were no significant differences revealed by qRT-PCR analysis. This study provides essential information for future research on the hexythiazox-resistance mechanism of *P. citri*. More methods are needed to further elucidate the molecular mechanisms of resistance to hexythiazox in *P. citri*.

Key Words: citrus red mite; differential analysis; cytochrome P450; hexythiazox resistance; miticide resistance; RT-PCR analysis

Resumen

El ácaro rojo de los cítricos, *Panonychus citri* (McGregor), es una plaga muy destructiva en plantaciones de cítricos por todo el mundo y ha desarrollado resistencia a muchos acaricidas registrados. Hexitiazox es un acaricida selectivo que ha sido ampliamente utilizado para controlar los ácaros de cítricos en una variedad de cultivos. Se obtuvieron cuarenta y seis genes del citocromo P450 monooxigenasa relacionados con la resistencia general de pesticidas en especies a partir de los transcriptomes de una cepa de *P. citri* resistente al hexitiazox (RR) y una cepa de *P. citri* susceptible al hexitiazox (SS) y fueron divididos en 4 clanes, 15 familias y 24 subfamilias. Los análisis de secuencias de cada CYP resultó en la detección de 3 mutaciones en el gen *CYP307A1* (841 - A a C, 1395 - T a C, 1491 - T a C) que difieren entre las 2 cepas. Sólo el primer cambio resultó en una mutación de sentido en una posición de aminoácido (278-lisina a glutamina). También, un sitio de SNP fue detectado en *CYP381A2* (40-A a T) que causa una mutación de sentido de aminoácidos (14-treonina a serina). Siete de estos P450 pertenecían al clan CYP2, CYP3 y CYP4 basado en la secuenciación de biblioteca de la expresión de genes digital (EGD) con un valor $|\log_2 \text{ratio}|$ superior de 2, pero no hubo diferencias significativas reveladas por el análisis de RT -PCR. Este estudio provee información esencial para investigaciones en la futura sobre el mecanismo de resistencia para hexitiazox por *P. citri*. Más métodos para dilucidar los mecanismos moleculares de resistencia a hexitiazox en *P. citri* son necesarios.

Palabras Clave: ácaro rojo de los cítricos; análisis diferencial; citocromo P450; resistencia a hexitiazox; resistencia a acaricida; análisis de RT-PCR

The citrus red mite, *Panonychus citri* (McGregor) (Trombidiformes: Tetranychidae), is a worldwide pest of citrus causing significant yield losses annually. It has a short life cycle, a high reproductive rate and infests over 80 species of plants, e.g., citrus, rose, almond, pear, castor bean, and several broadleaf evergreen ornamentals (Lee et al. 2000; Zhang 2003). The citrus red mite is quite difficult to manage because of improper application of acaricides, and its capacity to rapidly develop resistance to many registered acaricides (Furuhashi 1994; Masui et al. 1995; Meng et al. 2000; Ran et al. 2008; Hu et al. 2010; Osakabe et al. 2010). Hexythiazox is a selective miticide that is active against various phytophagous mites. It has been widely used in integrated pest management programs on various crops, especially against the citrus red mite. However, phytophagous mites are capable of rapidly developing resistance to hexythiazox. *Tetranychus urticae* has devel-

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oped a > 1,000-fold resistance in Australia (Gough 1990), and *P. citri*, developed > 23,000-fold resistance in Japan (Yamamoto et al. 1995) and > 3,500-fold resistance in Chongqing, China (Ran. et al. in press). In a previous study, we increased the resistance of *P. citri* to 3,532-fold by continuous selection with hexythiazox for 20 generations (Liu et al. 2011a). Nevertheless, the molecular mechanisms of resistance to hexythiazox in *P. citri* remain unknown.

Cytochrome P450s are a very large and diverse group of enzymes found in all domains of life. They are an extremely important system involved in the metabolism of endogenous compounds and xenobiotics such as drugs, pesticides, plant toxins, chemical carcinogens, mutagens, hormones, fatty acids and steroids (Guengerich et al. 1999; Waxman 1999; Eaton 2000; Ingelman-Sundberg 2001; Zhang et al. 2004; Kretschmer & Baldwin 2005; Li et al. 2007; Strode et al. 2008). Down- or up-regulation of P450 gene expression can significantly affect the disposition of xenobiotics or endogenous compounds in the tissues of organisms, and thus alter their pharmacological/toxicological effects (Liu et al. 2011b). In arthropods, an increase in P450 activity is associated with the enhanced metabolic detoxification of insecticides; and a constitutive over expression of P450 genes has been implicated in the evolution of resistance to insecticides (Cariño et al. 1994; Feyereisen 2005; Zhu et al. 2008a) and tolerance to plant toxins (Wen et al. 2003). The isolation, characterization, classification and nomenclature of specific insect P450s are critical first steps towards understanding their involvement in these important metabolic processes. Accumulating genomic and post-genomic technologies have made it easier to study large and complex gene families such as the cytochrome P450 superfamily. To date, more than 2000 insect P450s have been recorded in the National Center for Biotechnology Information (NCBI), most of which were described from *Drosophila melanogaster* Meigen (Amichot et al. 2004; Tijet et al. 2001), *Culex quinquefasciatus* (Liu et al. 2011b), *Musca domestica* (Markussen & Kristensen 2010), *Helicoverpa armigera* (Hübner) (Brun-Barale et al. 2010; Pittendrigh et al. 1997), *Bombyx mori* (L.) (Ai et al. 2011), *Manduca sexta* (L.) (Rewitz et al. 2006), *Plutella xylostella* (L.) (Bautsita et al. 2009) and *Apis mellifera* (Johnson et al. 2006).

The global transcriptome of resistant (RR) and susceptible (SS) strains of *P. citri* has been sequenced (Liu et al. 2011a). A total of 34,159, 30,466 and 32,217 transcripts were identified by assembling SS reads, RR reads and SS plus RR reads after filtration of the low quality reads, respectively, in which 121 unigenes were related to cytochrome P450 monoxygenases. Only 46 P450s were functionally annotated according to their degree of sequence matching, owing to the low sequence similarity of other P450s (Liu et al. 2011a). To clarify the relationship between resistance of *P. citri* and P450 genes, we compared the sequences of P450 genes between RR and SS strains. Furthermore, we compared the gene expression profiles of *P. citri* among different strains using a digital gene expression system and quantitative RT-PCR.

Supplemental File is displayed in **s**upplementary material for this article online in Florida Entomologist 98(1) (March 2015) at http://purl.fcla.edu/fcla/entomologist/browse together with color versions of Figs. 4, 5, 6 and 7.

Materials and Methods

CITRUS RED MITE REARING AND RESISTANT MITE SELECTION

A colony of citrus red mite, *P. citri*, was collected from citrange (*Citrus sinensis × Poncirus trifoliata*) next to citrus orchard fences (Citrus Research Institute, Chinese Academy of Agricultural Sciences), which had not been exposed to acaricides for more than 10 years. These mites were reared on citrange for 3 years under acaricide-free conditions (25 ± 1°, 80 ± 5% RH and 14:10 h L: D and were considered to be a susceptible strain (SS). The SS strain was treated with hexyzhiazox (about a 70% population mortality rate was observed at every spray application) and was continuously screened for 20 generations until it was regarded as a fully resistant (RR). The selection process and the resulting changes in resistance were shown in a previous study (Liu et al. 2011a). A mixture of eggs, larvae and adults was collected in PerkinElmer (PE) tubes with one sample for SS and RR respectively. The 2 samples were stored at -80° until use.

