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Transcriptomic responses of the aphid *Myzus persicae nicotianae* Blackman (Hemiptera: Aphididae) to insecticides: Analyses in the single Chilean clone of the tobacco aphid

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The tobacco aphid *Myzus persicae nicotianae* Blackman is a subspecies of the highly polyphagous and agricultural pest *Myzus persicae* (Sulzer). For its control, insecticide applications are widely used, but resistance to numerous molecules has been reported, displaying at least three insecticide resistance mechanisms, including: (i) elevated carboxylesterases (E-Carb), (ii) modification of the acetylcholinesterase (MACE), and (iii) *kdr* and *super-kdr* insensitivity mutations. In Chile, populations of the tobacco aphid are characterized by the presence of a single predominant clone, which is also present in high proportions in other countries of the Americas. This aphid clone exhibits low levels of carboxylesterase activity and is *kdr* susceptible, but the MACE mechanism of insecticide resistance has not been studied. In order to characterize the tobacco aphid in terms of the MACE mechanism and to identify a preliminary group of aphid genes putatively involved in insecticide resistance, a cDNA microarray was used to study the transcriptomic responses when aphids are sprayed with a carbamate insecticide. The single Chilean clone of the tobacco aphid was characterized as MACE susceptible, but we found 38 transcripts significantly regulated by insecticide exposure (13 up- and 25 down-regulated genes). The expression of six of them was validated by qRT-PCR experiments at several time points (6, 12, 18, 24, 30, 36, and 42 h) after insecticide application. This mutational and transcriptomic characterization of the tobacco aphid responding to insecticide spray opens new hypotheses in the understanding of the molecular mechanisms underlying insecticide resistance.

Key words: Insecticide resistance, MACE mechanism, *Myzus persicae*, transcriptomic analysis.

INTRODUCTION

Since the apparition of the first synthetic insecticide (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane, DDT) in 1939, human efforts have been directed to lessen the resistance that sooner or later, most arthropod species develop (Yu, 2008). In fact, the systematic use of a wide array of insecticides, with variable dosage and at various frequencies of applications, represents a broad range of selective regimes on insect pest populations. This scenario can ultimately lead to the appearance of resistance to multiple insecticide families and concentrations, causing an army race between insects and humans that have caused a number of non-desirable effects such as decreased crop yields, accumulation of pesticide residues in foods, negative environmental impacts on beneficial insects, and increased pollution in air, soil and water (Chowdhury et al., 2012). Most insecticides affect the insect nervous system, resulting in uncontrollable muscular contractions and lactic acidosis (Yu, 2008). So far, two main mechanisms that confer insecticide resistance in insects have been reported: (i) insensitivity resistance, referred to a modification of insecticide target-sites that reduce or abolish insecticide sensitivity, and (ii) metabolic resistance through increased activities of detoxifying enzymes (Ffrench-Constant et al., 2004).

The study of the molecular mechanisms involved in insecticide resistance began in the early 90's, revealing enzymatic mechanisms of sequestration and degradation of insecticides (Raymond et al., 1998) and target site mutations (Li et al., 2007). Recent advances in genomics and post-genomics allowed significant progress in the field of insecticide resistance by especially facilitating the study of more complex metabolic pathways and greatly expanding our views on the range of options available for insects to develop insecticide resistance (Ffrench-Constant et al., 2004).

Aphids (Hemiptera: Aphididae) are herbivorous insects distributed worldwide that feed exclusively from phloem sap. Aphids are important pests of main cultivated crops causing significant economical loses due to direct damages by feeding and indirect damages

