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Molecular characterization of western corn rootworm pyrethroid resistance

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Abstract

Background: Western corn rootworm (WCR) pyrethroid resistance has been confirmed in the western US Corn Belt. Toxicological and biochemical studies indicated that multiple mechanisms of resistance might be involved in the resistance trait, such as enhanced metabolism and/or kdr target-site mutation(s) in the voltage-gated sodium channels. To characterize the mechanisms of WCR pyrethroid resistance at the molecular level, pairwise comparisons were made between RNA-Seq data collected from pyrethroid-resistant and -susceptible WCR populations. Gene expression levels and sodium channel sequences were evaluated.

Results: Seven transcripts exhibited significantly different expression ($q \leq 0.05$) when comparing field-collected pyrethroidresistant (R-Field) and -susceptible (S-Field) WCR populations. Three of the differentially expressed transcripts were P450s overexpressed in R-Field (9.2–26.2-fold). A higher number (99) of differentially expressed transcripts was found when comparing laboratory-derived pyrethroid-resistant (R-Lab) and -susceptible (S-Lab) WCR populations. Eight

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of the significant transcripts were P450s overexpressed in R-Lab (2.7–39.8-fold). This study did not detect kdr mutations in pyrethroid-resistant WCR populations. Other differentially expressed transcripts that may play a role in WCR pyrethroid resistance are discussed.

Conclusion: This study revealed that P450-mediated metabolism is likely to be a major mechanism of WCR pyrethroid resistance, which could affect the efficacy of other insecticides sharing similar metabolic pathways. Additionally, results suggested that although laboratory selection of a pyrethroid-resistant WCR population may help to characterize resistance mechanisms, a field-selected population provided rare and perhaps major variants corresponding to the resistance trait.

Keywords: *Diabrotica virgifera virgifera,* western corn rootworm, pyrethroid resistance, insecticide resistance, RNA-Seq, P450

1 Introduction

Yield losses and management costs associated with the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), have been estimated in excess of 1 billion dollars per year in the USA.[1–3] Populations of this major pest of maize (*Zea mays* L.) have been continuously adapting to a variety of control tactics.[4] Particularly in the western US Corn Belt, limited rotation of WCR management strategies and increased reliance on aerial applications of pyrethroid insecticides for adult suppression in addition to the use of soil insecticides for larval control have been imposing high selection pressure on WCR populations.[5–8] As a result, multiple WCR populations in southwestern areas of Kansas and Nebraska have developed resistance to pyrethroids used in foliar (bifenthrin) and soil (bifenthrin, tefluthrin and cyfluthrin) formulations significantly impacting the performance of major insecticide products used for WCR control.[9–11]

The toxicity of pyrethroids depends mainly on the level of exposure and their ability to bind and disrupt voltage-gated sodium channels, although secondary target sites like voltage-gated calcium and chloride channels have also been reported.[12–16] Mutations in the insect voltage-gated sodium channels can lead to reduced sensitivity to pyrethroid insecticides, and consequently to what is commonly referred to as knockdown resistance (*kdr*).[16,17] A variety of nonsynonymous amino acid substitutions in the sodium channel gene have been identified as *kdr* mutations conferring pyrethroid resistance in insects.[18] Since pyrethroids and dichloro-diphenyl-trichloroethane (DDT) share

the same target site, *kdr* mutations provide cross-resistance between these two insecticides,[19–24] which could explain the DDT cross-resistance observed in pyrethroid-resistant WCR populations.[25] However, an investigation of pyrethroid-resistant WCR sodium channels did not detect mutations that are commonly associated with *kdr* resistance in other insect species, suggesting that if target-site insensitivity were associated with the resistance trait, it could represent a novel mutation not previously identified within Chrysomelidae.[10]

In addition to changes in target site sensitivity as described above, an elaborate system of enzymes such as cytochrome P450 microsomal monooxygenases (P450s), esterases and glutathione *S*-transferases act to metabolize and detoxify xenobiotic compounds[26] and have commonly been associatedwith resistance in pest species. A number of instances of pyrethroid resistance in arthropods are related to enhanced metabolism involving esterases[27–31], P450s,[32–40] and glutathione *S*-transferases.[41–44] It was previously reported that WCR resistance to pyrethroids was partially suppressed by inhibitors of esterases and P450s,[25] and that the activity of these enzymes was higher in pyrethroid-resistant WCR populations.[10] Collectively, these investigations suggest that enhanced metabolism may be a major contributor to pyrethroid resistance in WCR.[10,25] The current study was conducted to test this hypothesis at the molecular level and to characterize the differential gene expression of pyrethroid-resistant WCR individuals.