RNA ISOLATION AND INTEGRITY EXAMINATION

Total RNA was isolated from the following 2 samples: RR and SS of *P. citri* to hexythiazox. From each sample, approximately 8 mg of mites were homogenized with liquid nitrogen in a mortar. RNA was extracted with TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions and was treated with RNase-free DNase I (Takara Biotechnology, China). RNA integrity was confirmed with a minimum RNA integrated number value of 8 by the 2100 bioanalyzer (Agilent).

DIGITAL GENE EXPRESSION (DGE) LIBRARY CONSTRUCTION AND SEQUENCING

Approximately 10 µg of total RNA from each specimen (a mixture of RNA from eggs, third-instar larvae, pupae, and adults at equal ratios) was used to construct the DGE libraries. Poly(A) mRNA was isolated with oligo-dT beads and then treated with the fragmentation buffer. The cleaved RNA fragments were then transcribed into first-strand cDNA using reverse transcriptase and random hexamer-primers. This was followed by second strand cDNA synthesis using DNA polymerase I and RNase-H. The double stranded cDNA was further subjected to end-repair using T4 DNA polymerase, Klenow fragment, and T4 polynucleotide kinase followed by a single <A> base addition using Klenow 3' to 5' exo-polymerase. It was then ligated with an adapter or index adapter using T4 quick DNA ligase. Adaptor ligated fragments were selected according to size and the desired range of cDNA fragments were excised from the gel. We performed a PCR reaction to selectively enrich and amplify the amount of the fragments. Finally, after validation on an Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System, the DGE libraries was sequenced on a flow cell using an Illumina HiSeq2000.Bioinformatics analysis process of sequence data were presented in Supplemental File 1. In particular, the raw reads were cleaned up by removing adapter sequences, reads in which unknown bases are more than 10% and low quality reads of quality value ≤ 5 as described by Liu (Liu et al. 2011a).

MANUAL CURATION OF CYPS IN *P. CITRI*

We blasted *P. citri* transcriptomes with sequences from the NCBI nucleotide database and obtained genes related to general pesticide resistance in other insect species. In total, there were 121 related to cytochrome P450 monooxygenase (CYPs). We chose only 46 of these for functional annotation according to sequence matching (E-value< le-5) owing to low sequence similarity of other P450s. Nucleotide sequences were dynamically translated using the EXPASY Proteomics Server (http://www.expasy.ch/tools/dna.html, Swiss Institute of Bioinformatics). All the identified sequences were searched with BLASTx against all the assembled contigs in the iceblast server using an E-value cut-off of 1e-5 and the results with more than 99% similarity with the query sequence were eliminated as allelic variants (note that from those sequences, only the longest contigs with the best coverage were manually curated). We also searched the *P. citri* P450s on the NCBI network and obtained 2 amino acid alignments of *P. citri* CYPs. Next, we manually curated each of the CYPs with the help of comparison with the CYP

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genome of the two-spotted spider mite (http://drnelson.uthsc.edu/Two.spotted.spider.mite.htm). Then, we assigned gene names based on the homology of *P. citri* CYPs to *T. urticae* using defined nomenclature and naming rules (Nelson 2006).

PHYLOGENETIC ANALYSIS OF CYPS IN *P. CITRI*

MEGA 4.0 software (Tamura et al. 2007) was used to analyze the phylogenetic relationships between P450 of *P. citri* and several CYPs in *T. urticae* to predict their classification. The neighbor-joining method was used to create phylogenetic trees (Saitou & Nei 1987). Positions containing alignment gaps and missing data were eliminated with pairwise deletion. Bootstrap analysis of 1,000 replication trees was performed to evaluate the branch strength of each tree (Efron et al. 1996). The NJ tree included bootstrap confidence levels at nodes, which reflected the confidence of trees sampled during the reconstruction that included each particular branch. Only clades with a bootstrap value higher than 50 were selected for the bootstrap consensus tree (Soltis & Soltis 2003).

SEQUENCE ALIGNMENT AND VERIFICATION OF MUTATIONS IN CYPS IN *P. CITRI*

Multiple nucleotide sequence alignment of each CYP between the resistant and susceptible haplotypes of *P. citri* was performed using ClustalX (1.83) software (Thompson et al. 1994) and followed methods of Liu et al. 2011a. This alignment process was also based on the Feng & Doolittle (1987) algorithm, but with an improved choice of alignment parameters using dynamic assignment of penalties. Two pairs of specific Primers (Table 2) for surveying the nucleotide differences between RR and SS were designed by using Primer Premier 5.0 software based on the transcriptome of *P. citri*. Analyses using PCR were carried out in a total volume of 25 µL and performed in a thermocycler(GeneAmp PCR system 9600; Perkin-Elmer Corporation, Norwalk, Connecticut). The PCR reaction contained 12.5 µL of 1×Taq MasterMix from (Tiangen Biotech), 10 pmol of each primer, 30 ng of cDNA. PCR was performed using a Mastercycler® Gradient using the following protocol: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, with a final extension step of 72 °C for 10 min. Samples (5 µL) of reaction mixtures were examined by electrophoresis through 2% agarose gels in TAE buffer. Bands were revealed by visualization with UV light after ethidium bromide staining. PCR products were purified and sequenced by Shanghai Sangong Biological Engineering Technology &Service CO., LID. All these primers mentioned above were also used as sequencing primers. Once a mutation was confirmed, both nucleotide sequences from the 2 strains were dynamically translated using the EXPASY Proteomics Server (http://www.expasy.ch/tools/dna.html, Swiss Institute of Bioinformatics). Amino acid sequence alignment was performed by ClustalX (1.83) software to confirm whether the mutation was a sense mutation.

DIFFERENTIAL EXPRESSION OF CYPS IN *P. CITRI*

The uniquely mapped reads for a specific transcript were counted by mapping reads to assembled sequences using SOAP (Li et al. 2009). Then the RPKM value for each transcript was measured in reads per kilobase of transcript sequence per million mapped reads (Mortazavi et al. 2008). The transcript fold change was calculated by the formula of $\log_2$(RR_ RPKM / SS_RPKM). If the value of either RR_ RPKM or SS_ RPKM was zero, we used 0.01 instead of 0 to calculate the fold change. We modified Audic's (Audic & Claverie 1997) method to analyze differential expression. The probability of a specific gene being expressed equally between the 2 samples was defined by the following formula:

$$p(y\backslash x) = \left(\frac{N2}{N1}\right)^{y} \frac{(x+y)!}{x!y!\left(1+\frac{N2}{N1}\right)^{(x+y+1)}}$$

In this equation, N1 and N2 indicate the total number of clean reads in the SS and RR haplotypes and x and y represent the mapped clean read counts of one transcript in the 2 samples. The false discovery rate (FDR) method was used to determine the threshold of the P value in multiple tests. In this study, we used 'FDR ≤ 0.001 and the absolute value of the $\log_2$ ratio ≥ 1' as the threshold to judge the significance of differential gene expression.