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by transmitting phytoviruses and contributing to fungus proliferation (Blackman and Eastop, 2000; Moury et al., 2007). Systematic insecticide applications are used to control aphid populations, but resistance to a wide range of insecticides (i.e., organophosphates, carbamates, pyrethroids, organochlorines, and neonicotinoids) has been reported in approximately 20 aphid species (van-Emden and Harrington, 2007). Among aphids, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) is a cosmopolitan and highly polyphagous pest that can provoke significant economic impacts (Blackman and Eastop, 2000; van-Emden and Harrington, 2007). *Myzus persicae nicotianae* is a subspecies of *M. persicae*, particularly well adapted to tobacco (*Nicotiana tabacum* L.) (Solanaceae) (Blackman and Eastop, 2000; Margaritopoulos et al., 2000). This tobacco aphid has developed at least four mechanisms of insecticide resistance, which are also found in *M. persicae*, including: (i) elevated carboxylesterases (E-Carb) levels, which confers resistance to organophosphates, carbamates and pyrethroids (Criniti et al., 2008), (ii) modification of the acetylcholinesterase (MACE), which confers resistance to organophosphates and carbamates (Anstead et al., 2008), (iii) *kdr* and *super-kdr* mutations in the voltage-gated sodium channel, which confers resistance to pyrethroids and organochlorines (Criniti et al., 2008), and (iv) a mutation of a nicotinic acetylcholine receptor β subunit that is associated with resistance to neonicotinoid insecticides (Bass et al., 2011). More recently, a new metabolic mechanism based on the amplification of a cytochrome P450 monooxygenase gene has been reported in *M. persicae* sampled on tobacco, which confers resistance to neonicotinoids, the most prominent class of insecticides currently used in the field (Puinean et al., 2010).

In Chile, the tobacco aphid populations are characterized by the presence of one single predominant clone (Fuentes-Contreras et al., 2004), which is also widespread in USA, Brazil, and Argentina (Zepeda-Paulo et al., 2010), being considered as a "superclone" (a predominant and time persisting aphid clone characterized by its ecological success in terms of geographic distribution and abundance) (Vorburger et al., 2003). This tobacco aphid clone in Chile exhibits low levels of carboxylesterase activity, being classified as R1 (moderately resistant) and does not exhibit the mutation in the sodium channel (i.e. susceptible *kdr*) (Fuentes-Contreras et al., 2004), but the MACE mechanism of insecticide resistance has not been studied yet. Despite the apparent insecticide susceptibility exhibited by this aphid clone, its large ecological success is intriguing considering its high frequency in populations and its wide distribution range in The Americas (Zepeda-Paulo et al., 2010), where insecticides such as neonicotinoid, pyrethroids, organophosphates, and carbamates are commonly used (Fuentes-Contreras, 2013, Universidad de Talca, personal communication).

This work aims at characterizing the tobacco aphid in Chile in terms of the MACE mechanism of insecticide resistance and identifying a preliminary group of RNAs differentially expressed in response to insecticide spray. To search for those genes, a heterologous cDNA microarray containing around 7000 transcripts of the pea aphid (*Acyrthosiphon pisum*, Harris, 1776) or the green-peach aphid (*Myzus persicae*) was used to study the transcriptomic response of tobacco aphids exposed to Pirimor (pirimicarb, 2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate), a carbamate insecticide. Resulting in MACE insecticide susceptibility, the Chilean superclone of the tobacco aphid significantly regulated 38 transcripts by insecticide application. The expression of six of those genes was further studied at several time points after insecticide application using qRT-PCR. Putative metabolic insecticide resistance mechanisms are discussed.

MATERIALS AND METHODS

Aphid rearing

Aphids were individually reared on *N. tabacum* var. Burley 'BY64' (Profigen do Brazil, Brazil) under controlled conditions (20 ± 0.1 °C; 16:8 h photoperiod) to ensure the parthenogenetic reproduction of aphids. All the experimental aphid lineages were genotyped with seven microsatellite *loci* to verify its genetic identity (i.e., they belong to the reported Chilean tobacco aphid superclone) (Fuentes-Contreras et al., 2004; Zepeda-Paulo et al., 2010). Only wingless aphids were used.

MACE mutation identification

Five individual aphids were used to characterize the MACE mutation (S431F) (Nabeshima et al., 2003) using quantitative PCR, as described in Anstead et al. (2008). The test consists in an allelic discrimination using the TaqMan PCR assay (Livak, 1999) and requires two specific primers to amplify a section of the DNA containing the mutation (Silva et al., 2012). Two reporter probes differentially labeled with VIC and FAM dyes were used to detect DNA fragments containing the MACE mutation. During each PCR cycle, the probes hybridize with their complimentary PCR products, which ultimately results in an increase in one or two fluorescent products, according to allelic distribution (i.e., heterozygous or homozygous, respectively).

Insecticide bioassays

For the preliminary identification of putatively regulated genes using cDNA microarrays, 20 synchronized wingless adult individuals of the Chilean tobacco aphid superclone were placed on tobacco foliar discs and sprayed with 1 mL at 20 mg L^{-1} (LC_{50} previously determined by the Probit method) of the commercial carbamate insecticide Pirimor (Syngenta) dissolved in water using a Potter Tower.