2 Materials and methods

2.1 WCR populations

Four independent WCR adult populations were tested throughout this study. In 2016, WCR beetles were collected from Saunders County (S-Field) and Keith County (R-Field) (Nebraska, USA) representing field-derived pyrethroid-susceptible and -resistant populations, respectively. The Keith County collection site represented a commercial field where continuous maize production plus annual soil- and aerial pyrethroid applications had been used for at least five consecutive years prior to this study. The Saunders County field was located at the University of Nebraska Eastern Nebraska Research and Extension

Center, which is surrounded by a large area of continuous maize that had not received insecticide aerial applications for over 10 years. Pyrethroids had only been soil-applied at that site in a few small-plot trials. A pyrethroid-resistant nondiapausing population reared for nine generations under adult bifenthrin selection (R-Lab)[10] and a nondiapausing control purchased from Crop Characteristics, Inc., Farmington, MN (S-Lab) were also used to represent lab-derived genotypes. R-Lab was originally established in 2014 by mating pyrethroid-resistant males from Perkins County, NE with susceptible non-diapausing females from S-Lab.[10] Susceptibility levels against bifenthrin, tefluthrin and cyfluthrin had been previously documented for the WCR populations tested in this study at the adult[25,45] and larval stages[11,25]. Relative to S-Lab, bifenthrin resistance ratios (RR_{50}) estimated for S-Field, R-Field and R-Lab adults were 13.1, 39.6 and 37.9, respectively.[45] WCR control failure had been observed for R-Field and R-Lab, but not for S-Field or S-Lab.[11,45] Both field collected and laboratoryderived populations were reared simultaneously for one generation prior to nucleic acid extractions using standard laboratory rearing procedures.[5] All populations were maintained under laboratory conditions of 23 ± 1 °C and 13 ± 1 h photophase in the Department of Entomology, University of Nebraska, Lincoln, NE. In 2017, WCR beetles (48 h-old) were transferred from rearing cages to individual microcentrifuge tubes, flash-frozen in liquid nitrogen immediately and stored at −80°C until RNA and DNA extractions.

2.2 RNA extraction and library preparation

Total RNA was individually extracted from whole bodies of 10 adults (n = 10 biological replicates) of each WCR population (1:1 sex ratio) using a Qiagen RNeasy mini kit (Cat No. 74104, Germantown, MD, USA) according to the manufacturer's instructions. RNA concentration and quality were determined by spectrophotometry using a Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA) and fluorimetry using Qubit (Thermo Scientific). In addition, an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) was used for assessment of the relative RNA Integrity Number equivalent (RINe) as a quality metric. Barcoded mRNA-Seq libraries (n = 40) were prepared by polyA selection and sequenced in three lanes of Illumina HiSeq 4000 to an

expected sequencing depth of ~26 million reads per sample. Quality assessment of samples and sequencing were performed by Genewiz, South Plainfield, NJ, USA.

2.3 Gene expression analysis

A web interface provided by the national cyberinfrastructure CyVerse in the Discovery Environment[46,47] was used for the RNA-Seq analyses. Data analysis of laboratory and field populations followed the same workflow in separate pairwise comparisons. Low quality bases and Illumina adapter sequences were trimmed from paired reads using the Trimmomatic 0.36 program,[48] with the following parameters: ILLUMINACLIP:contaminants:2:30:10, LEADING:3, TRAILING:3, HEADCROP:7, CROP:138, SLIDINGWINDOW:5:20, MINLEN:50. FastQC 0.11.5[49] was used to evaluate the qualities of raw and trimmed sequencing data. Trimmed reads were aligned to the WCR genome (NCBI, BioProject: PRJNA432972) using HISAT2 2.1[50] and transcripts assembled with StringTie 1.3.3.[51] A single transcript sequence was obtained using StringTie 1.3.3-merge[52] and used to build an index file in Kallisto 0.42.3-index.[53] An alignment-free transcript quantification approach was then performed for each paired-read using Kallisto 0.42.3-quant[53] with 100 bootstrap iterations to provide a measure of the accuracy of the quantification by random resampling with replacement. Pairwise comparisons of Kallisto quantification outputs were made between pyrethroidresistant and pyrethroid-susceptible WCR populations using sleuth 0.29.0 package[54] in R 3.5.0 software (R Foundation for Statistical Computing, Vienna, Austria). A likelihood ratio test (LRT) was performed to verify goodness of fit of transcripts to the full statistical model that considers the pyrethroid resistance status as a factor. Considering a correction of *P* values for type I error of false positives, that is false discovery rate (FDR) correction, expression levels with $q \leq 0.05$ were considered significant. Transcripts expressed significantly differently were translated into amino acid sequences using EMBOSS Transeq — https://www.ebi.ac.uk/Tools/st/emboss_transeq/ .[55] Sequence similarity searches were performed for the longest open reading frames (ORFs) of significant transcript sequences on NCBI BLASTx/BLASTp,[56] Inter-Pro Scan[57] and i5K Workspace@NAL.[58] Also, pairwise local protein sequence alignment between

some significant transcripts were performed in EMBOSS Water — https://www.ebi.ac.uk/Tools/psa/emboss_water/ [55] — with default settings (matrix:BLOSUM62; gap penalty:10; and extension penalty:0.5) to verify similarity between them.