QUANTITATIVE RT-PCR ANALYSIS

Quantitative Reverse transcription polymerase chain reaction (RT-PCR) detection was used to verify 5 differentially expressed genes (2 up-regulated and 3down-regulated genes), e.g., *CYP389A6*, *CYP385A2*, *CYP307A1*, *CYP307A2*, *CYP389A1*. Multiple specific Primers (Table 2) were designed by using Primer Premier 5.0 software (PREMIER Biosoft International, California). The expression study was performed on an Mx3000P™ Multiple Quantitative PCR System (Stratagene, La Jolla, California, USA) with ELF1A, elongation factor-1 alpha, used as reference gene (Niu et al. 2012). The reaction system and reaction parameters were same as Niu et al. (2012) described earlier. After collecting the Ct values, the relative quantitative expression of these 5 genes were calculated by the $2^{-\triangle\triangle CT}$ method (Livak & Schmittgen 2001).

Results

DIGITAL GENE EXPRESSION (DGE) LIBRARY SEQUENCING

Based on the transcriptome sequence data, 2 DGE libraries were constructed to identify the expression profiles of the various strains. After removing the low-quality reads, each library generated more than 7 million clean reads. Among these clean reads, approximately 2.8 million and 2.9 million (36.92% and 40.36%, respectively) were mapped to unigenes in each library (Table 3). The percentage of clean reads ranged from 98.68% to 99.70%, reflecting a high quality of sequencing.

MANUAL ANNOTATION IDENTIFIED 48 CYPS IN *P. CITRI*

Manual annotation and curation of the CYPs in the *P. citri* transcriptome sequence assembly (http://www.ebi.ac.uk/ena/data/view/ERP000885) produced 121 sequences related to cytochrome P450 monooxygenase (CYPs). Of these, 46 were manually curated, as the remainder was found to have either low sequence similarities to other P450s according to sequence matching (E-value< le-5), or they contained too many sequencing errors. In addition, 2 full length P450s were detected by Jiang et al. (Jiang et al. 2010), which were the first detected P450 genes of *P. citri*. The latter were added to our data, and these 48 P450 sequences were named by D. R. Nelson in accordance with the P450 nomenclature committee conventions (http://drnelson.uthsc.edu/cytochromeP450.html). Based on the closest BLASTX matches in the NCBI nr database first, and when possible, by phylogenetic analyses with CYPs in *T. urticae* (http://drnelson.uthsc.edu/Two.spotted.spider.mite.htm), the P450s of *P. citri* were assigned to all 4 major insect CYP clans (CYP2, CYP3, CYP4 and mitochondrial). The 4 clans can be further subdivided into 15 families, 24 subfamilies, and 48 putatively functional isoforms (Fig. 1). Comparison of the number of functional CYP genes in different

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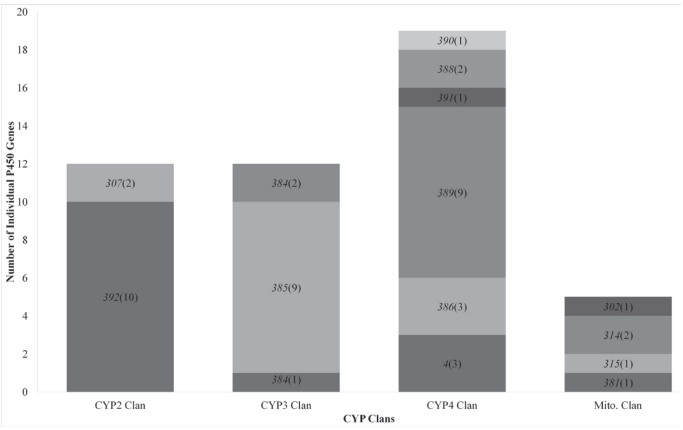


Fig. 1. Number, family and clan distribution of cytochrome P450 genes in *Panonychus citri*. The number shown along each column represents the P450 family and the number in parenthesis is the number of individual genes in the corresponding family. The P450 gene sequence information generated is from the VectorBase of the *P. citri* transcriptome sequence.

transcriptomes and genomes (Table 1) shows that the CYP number is lower than in the typical invertebrate or insect with the exception of the honeybee (Claudianos et al. 2006) and the New World screwworm (Carvalho et al. 2010).

Table 1. Comparison of the number of functional CYP genes in different genomes or transcriptomes of various arthropods.

Genus/Species	Database	Approximate total*	DP**
Panonychus citri (Liu et al. 2011a)	Transcriptome	48	Dec 2011
Bactrocera dorsalis (Shen et al. 2011)	Transcriptome	51	Dec 2011
Cochliomyia hominivorax (Carvalho et al. 2010)	Transcriptome	28	Dec 2010
Cimex lectularius (Bai et al. 2011)	Transcriptome	73	Jan 2011
Trialeurodes vaporariorum (Karatolos et al. 2011)	Transcriptome	57	Nov 2011
Tribolium castaneum (Zhu et al. 2013)	Genome	143	Mar 2013
Tetranychus urticae (Grbić et al. 2011)	Genome	83	Nov 2011
Acyrthosiphon pisum (IAGC 2010)	Genome	58	Feb 2010
Daphnia pulex (Baldwin et al. 2009)	Genome	75	Apr 2009
Nasonia vitripennis (Oakeshott et al. 2010)	Genome	106	Sept.2007
Solenopsis invicta (Wurm et al. 2011)	Genome	152	May 2011
Apis mellifera (Claudianos et al. 2006)	Genome	46	Sep 2005
Drosophila melanogaster (Tijet et al. 2001)	Genome	90	Jan 2000
Anopheles gambiae (Holt et al. 2002)	Genome	106	Oct 2002
Bombyx mori (Bin et al. 2005)	Genome	81	Oct 2002

*Includes partials that are expected to be functional (e.g., *CYP302A1* in *Tetranychus urticae*).

**Dates are for major genome publications, most recent genome or transcriptome assembly or data release.

PHYLOGENETIC ANALYSIS OF CYPS IN *P. CITRI.*

Repeated and exhaustive searching for *P. citri* transcriptome assemblies and nucleotides in the NCBI uncovered a total of 48 CYP genes. Based on inferred amino acid sequences, these genes fell into 14 CYP gene families. Molecular phylogenetic analysis (Fig. 2) by the neighbor-joining method (Saitou & Nei 1987) shows the relationships among CYP genes and gene families in *P. citri* and several *T. urticae* P450s. Posi-