Another batch of aphids on foliar discs was sprayed only with H_2O as a control. After 12 h of application, all living aphids were removed and immediately frozen in liquid nitrogen for subsequent RNA isolation. This experiment was repeated three-times in order to obtain three independent biological replicates per condition.

For the quantification of the transcripts found as regulated in the microarray experiment, another two independent biological replicates were prepared following the same protocol described above. To improve the accuracy and reliability of the expression profiles, however, the commercial insecticide Pirimor was replaced by the pure active ingredient 2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate. For each biological replicate, 300 synchronized wingless adult aphids were placed in groups of 20 individuals, and sprayed either with the pure insecticide or water as a control. After 6, 12, 18, 24, 30, 36, and 42 h of spraying, living aphids were removed and frozen in liquid nitrogen for subsequent RNA isolation.

RNA isolation and cDNA preparation

Total RNAs were extracted from whole bodies of treated (T) and control (C) aphids using the RNeasy Plant Mini kit (Qiagen, Germantown, Maryland, USA). The RNAs were resuspended in RNase-free water, and the RNAs integrity checked using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). The T and C samples were then split into two technical replicates of 1 μg total RNA. For these four samples (T1, T2; C1, C2), the mRNAs were amplified using MessageAmp aRNA kit (Ambion, Austin, Texas, USA), and the RNA quality checked again. From each technical replicate, 5 μg of aRNA were used as template for cDNA synthesis. The cDNAs were then labeled with Cy3 and Cy5 dyes using ChipShot TM Indirect Labeling and Clean UP system (Promega, Madison, Wisconsin, USA), and purified using the CyScribe GFX Purification kit (Amersham Biosciences-GE, Fairfield, Connecticut, USA).

Microarray hybridizations

The cDNA microarray used for hybridizations contained probes for 7166 aphid genes (6776 ESTs cloned from *Acyrthosiphon pisum* and 390 from *Myzus persicae*), as described in Le Trionnaire et al. (2009). Pre-hybridization was performed at 42 °C for 1 h in 2 mL ChipSpread buffer containing 4x SSC (NaCl, sodium citrate) and 0.2% SDS. Hybridizations were then performed using the labeled cDNA T1, T2, C1, and C2 samples. Each treated sample was mixed with control samples (T1Cy3 *vs*. C1Cy5; T1Cy5 *vs*. C1Cy3; T2Cy3 *vs*. C2Cy5, and T2Cy5 *vs*. C2Cy3) with opposite dye swap to take into account the dye incorporation variability. This resulted in four hybridizations performed at 42 °C during 6 h (Ventana Medical Systems, Tucson, Arizona, USA) on a Discovery XT System hybridization robot with the ChipMap 80 kit (Ventana Medical Systems) at INRA-SCRIBE transcriptomic facilities (IFR 140 GFAS, Rennes, France). Hybridized slides were washed manually at room temperature for 2 min with a RiboWash solution (twice) and with a 0.1× SSC solution (one time).

Microarray data analysis

All fluorescent images from the microarrays were analyzed using the Genepix 4000B scanner and the Genepix Pro software v6.0 (Axon Instruments, Molecular Devices Co., Sunnyvale, California, USA). Raw data were corrected using MADSCAN software (Le Meur et al., 2004). After subtraction of the background fluorescence, a rank-invariant method and a spatial normalization were performed before scaling of the variance within each slide and between all the slides at the same rank. The corrected values of fluorescence were used to perform statistical analysis using the GeneANOVA software (Didier et al., 2002) considering the three following factors: gene (fluorescence level), treatment (insecticide or water), dye (Cy3 or Cy5) and technical replicates (T1; T2 and C1; C2). Fold-change values over 1 were considered as up-regulated gene expression, while fold-changes under 1 as down-regulated.

Gene ontology analysis

In order to know which biological processes were over-represented among regulated transcripts, a gene ontology (GO) analysis was performed using the online available software Blast2GO (Conesa et al., 2005). After the identification of the putative roles for each predicted protein, the regulated transcripts were grouped in the three domains of molecular biology: Biological Processes, Molecular Functions and Cellular Components.