2.4 WCR voltage-gated sodium channel gene screening

Voltage-gated sodium channel amino acid sequences from Colorado potato beetle (CPB) *Leptinotarsa decemlineata* (Say) and Asian longhorned beetle (ALB) *Anoplophora glabripennis* (Motschulsky) were used to localize homologous sequences in the WCR transcriptome. Similarity searches were performed with local tBLASTn where the CPB and ALB protein sequences available from NCBI (Accessions: XP_023023069.1 and XP_018568941.1, respectively) were used as queries and the WCR genome was used as database. Sequences identified in tBLASTn were filtered from HISAT2 alignment *bam* files and then merged using Samtools 1.7[59] available in the Discovery Environment of CyVerse.[46,47] The resulting merged file was screened for nonsynonymous nucleotide substitutions in Tablet 1.17.08.17.[60] using the visual application for tag variants. In addition, the sequences from CPB and ALB used earlier in tBLASTn were aligned to sequences extracted from the WCR sodium channel scaffold and to the sodium channel sequence of housefly *Musca domestica* L. (*Vssc1*) in GenBank (Acession No: AAB47604) using T-Coffee (https://www.ebi.ac.uk/ Tools/msa/tcoffee/).[61] The predictions of WCR sodium channel domain structures and transmembrane segments were performed with SMART (http://smart.embl-heidelberg.de/),[62] HMMER 3.2.1 (https://www.ebi.ac.uk/Tools/hmmer/)[63,64] and TMHMM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/).[65]

2.5 DNA sanger sequencing kdr investigation

Two putative nonsynonymous substitutions found in the voltagegated sodium channel screening, numbered L1422 and A1558 in *Vssc1*, were investigated in WCR populations by Sanger sequencing. Genomic DNA was extracted individually from 10 adults (1:1 sex ratio) of each WCR population using a Qiagen DNeasy kit (Cat No. 69504) according to the manufacturer's instructions. Polymerase chain reactions (PCR) were performed in a 50 µL final reaction volume containing 100 ng of

template DNA, 1 unit of GoTaq® Flexi DNA polymerase, 1× Colorless GoTaq® Flexi buffer (Cat No. M8291, Promega, Madison, WI, USA), 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 0.2 µM of each primer. The PCR temperature profile for each fragment included an initial heating step at 95 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, annealing temperatures for 90 s, 72 °C for 1 min, and a final extension of 60 °C for 30 min. The forward (5′-CCTTAAACCGTCACTGGCAG-3′) and reverse (5′-ACAAGCATTCACATCAGGGA-3′) primers were used with an annealing temperature of 59 °C to amplify a 492 bp region surrounding the site L1422. Also, forward (5′-TGAGCAGATGGGACGTGAAT-3′) and reverse (5′-AATCGCAACTTTTCCGCACT-3′) primers were used with an annealing temperature of 56 °C to amplify a 411 bp region surrounding the A1558 location. Primers were designed in Primer3web version 4.0.0.[66,67] DNA concentration and quality before and after PCR were determined by Nanodrop 2000 (Thermo Scientific). PCR products were analyzed by 1.3% agarose gel electrophoresis, purified with QIAquick PCR Purification Kit and sequenced by Genewiz, South Plainfield, NJ with the Applied Biosystems (ABI) 3730 DNA Analysis Instrument (Life Technologies, Grand Island, NY, USA). Sequences were aligned and mapped to the WCR sodium channel gene contig using Geneious Basic 5.6.7[68], and screened for targeted *kdr* polymorphisms.

3 Results

3.1 RNA extraction and library preparation

All RNA samples used for library preparation were of high integrity with an average A260/A280 = 2.12 and RINe = 10. The Illumina HiSeq platform produced a total of 1.29 billion paired-reads of 150 bp in length, yielding 389 876 Mbases with a mean quality score of 37.96 (91% bases ≥ Q30). An average of 32.3 ± 0.5 (SE) million reads were produced per library. Illumina sequencing data from this study have been submitted to the NCBI BioProject (https://www.ncbi.nlm.nih.gov/bioproject) under accession number PRJNA430262. These data are also available through the NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) under accession numbers that go from SRX3594800 through SRX3594839.