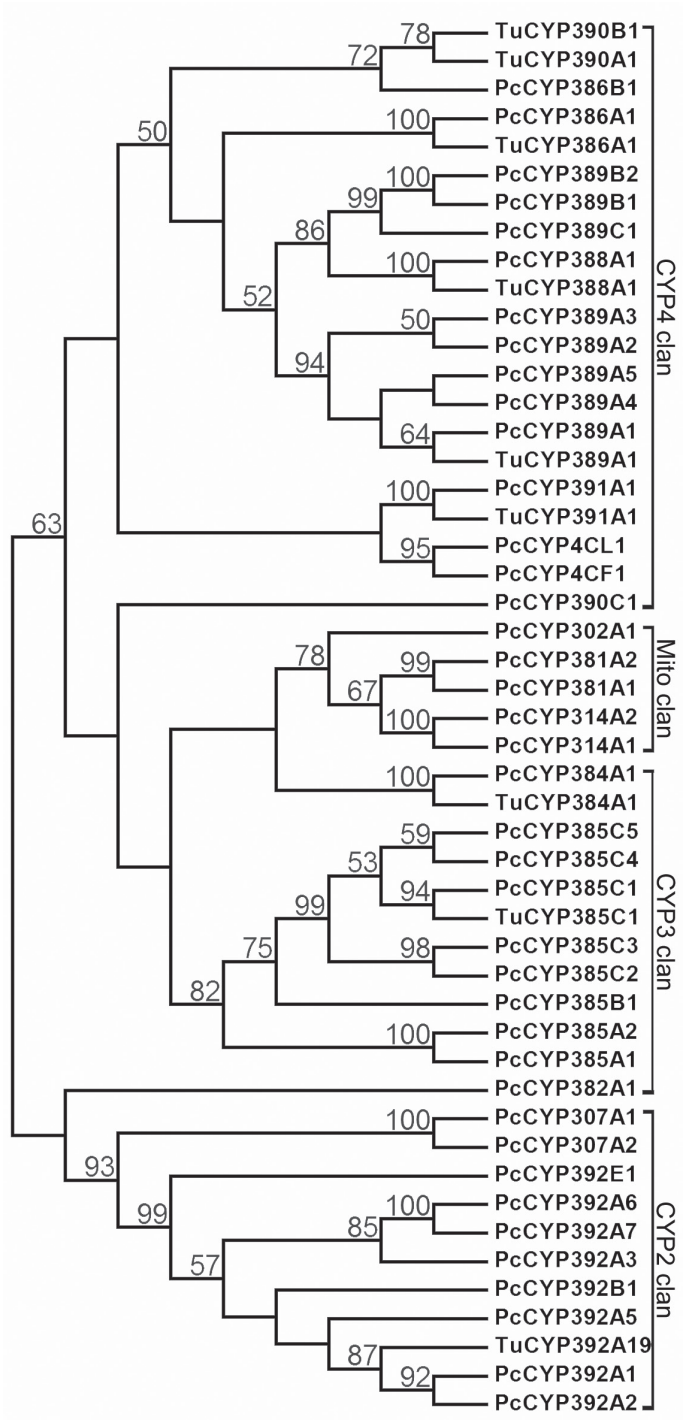


Fig. 2. Neighbor-joining phylogenetic analysis of cytochrome P450 from *Panonychus citri* and *Tetranychus urticae*. 4clans were observed. There are species (*P. citri* and *T. urticae*) in the phylogenetic tree. Only 10 sequences belong to *T. urticae*; A (Pc) before the CYP name denotes *P. citri*, a (Tu) before the CYP name denotes *T. urticae*. Numbers at nodes are bootstrap values.

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Table 2. Specific RNA primers used in sequencing and quantitative RT-PCR of *Panonychus citri*.

Application	Gene	Primer (5'-3')	Amplicon
Sequencing	*S-CYP307A1*	Forward: CAATTAACTCTATTAAAAGCTTGC Reverse: CTTTTACTCAAATACAAGGTC	787
	S-CYP381A2	Forward: AACTCTATTAAAAGCTTGCCTC Reverse: ATCGATGAACATTATTGCCTATC	392
RT-PCR	*RT-CYP389A6*	Forward: TTTGAATGAGAGCGATAAGTCTG Reverse: CCTTTGTTAGATGTGAGCAAC	85
	RT-CYP385A2	Forward: CGGAGACTTTCATTAATCACC Reverse: TAATCCCGAATGTCAAAATCG	114
	RT-CYP307A1	Forward: TATCGCACAAAGGACACGAC Reverse: CGATATCATTTAATTTTCGGAGG	96
	RT-CYP307A2	Forward: TAACCTTGAAGCACCGACCT Reverse: GAGGGACGATTGGTGAAGAT	87
	RT-CYP389A1	Forward: CACCGAGATACTGTATTGGTT Reverse: TGACGGAAACATCTTCGAGT	117
	RT-ELF1A	Forward: GGCACTTCGTCTTCCACTTC Reverse: ATGATTCGTGGTGCATCTCA	164

tions containing alignment gaps and missing data were eliminated with pairwise deletion. Bootstrap analysis of 1,000 replication trees was performed to evaluate the branch strength of each tree (Efron et al. 1996). The NJ tree included a bootstrap confidence level at nodes that reflects the confidence of trees sampled during the reconstruction that included each particular branch. In general, the tree demonstrates that the 4 major clans found in insects (Feyereisen 2006), which include CYP2, CYP3, CYP4 and mitochondrial clans, encompass all of the CYPs in *P. citri*. All nucleotide and amino acid sequences of cytochrome P450 monooxygenase genes in *P. citri* are available in the database (http://www.ebi.ac.uk/ena/data/search?query=Panonychus citri).

The CYP2 clan of *P. citri* contains 12 members, which can be separated into 2 distinct families (*CYP307*, *CYP392*). The *spook* gene *CYP307A1*, which is expressed in the prothoracic gland (Niwa et al. 2005), is a conserved Halloween gene involved in the early stages of ecdysone synthesis (Rewitz et al. 2006; Rewitz & Gilbert 2008), and can convert