Quantitative RT-PCR

Total RNAs were isolated from three aphids with the RNeasy Plant Mini Kit, resulting in samples containing between 100 and 400 ng μL^{-1} according to Nanodrop quantifications (Nanodrop Technologies, USA). A reverse transcription using AffinityScript QPCR cDNA Synthesis kit (Agilent) was conducted using 1.5 μg of total RNA previously treated with DNAse (DNA-*free*™ Kit, Ambion, Canada). The cDNA was then diluted 10 times, using 2 μL for PCR in 15 μL reaction volume containing: 1 μL (10 μmol) of each primer, 12.25 μL of SYBR Green PCR Master Mix (Applied Biosystems), 0.375 μL of Rox Dye (1:500) and 4.374 μL of water. All qPCRs assays were performed in triplicate on a Mx3000P QPCR System (Stratagene) under the following conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 57 °C, 20 s a 72 °C. To confirm the absence of nonspecific amplicons, a melting curve was performed immediately after the PCR containing a ramp of 65 to 95 °C. The level of expression for six genes was tested. Specific primers were designed for these genes using the

available online softwares FastPCR v5.4.30 and AmplifX v1.3.7. An annealing temperature of 60 °C, an amplicon size of 80-120 bp, and 45%-55% GC content were selected as parameters. PCR efficiency was determined by performing standard curves in triplicate for all primers sets with selected serial dilutions of a standard batch of cDNA. Primers sequences, GeneBank accession numbers and PCR efficiency values are reported in Table 1.

qRT-PCR data analysis

Quantitative analyses (two biological and three technical replicates) were conducted using the Ct comparative method (Applied Biosystems User Bulletin nr 2, P/N 4303859) (Livak and Schmittgen, 2001), using glyceraldehyde 3-phosphate dehydrogenase (GADPH) as reference gene for normalization of input cDNA (Wang and Xu, 2010). Comparisons for expression fold-changes were performed between treatments (pure insecticide *vs*. water), at seven time points (6, 12, 18, 24, 30, 36, and 42 h) and within each biological replicate. The qRT-PCR data were analyzed using a *t*-test for independent samples, using 1 as a reference value for no change in gene expression (Silva et al., 2012). The results are expressed as mean ± standard error (SE) of two biological and three technical replicates.

RESULTS

MACE mutation

The MACE mutation was absent from the different genomic sequences of analyzed aphids, indicating that the predominant clone of the tobacco aphid in Chile lacks the main mutation that confer resistance to organophosphates and carbamates.

Microarray and gene ontology analyses

Following the normalization by MADSCAN software and the statistical analysis by GeneANOVA, 38 transcripts appeared to be significantly regulated ($p < 0.05$) in response to insecticide treatment: 13 transcripts were found to be up-regulated (including two unknown transcripts) and 25 transcripts were shown to be down-regulated (including eight unknown transcripts) (Table 2). These 28 known regulated mRNAs were then functionally classified using a gene ontology analysis (GO). Fifteen GO terms were associated with sixteen the regulated genes (Table 3). These GO terms were grouped in three Biological Processes: reproduction (three terms), metabolism (six) and cellular proliferation (six) (Table 3).

Three up-regulated genes: Acyl-CoA-binding protein (Acyl CoA BP) (Fold-change (FC): 1.57), glutathione S-transferase (GST) (FC: 1.30) and dihydrolipoyllysine-residue acetyltransferase (PDC-E2) (FC: 1.28), and three down-regulated genes: heat shock protein (HSP) (FC: 0.74), ubiquinone NADH dehydrogenase (UBIQ) (FC: 0.77) and hypothetical protein RNA binding protein (RNBP) (FC: 0.69), were selected to quantify their relative expression (see marked genes in Table 3) using new independent biological replicates. These genes were selected based on their putative participation in a metabolic response to xenobiotics (Figueroa et al., 2007).

Quantification of the expression levels

The three selected up-regulated RNAs GST, Acyl CoA BP, and PDC-E2 showed an increasing positive fold-change value during the first half of the time-course of response to insecticide with a peak of expression at 24 h after insecticide pure active ingredient exposure, and decreasing levels of gene expression at later time points (Figure 1). For the selected down-regulated RNAs, UBIC and HSP, we observed lower levels of expression at 24 h after the exposure to the insecticide. RNABP exhibited a lower level of gene expression at 18 h after insecticide exposure. The three down regulated RNAs HSP, UBIC and RNABP, showed an increase in expression until they reach 42 h after insecticide treatment.