3.2 Gene expression analysis

Out of 53 929 assembled transcripts from WCR field populations, seven were differentially expressed and expressed at a higher level in the pyrethroid-resistant R-Field compared to the -susceptible S-Field population ($q \leq 0.05$) (Tables S1 and S3). Similarity searches of translated amino acid sequences predicted that three of the transcripts were cytochrome P450 monooxygenases ($P450_{(1\text{-}3)Field}$), two were transcription factors and one was a junctophilin (**Figure 1**). The

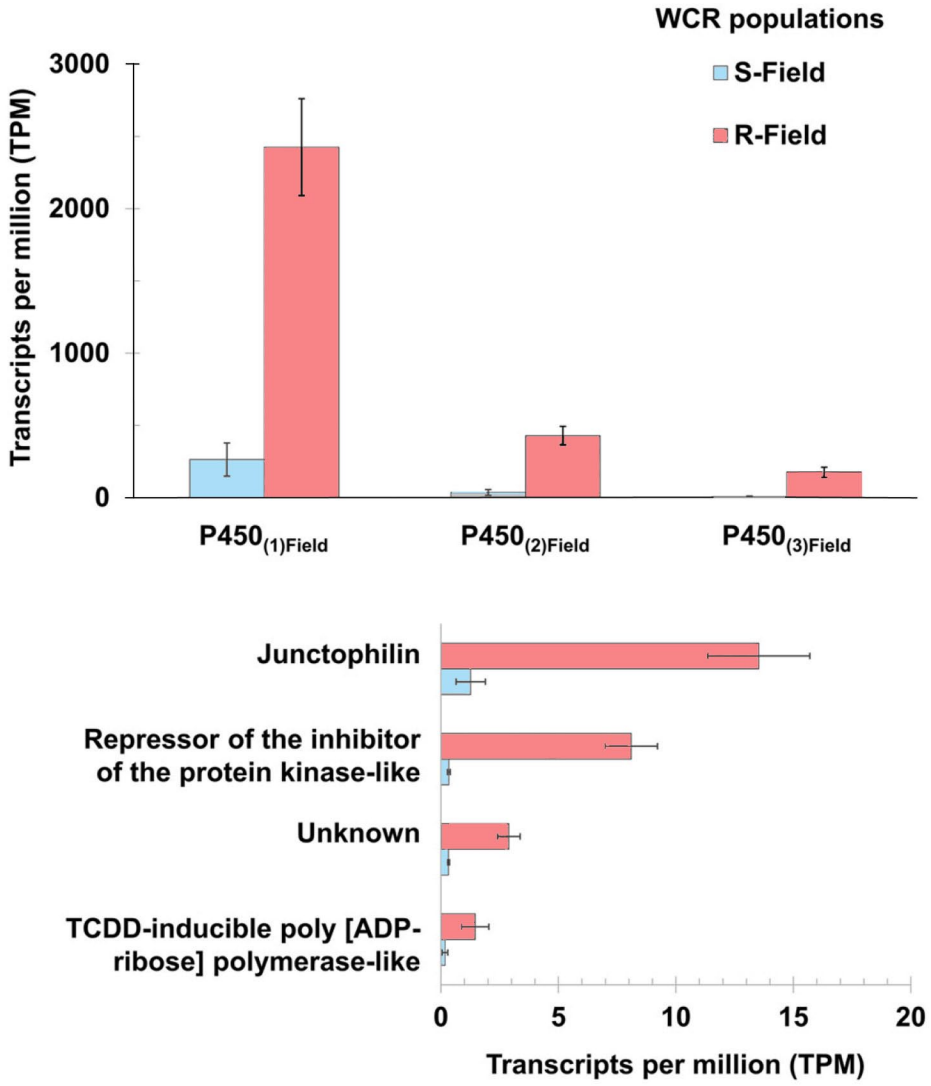

Figure 1. Transcripts differentially expressed ($q \leq 0.05$) in pairwise comparisons between field-derived WCR populations known to be susceptible (S-Field) and resistant (R-Field) to pyrethroids. Graphs show the transcript mean abundance in transcripts per million (TPM) units ± SE.

Table 1. Abundance ratios of P450 transcripts differentially expressed ($q \leq 0.05$) in pairwise comparisons within laboratory- or field-derived WCRpopulations known to be susceptible (S-) and resistant (R-) to pyrethroids

P450 candidates	*ORF size (aa)*	*WCR genome top hit ID (E-value)*[a]	*Abundance ratio* $R_{-(TPM)}/S_{-(TPM)}$ [b]
$P450_{(1)Field}$	146	XM_028285602.1 (1.17e-50)	9.17
$P450_{(2)Field}$	158	XM_028294106.1 (6.61e-103)	11.77
$P450_{(3)Field}$	166	XM_028285602.1 (4.03e-51)	26.18
$P450_{(1)Lab}$	182	XM_028285602.1 (5.55e-51)	39.06
$P450_{(2)Lab}$	120	XM_028285602.1 (5.59e-75)	28.14
$P450_{(3)Lab}$	499	XM_028285602.1 (1.27e-100)	22.32
$P450_{(4)Lab}$	158	XM_028285602.1 (1.92e-102)	47.18
$P450_{(5)Lab}$	158	XM_028294106.1 (6.61e-103)	39.84
$P450_{(6)Lab}$	499	XM_028289555.1 (6.4e-180)	2.70
$P450_{(7)Lab}$	288	XM_028297637.1 (0)	8.71
$P450_{(8)Lab}$	442	XM_028290131.1 (0)	7.28
$P450_{(9)Lab}$	292	XM_028294105.1 (0)	0.04
$P450_{(10)Lab}$	139	XM_028289556.1 (2.11e-49)	0.12

a. i5k Workspace@NAL (https://i5k.nal.usda.gov/webapp/blast/) tBLASTn; Database: *Diabrotica virgifera* genome assembly GCA_003013835.2.
b. TPM, mean abundance in transcripts per million (TPM) units.