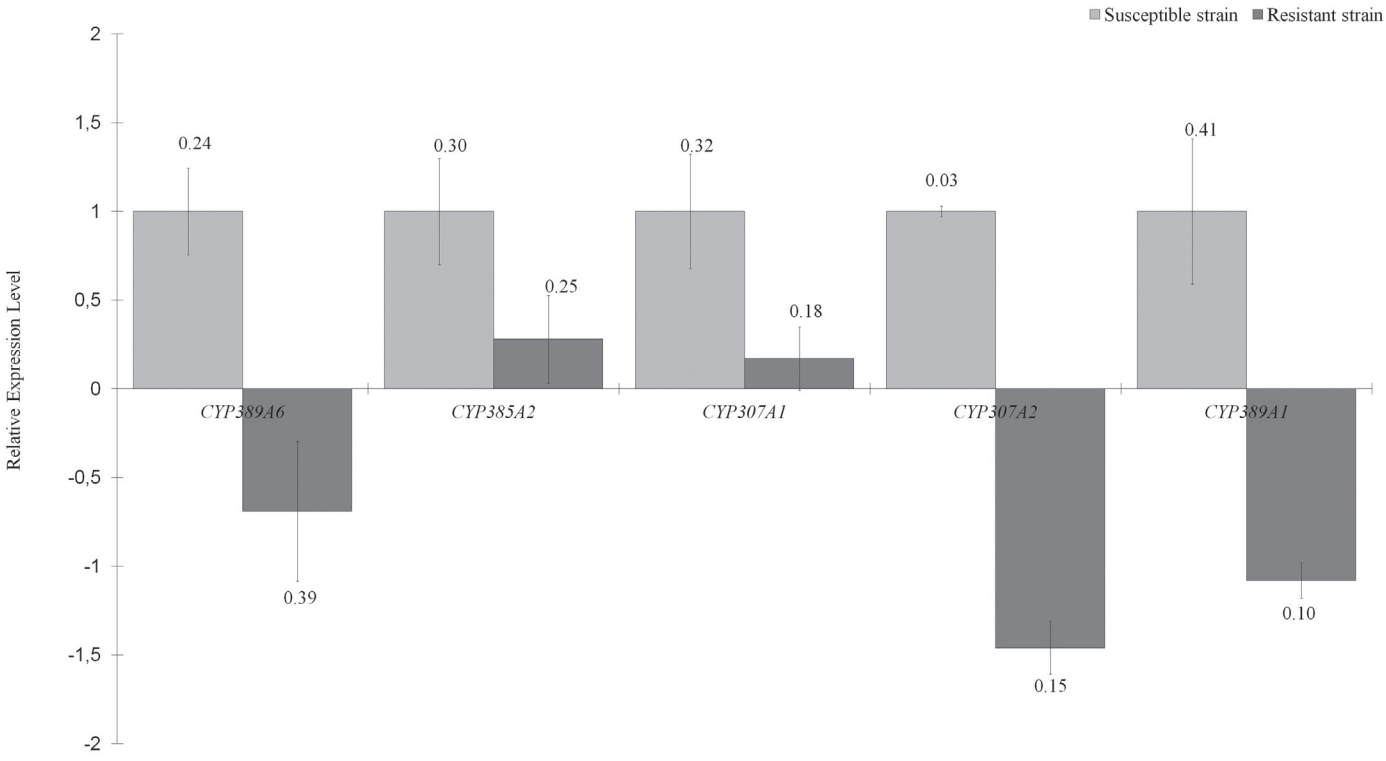


Fig. 3. Quantitative Real-time PCR analysis of CYPs in *Panonychus citri* between the hexythiazox-resistant (RR) and susceptible (SS) strains. The numbers of genes down-regulated and up-regulated in the RR relative to the SS are indicated above or below the X axis. The light or dark gray was susceptible strain and resistant strain, respectively.

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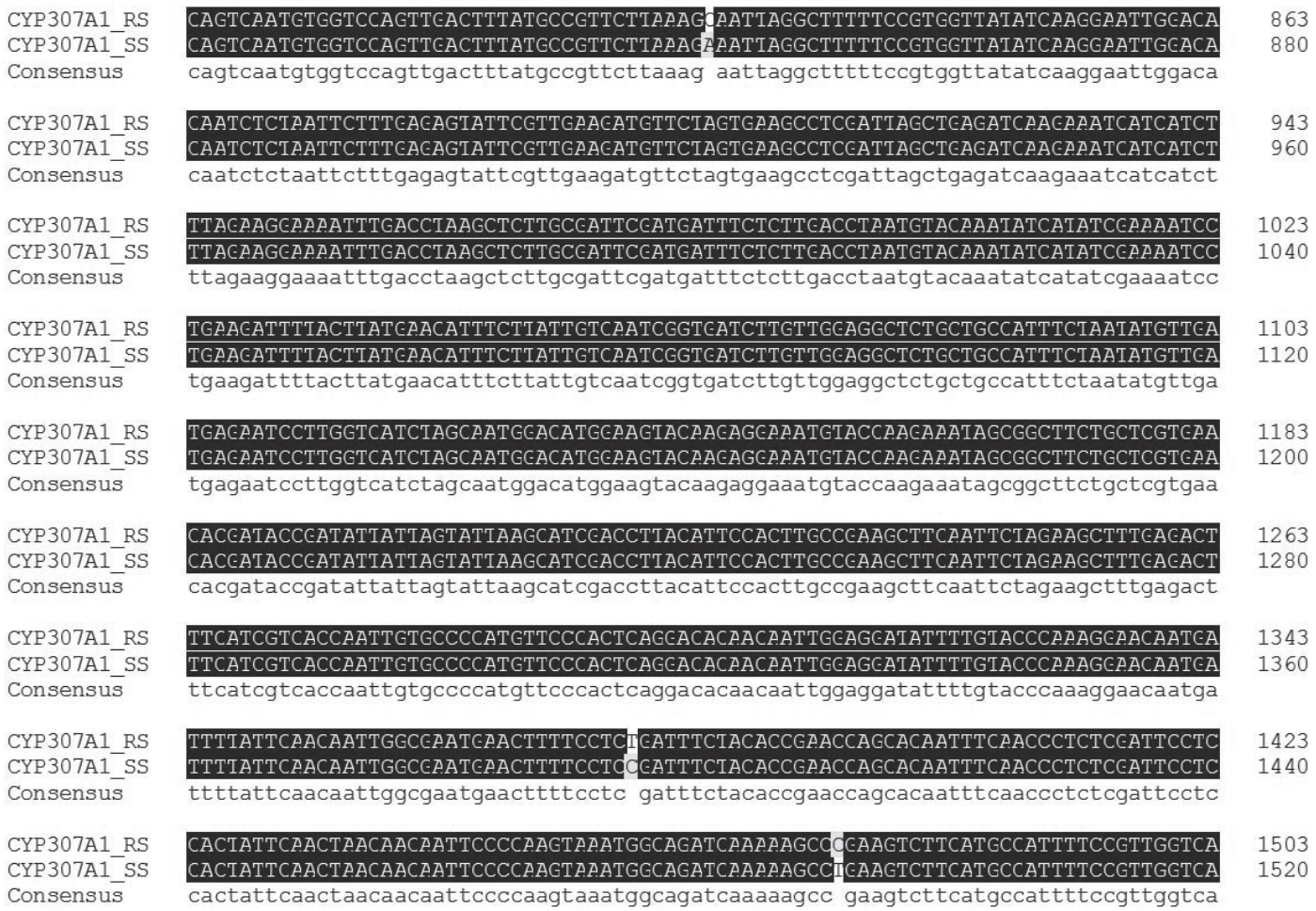


Fig. 4. Nucleotide sequence comparison of the *CYP307A1* in *Panonychus citri* between the hexythiazox-resistant (RR) and susceptible (SS) strains. Mazarine shading indicates identities and different color shading represents mutations. "-" represents no sequence to compare. Three SNP sites were detected in all. The first nucleotide mutation (A to C) is located at 841, the second mutation is 1395-T to C, and the final mutation is 1491-T to C. . This figure is shown in color in a supplementary document online as Suppl. Fig. 4 in Florida Entomologist 98(1) (March 2015) at http://purl.fcla.edu/fcla/entomologist/browse.

7-dehydrocholesterol to Δ^4-diketol with the gene product of *spookier* (*CYP307A2*) (Gilbert 2008). These 2 genes are close paralogs that are believed to mediate the same enzymatic reaction at different stages of development (Rewitz & Gilbert 2008). The other CYP2 clan family in *P. citri* (*CYP392*) is divided into 3 subfamilies.

The CYP3 clan of *P. citri* contains 12 CYPs. Genes in the CYP3 clan are the most numerous among insect P450 genes, and are often found in large clusters associated with oxidative detoxification of xenobiotics (Kretschmer & Baldwin 2005; Li et al. 2009; Waxman 1999) and endobiotics (Strode et al. 2008; Zhang et al. 2004). The first insect P450 gene (*CYP6A1*) was isolated from an insecticide-resistant strain of the housefly, *M. domestica* L. (Feyereisen et al. 1989). The first P450 gene (*CYP6A2*) was isolated from *D. melanogaster,* and is also associated with resistance to insecticides (Waters et al. 1992). In *P. citri*, the CYP3 clan contains 12 genes and 5 subfamilies. Eight genes belong to the CYP385 family and just 1 gene (*CYP382A1*) is part of the CYP382 family.

Table 3. Alignment statistics of the DGE-SEQ analysis of *Panonychus citri* RNA extracted from the hexythiazox-resistant (RR) and susceptible (SS) strains.

Summary	Resistant strain (RR)	Susceptible Strain (SS)
Total reads	7504330	7108109
Total base pair	367712170	348297341
Total mapped reads	2770716	2868622
Perfect match	2391472	2439559
<=2bp Mismatch	379244	429063
Unique match	2610289	2703250
Multi-position match	160427	165372
Total unmapped reads	4733614	4239487

The CYP4 clan of *P. citri* contains 19 genes and can be divided into 6 families and 10 subfamilies. Interestingly, the number of CYP4 clan genes varies in different species. For example, there are 32 in *D. melanogaster* (Tijet et al. 2001), 45 in *Anopheles gambiae* Giles (Holt et al. 2002), 44 in *Tribolium castaneum* (Herbst) (Richards et al. 2008), 32 in *B. mori* (L.) (Bin et al. 2005), 59 in *Aedes aegypti* L. (Strode et al. 2008), and only 4 in *Apis mellifera* L. (Claudianos et al. 2006). The great diversity of genes in the CYP4 clan was also reflected in a great diversity of functions. CYP4s in other insects have been implicated in functions as diverse as 20-hydroxyecdysone biosynthesis (Maibeche-Coisne et al. 2000), pheromone metabolism (Maibeche-Coisne et al. 2000) and pyrethroid insecticide resistance (Pridgeon et al. 2003).

The mitochondrial clan of *P. citri* probably originated in the CYP2 clan, which includes P450s with essential physiological functions, and contains 5 members in 3 families and 3 subfamilies. Two of the

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Fig. 5. Alignment of the predicted amino acid sequences of *CYP307A1* in *Panonychus citri*between the hexythiazox-resistant (RR) and susceptible (SS) strains. Mazarine shading indicates identities and different color shading represents mutations. "-" represents no sequence to compare. Only one amino acid mutation (278-lysine to glutamine) was detected. . This figure is shown in color in a supplementary document online as Suppl. Fig. 5 in Florida Entomologist 98(1) (March 2015) at http://purl.fcla.edu/fcla/entomologist/browse.

members, *CYP302A1* and *CYP314A1*, are highly conserved *halloween* genes, involved in ecdysone synthesis (Rewitz et al. 2006, 2008). The *disembodied* (*dib*) gene (*CYP302A1*) codes a cytochrome P450 enzyme that adds a hydroxyl group to the carbon-22 position of 2, 22, dE- Ketotriol to make 2- Deoxyecdysone (Gilbert 2004). The *dib* mutants are defective in producing cuticle and have severe defects in morphological processes such as head involution, dorsal closure and gut development (Chávez et al. 2000). The *shade* gene (*CYP314A1*) codes for a P450 enzyme which reportedly adds hydroxyl group to the 2C- position of ecdysone to make 20-hydroxyecdysone, as the final step in the biosynthetic pathway (Petryk et al. 2003). *CYP381* was a single family in *P. citri*.

SEQUENCE ALIGNMENT AND VERIFICATION OF MUTATIONS IN CYPS OF *P. CITRI*

ClustalX (1.83) software (Thompson et al. 1994) was used to analyze alignment differences in each CYP between the RR and SS of *P. citri*. After sequence verification, 3 SNP sites were detected in*CYP307A1* (1688 bp, Fig. 4). The first nucleotide change located at 841(A to C), the second change located at 1395 (T to C), and the final change located at 1491 (T to C). With alignment of the 2 predicted amino acid sequences of the *CYP307A1* (Fig. 5), a mutation was detected for one amino acid (278-lysine to glutamine). Therefore, the mutation was a sense mutation and the other 2 were nonsense mutations. A single SNP site, 40 (A to T), was detected in *CYP381A2* (619 bp, Fig. 6). With alignment of the 2 predicted amino acid sequences of the *CYP381A2* (Fig. 7), there was a sense amino acid mutation detected (14-threonine to serine). In insects, steroidogenic CYPs are products of the Halloween genes *phantom* (*CYP306A1*), *disembodied* (*CYP302A1*), *shadow* (*CYP315A1*) and *shade* (*CYP314A1*) and are responsible for the last 4hydroxylations in the pathway leading to 20E (Niwa et al. 2004; Niwa et al. 2005; Warren et al. 2002),which are biochemically similar to one that yields 20E in crustaceans (Lachaise et al. 1993). In *D. melanogaster*, mutations in these genes disrupt 20E production and cause the arrest of embryonic development and death. *Spook* (*CYP307A1*) is another member of this CYP group and, when mutated, results in low 20E mutants (Niwa et al. 2005). In *B. mori*, the nucleotide transition of C to T at position 1049 was found in *phantom* (*CYP306A1*), resulting in a nonsense mutation at amino acid position 286. The morphology of the *phantom* mutant is normal until stage 14 when there is a failure of dorsal closure and head involution and embryos become compacted. The *phantom* mutant exhibited severe reductions in the epidermal expression of these 20- hydroxyecdysone-inducible genes (Niwa et al. 2004).

DIFFERENTIAL EXPRESSION OF CYPS IN RR AND SS OF *P. CITRI*

A rigorous algorithm (see Methods) was developed to identify genes differentially expressed between resistant (RR) and susceptible (SS) mite populations treated with hexyzhiazox by referring to "The significance of digital gene expression profiles" (Audic & Claverie 1997). To explore the gene expression levels further, the reads per kb per million reads (RPKM) method (Mortazavi et al. 2008) was adapted to eliminate the influence of variation in gene length and the total reads number. Results showed 22 P450s were up-regulated and 24 were down-regulated (Table 4). Furthermore, the significance of gene expression differences were determined using the false discovery rate (FDR≤ 0.001) and the absolute value of the $\log_2$ ratio (≥ 1). Nine genes were differentially expressed between the 2 samples at significant levels, including 5 down-regulated and 4 up-regulated genes (Table 4). Seven members of P450s had a greater value than 2 based on a $\log_2$ ratio formula, and these members were distributed as 2, 2 and 3 in the CYP 2 clan, CYP 3 clan and CYP 4 clan, respectively. It was straightforward to identify the highest up-regulated expression gene ($\log_2$ ratio = 12.14) as *CYP389A6*, which belonged to the CYP4 clan. The gene of highest expression might be analogous with those CYP4s in other insects that have been implicated in pesticide metabolism. For example, *CYP4G8* is over expressed in pyrethroid-