transcript mean abundances of $P450_{(1)Field}$, $P450_{(2)Field}$ and $P450_{(3)Field}$ were 9.2-, 11.8-, and 26.2-times greater in R-Field than in S-Field, respectively (**Table 1**). The two transcription factor candidates were 8.5- and 23.9-times more abundant in R-Field. The mean abundance of predicted junctophilin (100% identity and query cover to either ALB or CPB in BLASTp) was 10.7-times greater in R-Field than in S-Field. No sequence similarity was found for one of the significant transcripts overexpressed in R-Field.

For pairwise comparison between the laboratory unselected (SLab) and bifenthrin-selected (R-Lab) WCR populations, 47 255 transcripts were analyzed and 99 exhibited significantly different expression ($q \leq 0.05$). A total of 64 significant transcripts were expressed at a higher level in the pyrethroid-resistant R-Lab population whereas 35 were expressed at a lower level in the same population (Tables S2 and S3). Protein similarity searches suggested that 10 of the transcripts differentially expressed between laboratory-derived populations were P450s ($P450_{(1-10)Lab}$) (**Figure 2**) and for eight of them ($P450_{(1-8)Lab}$), the

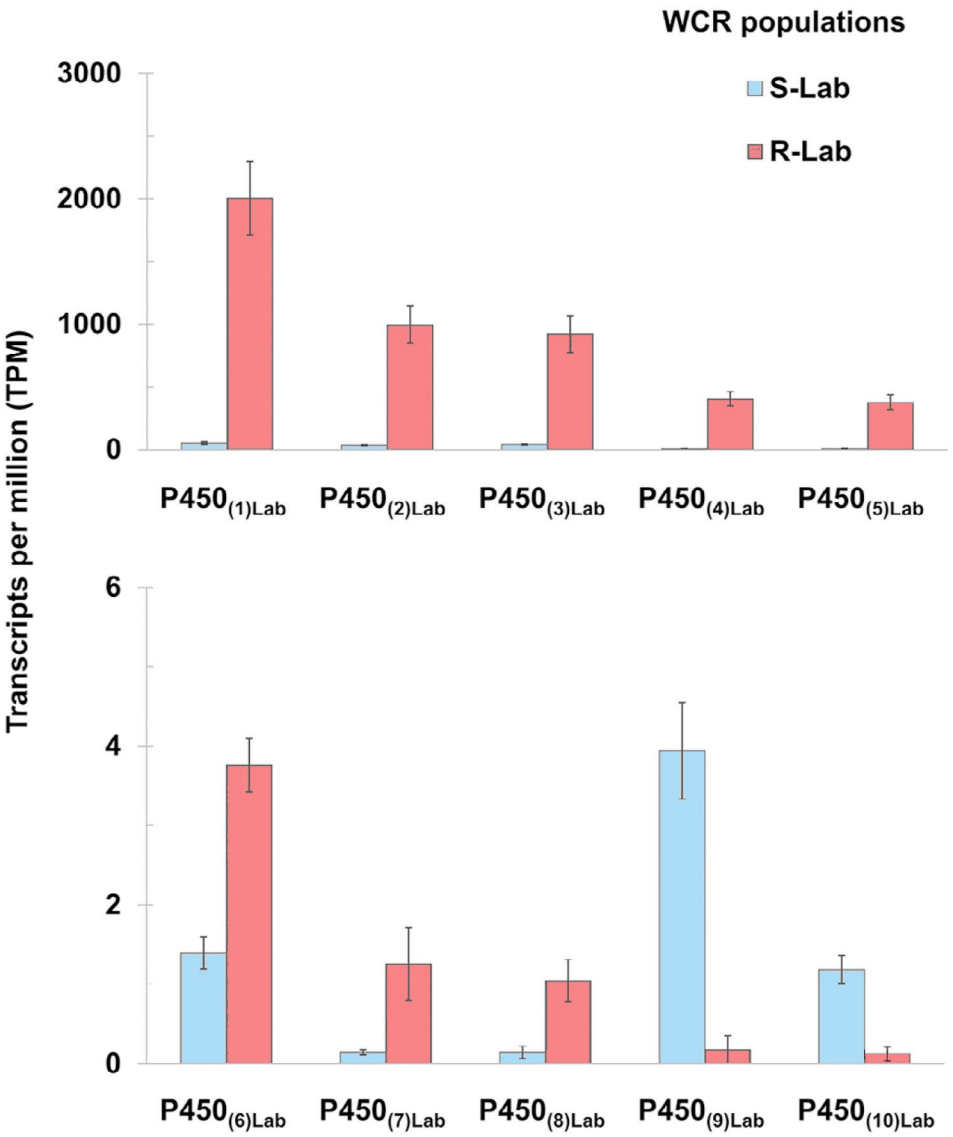

Figure 2. P450 transcripts differentially expressed ($q \leq 0.05$) in pairwise comparisons between laboratory-derived WCR populations known to be susceptible (S-Lab) and resistant (R-Lab) to pyrethroids. Graphs show the transcript mean abundance in transcripts per million (TPM) units ± SE.

mean abundance in RLab was 2.7 to 39.8 times greater than in S-Lab (Table 1). Two P450s ($P450_{(9\text{-}10)Lab}$) and three enzymes also involved in oxidative metabolic processes appear to be underexpressed in the R-Lab population along with 13 other predicted proteins (**Table 2**). The mean abundance of $P450_{(9)Lab}$ and $P450_{(10)Lab}$ was 22.6 and 9.5 times lower in R-Lab than in S-Lab, respectively (Figure 2 and Table 1).

Table 2. Predicted identity and function of transcripts differentially expressed ($q \leq 0.05$) in the pyrethroid-resistant laboratory population R-Lab

	Proteins predicted (no. of targets)[a]	
Predicted function	*Overexpressed*	*Underexpressed*
Oxidation	P450s (8)	P450s (2), lysyl oxidase (1), Glyoxylate/hydroxypyruvate reductase (1), Sorbitol dehydrogenase (1)
Hydrolysis	Carboxypeptidase (1), maltase (1), beta-hexosaminidase (1), chitinase (1), aldose-epimerase (1), myrosinase (1), glycoside hydrolase (1)	Myrosinase (1), beta ureidopropionase (1)