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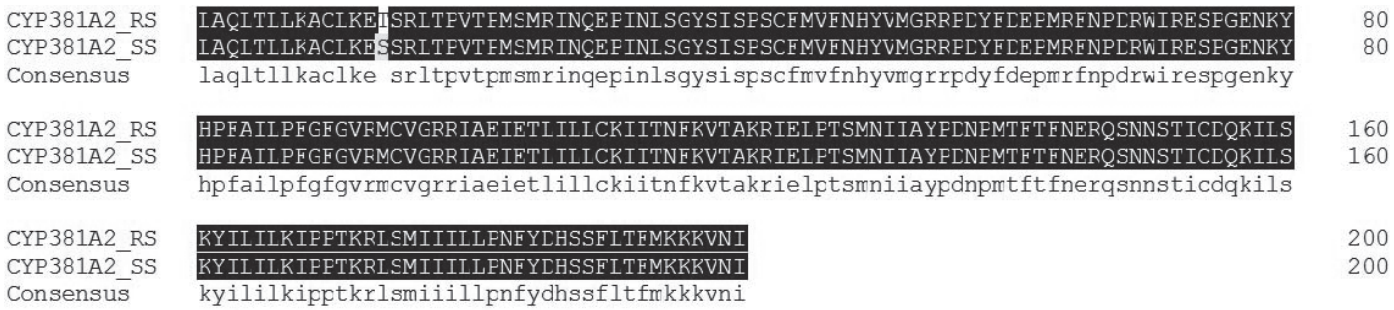


Fig. 6. Nucleotide sequence comparison of *CYP381A2* in *Panonychus citri* between the hexythiazox-resistant (RR) and susceptible (SS) strains. Mazarine shading indicates identities and different color shading represents mutations. "-" represents no sequence to compare. Just one SNP site was detected. The nucleotide transition of A to T was at position 40. . This figure is shown in color in a supplementary document online as Suppl. Fig. 6 in Florida Entomologist 98(1) (March 2015) at http://purl.fcla.edu/fcla/entomologist/browse.

resistant strains of *H. armigera* (Pittendrigh et al. 1997). *CYP4C27* in *A. gambiae* is over expressed in a DDT-resistant strain (David et al. 2005) and *CYP4G19* expression in *Blattella germanica* is correlated with pyrethroid resistance (Pridgeon et al. 2003). Several CYP4 genes are over expressed in pesticide resistant *C. pipiens* and *Diabrotica virgifera* (Scharf et al. 2001; Shen et al. 2003). The expression level of *CYP385A2* ($\log_2$ ratio = 9.46), belonging to the CYP3 clan, was a little lower than that of *CYP389A6*. The CYP3 clan is related to metabolism of pyrethroids, chlorinated hydrocarbons (e.g., DDT) and neonicotinoids (e.g., imidacloprid) (Daborn et al. 2002; Jiang et al. 2010; Karunker et al. 2008; Komagata et al. 2010; Nikou et al. 2003). The most down-regulated gene ($\log_2$ ratio = -11.816) was *CYP389A1*, which belonged to the CYP4 clan. The second lowest ($\log_2$ ratio = -11.079) was *CYP307A2*, which belonged to the CYP2 clan. The genes with the absolute value of the $\log_2$ ratio (≥ 2) might be related to the lower activity of hexythiazox-poisoned *P. citri* individuals (Liu et al. 2011a). However, quantitative RT-PCR results showed that the up-regulated value of $\log_2$ ratio (RR/SS) in *CYP385A2* and *CYP307A1* were only 0.28 and 0.17, the down-regulated value of $\log_2$ ratio (RR/SS) in *CYP389A6*, *CYP307A2* and *CYP389A1* were just -0.69, -1.46 and -1.08, respectively (Fig. 3). All sequences at http://www.ebi.ac.uk/ena/data/search?query=Panonychus citri



Fig. 7. Alignment of the predicted amino acid sequences of the *CYP307A1* in *Panonychus citri* between the hexythiazox-resistant (RR) and susceptible (SS) strains. Mazarine shading indicates identities and different color shading represents mutations. "-" represents no sequence to compare. We detected a sense amino acid mutation (14-threonine to serine). . This figure is shown in color in a supplementary document online as Suppl. Fig. 7 in Florida Entomologist 98(1) (March 2015) at http://purl.fcla.edu/fcla/entomologist/browse.

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Discussion

Insecticide resistance of the citrus red mite will seriously disrupt chemical control efficiency, and cross-resistance to various acaricides could further increase the difficulty of resistance management (Hu et al. 2010). Previously, only 2 P450 sequences of *P. citri* were available in GenBank. In this study, 46 additional unique sequences encoding P450 genes were selected. All 48 P450 (CYP) genes of the citrus red mite (Jiang et al. 2010; Liu et al. 2011a) were placed in the P450 repertoire for the insect genome or transcriptome that has been reported so far. The number of P450 sequences of the citrus red mite is fewer than the number found in other species (Table 1), which is more proof of the diversity of CYP.

Despite decades of study on P450 enzymes, the molecular mechanism mediated by this super-family of enzymes during metabolism is yet to be fully elucidated. Gene mutations and over-expression are considered to be responsible for resistance. Amichot et al. (2004) first demonstrated that point mutations were associated with insecticide resistance in the *Drosophila* cytochrome P450 and *CYP6A2* enabled DDT metabolism by the insect's cytochrome P450. Previously, sequence polymorphism of *CYP6A1* and *CYP6D1* had been documented in the house fly, but there was no link established between these polymorphisms and insecticide resistance (Kasai & Scott 2000; Scott et al. 1998). These results are in contrast with cytochrome P450 polymorphisms in humans, which are known to affect the metabolism of drugs (Guengerich et al. 1999; Ingelman-Sundberg 2001) and even pesticides (Eaton 2000). In fact, only 2 examples of pesticide resistance linked to mutations in a cytochrome P450 have been described. Thus, a single substitution in *CYP51* of the saccharomycete *Candida albicans* (C.P.Robin) Berkhout (T315A) (Lamb et al. 1997) and of the pathogenic fungus *Uncinula necator* (Schwein.) Burrill (F136Y) (Delye et al. 1998) confer resistance to the fungicides fluconazole and triadimenol respectively. Recently, 2 *CYP9M10* haplotypes were isolated from susceptible (JHB) and resistant (JPal-per) strains of *C. quinquefasciatus* Say to insecticides. The results showed that *cis*-acting mutations and duplications in the *CYP9M10* haplotype might be responsible for the insecticide resistance (Itokawa et al. 2011). In this study, sequence variation was detected in 2 CYPs. The A841C mutation on *CYP307A1* and A40T-mutation on *CYP381A2* caused the amino acid mutations *CYP307A1* (K278Q) and *CYP381A2* (T14S) respectively. These variations may be linked with metabolic resistance of *P. citri* to hexythiazox.

In many cases, the overexpression of P450 genes results in increased levels of total P450s and their activities are responsible for insecticide resistance (Cariño et al. 1994; Zhu et al. 2008b). Both constitutively increased expression (overexpression) and induction of P450s are thought to be responsible for increased levels of detoxification of insecticides (Pavek & Dvorak 2008). Expression of the P450 genes *CYP6AA7*, *CYP9J40*, *CYP9J34*, and *CYP9M10* isolated from *C. quinquefasciatus* were strongly correlated with levels of resistance to permethrin in the larval stage, with the highest expression levels identified in the most RR, suggesting the importance of *CYP6AA7*, *CYP9J40*, *CYP9J34*, and *CYP9M10* in permethrin resistance of larva mosquitoes (Hardstone et al. 2010; Liu et al. 2011b). The over expression of *CYP9M10* has also been reported in a resistant *C. quinquefasciatus* mosquito strain in Japan (Komagata et al. 2010) and has been linked with pyrethroid resistance in *C. quinquefasciatus* (Itokawa et al. 2010; Xu et al. 2005). Recent studies (Zhu et al. 2008b) indicated that several P450 genes were up-regulated in insecticide resistant house flies through a similar induction mechanism. However some researchers considered that a strain of *D. melanogaster* may not have the *CYP6A1* gene insertion and that *CYP6A1* may not have to be over expressed for DDT resistance to occur (Kuruganti et al. 2007). The results from this study supported that 22 cytochrome P450 genes were up-regulated and 24 cytochrome P450 genes were down-regulated in RR mites. *CYP389A6* (log_2 ratio [RR/SS] = 12.14) and *CYP307A2* (log_2 ratio [RR/SS] = -11.079) were the

Table 4. Cytochrome P450 gene expression differences between hexythiazox-susceptible and hexythiazox-resistant *Panonychus citri* strains.

Gene ID*	Gene name	Gene length	SS-RPKM**	RR-RPKM	log_2 ratio (RR/SS) ***	P-value	FDR§
U22268	*CYP389A6*	254	0.001	4.525	12.144	0.116	0.571
U20696	*CYP385A2*	545	0.001	0.703	9.457	0.483	0.878
U28087	*CYP389B2*	423	0.000	0.315	8.300	0.454	0.584
U12466	*CYP392A2*	296	39.992	84.127	1.073	0.000	0.016
U20374	*CYP385C2*	691	17.666	34.374	0.960	0.002	0.048
U4746	*CYP389A4*	339	4.365	6.781	0.635	0.510	0.879
U8588	*CYP385C4*	209	86.729	124.645	0.523	0.052	0.412
U8020	*CYP385B1*	1111	7.991	11.379	0.510	0.188	0.687
U25303	*CYP385C3*	583	31.726	44.027	0.473	0.079	0.481
U14605	*CYP386A1*	728	6.098	8.420	0.466	0.403	0.841
U32052	*CYP392A6*	742	6.481	8.777	0.438	0.415	0.852
U15991	*CYP386B1*	808	27.012	34.612	0.358	0.157	0.632
U10386	*CYP302A1*	426	9.552	11.691	0.291	0.626	0.899
U14234	*CYP385C6*	1027	32.058	38.049	0.247	0.238	0.722
U28154	*CYP314A1*	849	0.571	0.628	0.138	0.883	0.925
U4027	*CYP391A1*	799	314.830	346.180	0.137	0.076	0.478
U3795	*CYP389C1*	908	18.333	19.830	0.113	0.707	0.922
U16122	*CYP381A2*	619	41.236	43.942	0.092	0.707	0.922
U14035	*CYP392A4*	1159	159.907	167.916	0.071	0.437	0.860
U17307	*CYP385C5*	599	18.527	19.187	0.050	0.891	1.002
U2698	*CYP384A1*	833	127.897	131.532	0.040	0.737	0.929
U7720	*CYP388B1*	768	18.304	18.457	0.012	0.969	1.001
U7046	*CYP385C1*	1827	51.429	50.535	-0.025	0.846	0.976
U14772	*CYP392E1*	1527	39.245	38.134	-0.041	0.801	0.945
U17099	*CYP390C1*	1074	32.033	31.033	-0.046	0.834	0.968
U4449	*CYP392A3*	463	666.345	645.394	-0.046	0.522	0.849
U5774	*CYP381A1*	555	19.996	19.328	-0.049	0.900	1.006
U14294	*CYP389B1*	927	16.760	15.704	-0.094	0.775	0.940
U8409	*CYP314A2*	1219	11.228	10.371	-0.115	0.744	0.932
U3279	*CYP384A2*	495	351.989	321.958	-0.129	0.185	0.682
U400	*CYP392A1*	1058	32.167	29.330	-0.133	0.547	0.862
U12944	*CYP307A1*	1688	57.856	51.519	-0.167	0.200	0.703
U481	*CYP4CL2*	901	2.463	2.126	-0.213	0.822	0.963
U14677	*CYP392B1*	1670	26.360	22.022	-0.259	0.191	0.691
U9839	*CYP386A2*	403	28.456	21.864	-0.380	0.343	0.813
U12806	*CYP392A5*	659	42.662	31.973	-0.416	0.103	0.551
U3001	*CYP382A1*	496	6.712	4.634	-0.534	0.498	0.869
U12419	*CYP389A5*	380	10.708	7.057	-0.602	0.401	0.841
U18640	*CYP388A1*	1407	4.207	2.723	-0.628	0.287	0.757
U9228	*CYP392A7*	1384	10.424	6.643	-0.650	0.082	0.491
U29486	*CYP389A2*	225	6.576	3.405	-0.950	0.482	0.889
U18305	*CYP389A3*	209	15.930	5.499	-1.534	0.105	0.553
U16387	*CYP392E2*	579	8.306	1.323	-2.650	0.005	0.922
U31979	*CYP385A1*	621	4.766	0.617	-2.950	0.025	0.280
U19977	*CYP307A2*	342	2.163	0.001	-11.079	0.263	0.751
U4854	*CYP389A1*	513	3.606	0.001	-11.816	0.035	0.331

*The gene ID number (e.g.: U22268, i.e., Unigene22268_All) from the vector base of the *P. citri* transcriptome sequence (http://www.ebi.ac.uk/ena/data/view/ERP000885).

**The calculation of unigene expression using the RPKM method (reads per kb per million reads; formula: $RPKM=10^6C/(NL/10^3)$, C is the number of reads that are uniquely aligned to unigene A, N are the total number of reads that are uniquely aligned to all unigenes, and L is the number of bases in unigene A.

***RR means resistance strain, SS means susceptible strain.

§FDR: The false discovery rate method.

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most up- and down-regulated genes, respectively, and are likely associated with resistance of *P. citri* to hexythiazox.

However, these gene expression differences were shown later to be negative to some extent by quantitative RT-PCR (Fig. 3). The amplification bias between Illumina sequencing and qRT- PCR was the major driving force to vary the amplification difference between the RS and SS strains; meanwhile the RPKM and the $2^{-\triangle\triangle CT}$ method were 2 different ways to analyze gene differential expression generated from Illumina sequencing and qRT-PCR. The results of qRT-PCR indicated that no obvious difference could be observed in the hexythiazox-resistance line, and the expression difference of CYPs was not closely related to resistance. Therefore, the primary goal of this study was to provide baseline information for future research on *P. citri*. The resistance ratios of mites to various pesticides have grown so dramatically that researchers would be perplexed by these differences between mites and insects. In establishing the molecular action and resistance mechanisms of these and other pesticides, a limiting factor has been the lack of genetic systems and accompanying genomic resources needed for efficient identification of resistance mutations that suggest molecular mechanisms (Heckel 2003). For these reasons, other researchers employed variety of methods, such as genome sequencing, functional genomics and RNAi methods to uncover the molecular mechanisms of deltamethrin resistance in the *T. castaneum* QTC279 strain (Zhu et al. 2010). Subsequently bulk segregant analysis (BSA) mapping, SNP detection and complementation assays were used to identify a locus for monogenic, recessive resistance to etoxazole in *T. urticae* (Van Leeuwen et al. 2012). These and other methods will need to be employed to more fully understand the genetic basis of chemical resistance in the red mite and other members of the Acari.

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