Conjugation		Glutathione *S*-transferase (1)
Proteolysis	Cysteine proteinases (2)	
Transporter	ABC transporters (4), sugar transporters (2), peptide transporters (4)	
Transcription factors	Zinc finger type proteins (4), nuclear factor NF-kappa-B p110 subunit-like (1), transcription activator MBF2 family (1)	Zinc finger type proteins (2)
Nervous system	E3 ubiquitin-protein ligase MYCBP2 (1), neprilysin (1)	
Immune response	Coleoptericin (1), leucine-rich repeat protein SHOC-2-like (1)	
Membrane barrier	Tetraspanin-2A (1)	
Catalysis	Uridine phosphorylase (1), glutamine-fructose-6-phosphate aminotransferase 2 (1)	
Cell division	Protein skeletor, isoform B/C (1)	Dynactin subunit 2 (1)
Other	Reverse transcriptase (3), laminin subunit gamma-1-like (1), galectin (1), mucin (1)	Reverse transcriptase (2), heat shock protein beta-1 (1), elongation factor Tu (1), retrovirus-related Pol polyprotein LINE-1 (1), alphatocopherol transfer protein-like (1), asialoglycoprotein receptor 2/C-type lectin precursor (1)

a. Sequences, similarity search results and putative GO annotations can be found in Table S2.

The longest and most complete P450 transcript sequences (~500 amino acids) differentially expressed among all WCR populations tested were $P450_{(3)Lab}$ (499 amino acids), $P450_{(6)Lab}$ (499 amino acids) and $P450_{(8)Lab}$ (442 amino acids). All remaining P450 transcripts were translated into shorter amino acid sequences (<300 amino acids) (Table 1). Sequence identity between the longest P450 transcripts and all remaining significant P450s ranged from 25% to 100% (**Table 3**). Protein search databases revealed that all P450s differentially expressed in either field-derived or laboratory-derived WCR populations presented >40% sequence identity to CYP6-like P450 proteins from other Coleoptera species (Tables S1 and S2).

Cellular functions predicted for remaining transcripts differentially expressed in laboratory-derived populations are described in Table 2. Among them, at least seven hydrolases, four ABC transporters, two sugar transporters, six transcription factors and two nervous system-related transcripts (E3 ubiquitin-protein ligase and neprilysin) were overexpressed in the pyrethroid-resistant WCR population R-Lab (Table 2). No sequence similarity was found in protein search databases for 23 significant transcripts (17 underexpressed and six overexpressed in R-Lab), and thus they are of unknown identity and function.

Table 3. Percentage identity matrix of local alignments performed between ORFs of P450 transcripts differentially expressed ($q \leq 0.05$) in WCR populations

		Longest ORFs (>400 amino acids)		
P450 candidate	*Transcript ID*[a]	*$P450_{(3)Lab}$*	*$P450_{(6)Lab}$*	*$P450_{(8)Lab}$*
$P450_{(1)Field}$	MSTRG.24908.1	99.3	51.7	50.3
$P450_{(2)Field}$	MSTRG.75696.1	93.7	47	45.6
$P450_{(3)Field}$	MSTRG.24907.1	88.9	48.5	50.3
$P450_{(1)Lab}$	MSTRG.19112.1	88.4	48.2	50.3
$P450_{(2)Lab}$	MSTRG.63174.1	100.0	29.2	25.8
$P450_{(3)Lab}$	MSTRG.22143.1	*	41.9	39.6
$P450_{(4)Lab}$	MSTRG.71693.1	98.7	47.7	46.2
$P450_{(5)Lab}$	MSTRG.59366.1	93.7	47.0	45.6
$P450_{(6)Lab}$	MSTRG.36287.1	41.9	*	51.7
$P450_{(7)Lab}$	MSTRG.3101.1	38.1	41.2	42.7
$P450_{(8)Lab}$	MSTRG.24813.1	39.6	51.7	*
$P450_{(9)Lab}$	MSTRG.56151.1	36.5	35.6	34.1
$P450_{(10)Lab}$	MSTRG.66987.1	51.9	98.5	53.1

a. Sequences, similarity search results and putative GO annotations of each transcript ID can be found in Tables S1 and S2.

3.3 WCR voltage-gated sodium channel gene screening

Two potential nonsynonymous substitutions were found in the voltage-gated sodium channel gene of some individuals from the R-Lab and R-Field populations when screening the RNA-Seq reads against the WCR genome. Locations of substitutions were L1422P and A1558I in *Vssc1* (Figure S1), which could be *kdr* mutations not reported previously in pyrethroid-resistant insects. However, the sequencing coverage at those regions was too low to enable confirmation. Voltage-gated sodium channel domain structures and transmembrane segments were predicted (Figure S1) along a 2040 amino acid sequence for WCR (Figure S2).

3.4 DNA sanger sequencing kdr investigation

Relative to the pyrethroid-susceptible individuals from populations S-Lab and S-Field, no mutations were found for the L1422 and A1558 loci in pyrethroid-resistant WCR. However, the 411 bp PCR product covering the A1558 locus did not amplify in all WCR samples (Figure S3) and therefore fewer sequences (14 out of 40) were compared at this locus.

4 Discussion

This study supports the hypothesis that constitutive enhanced metabolism of pyrethroid insecticides such as bifenthrin, tefluthrin and cyfluthrin is a mechanism of WCR pyrethroid resistance and suggests that P450s may play a major role in the resistance trait. Although several hydrolytic enzymes were differentially expressed in laboratory-derived WCR populations, P450s were the only metabolism-related genes exhibiting significantly different expression in field-derived WCR populations. In fact, nearly 50% of transcripts differentially expressed in the field-derived pyrethroid-resistant WCR populations were overexpressed P450s as well as ~13% of transcripts overexpressed in the laboratoryselected pyrethroid-resistant WCR population. Enhanced oxidative metabolism resulting from the overproduction of P450 enzymes has been commonly observed in

pyrethroid-resistant arthropods[33,34,36–39,69] since it can accelerate the production of metabolites that are less toxic, less stable and are unable to reach the target site.[26]

The overexpression of P450s observed in resistant insects may be derived from gene amplification and/or from up-regulation of expression caused by substitutions, insertions and deletions in promoter sequences and/or regulatory loci.[70–72] Further investigation is necessary to identify not only the mechanism behind P450s overexpression in pyrethroid-resistant WCR but also specific P450 genes involved. A detailed annotation and phylogeny of all WCR P450 genes is part of an ongoing effort to annotate the WCR genome and will be published elsewhere. Most P450 proteins are ~500 amino acids long,[73] and it is likely that the majority of differentially expressed P450 transcripts were fragments of longer gene sequences as the translated protein sequences were ~200 amino acids. Although incomplete sequences prevent the identification of specific encoding P450 genes, all differentially expressed P450 transcripts shared >40% similarity to Coleoptera CYP6 proteins in protein search databases. Results from local alignments and the variable expression observed between transcripts suggest that multiple P450 genes may be involved in WCR pyrethroid resistance. Also, differences in percent identity of amino acid sequences may suggest that P450s overexpressed in the field-derived pyrethroid-resistant population are related but not necessarily the same P450 genes, or are isoforms overexpressed in the pyrethroid selected laboratory population. These results support the variable activity of P450-mediated *O*-demethylation observed previously when comparing laboratory-selected and field-derived pyrethroid-resistant WCR populations.[10]

The number of transcripts differentially expressed between resistant and susceptible WCR populations was higher when comparing laboratory-selected individuals with the laboratorysusceptible individuals, which supports the suggestion that artificial insecticide selection favors a polygenic response.[74,75] Our results indicate that although laboratory selection of pyrethroidresistant WCR populations is useful for predictions and investigations of resistance mechanisms, the heterogeneity and population dynamics present in the field are more likely to provide conditions for detection of rare and perhaps major variants contributing to resistance.[76,77] Several hydrolytic enzymes

and ABC transporters commonly associated with insecticide resistance [28,39,78–80] were overexpressed in the laboratory-selected WCR population whereas their expression were not significantly different in the field-derived resistant population. However, the correlation between transcript levels and protein activity is not necessarily linear in resistant insects.[81–83] Qualitative changes in esterases are suggested by their higher biochemical activity in field-derived pyrethroid-resistant WCR populations[10] in which transcript levels remain unchanged. Insecticides such as pyrethroids, carbamates and organophosphates share the common structural feature of ester bonds between alcohol and acid moieties and are therefore susceptible to hydrolysis catalyzed by esterases.[84–86] Qualitative changes of esterases may play a role in pyrethroid enhanced metabolism, reduced efficacy of the organophosphate dimethoate and negative cross-resistance with indoxacarb previously observed in pyrethroid-resistant WCR populations,[45] and requires further investigation to fully elucidate their role in resistance.

A previous investigation showed that bifenthrin resistance levels estimated from adult bioassays using active ingredients were similar for both the laboratory-selected and field-collected pyrethroid-resistant WCR populations used in our study.[45] However, this same investigation revealed that their susceptibility to commercial rates of a bifenthrin foliar formulation differed under aerial application simulations. It was found that the lowest bifenthrin application rate failed to control either the laboratory- or field-derived resistant adults while the highest application rate failed to control only field-derived resistant populations.[45] Although both pyrethroid resistant WCR populations used in our study may share some mechanisms of resistance, gene variants and/or transcriptional levels present in the field-derived resistant WCR population could be more relevant in terms of practical resistance.[87]

Moreover, it should be noted that estimates of phenotypic and genotypic differences associated with a resistance trait are relative to the susceptible populations used for comparison. While the field-derived adult WCR population used in our study as a susceptible reference had previously shown to be effectively controlled by application rates of a bifenthrin foliar formulation, it was still approximately 10-fold more tolerant to bifenthrin active ingredient than laboratory-derived susceptible populations.[45] Thus, differences in expression between

laboratory- and fieldderived WCR adults may also reflect the differential response to pyrethroids of the susceptible populations used in each comparison. Variation in number, relative abundance and identity of transcripts differentially expressed should be expected for different WCR populations and pairwise comparisons.

Pyrethroids bind to voltage-gated sodium channels caused a delay in channel closing and prolonged sodium inactivation.[26] Repetitive action potentials are generated across neurons, ultimately disrupting the normal flow of information to the central nervous system. Some proteins with nervous system functions were also differentially expressed in the pyrethroid-resistant WCR populations tested and could play a role in the insecticide resistance trait. Junctophilin, overexpressed in the field-derived pyrethroid-resistant WCR population, is a protein commonly found in human neurons, where it is involved in coupling membrane neurotransmitter receptors and intracellular channels.[88] A study showed that a junctophilin gene from *Drosophila melanogaster* Meigen is functionally equivalent to mammalian homologues and that flies with altered junctophilin expression also have marked neuronal alterations.[89] The predicted E3 ubiquitinprotein ligase and neprilysin, overexpressed in the laboratoryselected pyrethroid-resistant WCR populations, also play a role in the nervous system of insects. Overexpression of the E3 ubiquitin-protein ligase gene in *Drosophila* glial cells causes synaptic impairments and down-regulation of Na+/K+ pumps,[90] whereas neprilysin modulates neuronal activity, viability and survival.[91–93]

This study did not detect *kdr* mutations in pyrethroid-resistant WCR populations. However, the low RNA sequencing coverage of the WCR sodium channel prevented a thorough screen. Previous research found DDT cross-resistance in pyrethroid-resistant WCR strains[25] and in most cases this is an indication of *kdr* target-site mutation(s) in the voltage-gated sodium channels.[19,94–96] Although *kdr* mutation(s) cannot be excluded as a mechanism of WCR resistance, our results indicate that one or more P450s overexpressed in pyrethroid-resistant WCR populations may be causing both pyrethroid resistance and DDT crossresistance. Overexpression of some P450s, such as *Cyp6g1*, have been associated with DDT cross-resistance in other species.[97–100] Understanding the molecular basis of WCR pyrethroid resistance is critical to implement efficient resistance management strategies and to

develop protocols for resistance detection. This study revealed that P450-mediated detoxification is probably a major mechanism positively selected in pyrethroid-resistant WCR populations, which could have an important impact on the efficacy of other insecticides sharing similar metabolic pathways. WCR pyrethroid resistance monitoring is recommended, and the P450 genes differentially expressed in this study could be further characterized and used for that purpose.

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Supporting information is attached to the archive record for this article.

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