Some differences between results obtained in the microarray and qRT-PCR assays were observed. For instance, the UBIQ was down-regulated at 12 h in the microarray, while in qRT-PCR was observed up-regulated. Similarly, the Acyl CoA BP gene, which was shown to be up-regulated in the microarray at 12 h, was noted down-regulated in the qPCR.

Table 1. Transcripts selected for qRT-PCR analysis. Primers sequences, GeneBank accession numbers, PCR efficiency values and R^2 standard curves values of the selected genes. GADPH was used as reference gene.

Gene name	GeneBank accession number	Primer sequence (5'-3')	R^2	PCR efficiency (%)
Acyl CoA-BP	EE262188.1	For: CACAAGCAACGATGAGGCTA Rev: GCCATTCGGTTTAAGCTTTG	0.999	98.3
GST	DW361988.1	For: CTATGGGCGAACCATGAGAT Rev: AATGGCATGGTAGGCTTGAG	0.998	91.3
PDC-E2	EE261058.1	For: GAGGAAACACCCCAGGAATTGA Rev: TTGTCTCCTGGTAGCTTATGCC	0.998	94.9
NADH	EE262674.1	For: TTGACAACAGAGAGCGTTGG Rev: GGCATCAGCTCTGTCAACAA	0.999	91.7
HSP	EE570079.1	For: AGCATCAACTCTCCGCTGAT Rev: ACCGTCTTGACGACGATTTC	0.997	92.9
RNABP	EE262993.1	For: GGCATAGAAATGGTCCTTGGCT Rev: GGGAAGTCAAACCAGGACACAT	0.996	93.9
GADPH	DW011095.1	For: TGGCTTTCAGAGTACCAGTTGC Rev: CTTCAGCGGCTTCCTTGACTTT	0.998	99.3

GADPH: Glyceraldehyde 3-phosphate dehydrogenase.

Table 2. List of differentially expressed transcripts detected by microarray experiments. Transcripts significantly regulated in the microarray are enlisted with their respective information (GeneBank accession number, fold change value in the microarray) and sequence blast results (species, protein, and E-value).

Accession number of EST	Fold change value	Species	Protein	E Value
ID0AAK4YG14*	1.57	*Acyrthosiphum pisum*	acyl-CoA-binding protein isoform 2	2,00E-42
ID0AFF2DD01	1.30	*A. pisum*	similar to AGAP000679-PA, partial length = 373	3,00E-95
MpW-IV-F01*	1.30	*A. pisum*	similar to glutathione S-transferase-like protein isoform 2	2,00E-18
ID0AFF14DB12*	1.28	*A. pisum*	similar to dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial precursor (E2)	9,00E-110
ID0AAK9YO06	1.28	*A. pisum*	similar to B-cell leukemia/lymphoma 6	1,00E-41
ID0AAK3YP12	1.25	*A. pisum*	Unknown	
ID0AFF11BA02	1.24	*A. pisum*	Unknown	
ID0AFF1DD09	1.22	*A. pisum*	similar to lethal (2) k14505 CG8674-PA	2,00E-52
ID0AFF4AE03	1.20	*Tribolium castaneum*	similar to N-acetyl-galactosaminyl-transferase	2,00E-78
ID0AAK3YM01	1.19	*A. pisum*	similar to Der1-like domain family member 1	8,00E-93
ApDT-XXXIII-C2	1.18	*A. pisum*	Acyrthosiphon pisum strain 5A mitochondrion, complete genome	0,00E+00
ID0AAK10YH08	1.16	*A. pisum*	Similar to CG7546 CG7546-PA (LOC100166345), mRNA	5,00E-10
ID0AAK3YL18	1.15	*A. pisum*	hypothetical protein	4,00E-69
ID0AFF10DF02	0.87	*A. pisum*	similar to ribosomal protein S27A	1,00E-59
ID0AFF5CG08	0.86	*A. pisum*	Unknown	1,00E-06
ID0AAK4YG24	0.84	*A. pisum*	Unknown	0.16
ID0AAK2YN05	0.84	*A. pisum*	similar to phosducin-like 3, partial	3,00E-68
ID0AAK6YB03	0.85	*A. pisum*	Unknown	1,00E-16
ID0AAK8YM07	0.83	*A. pisum*	hypothetical protein LOC100160539	3,00E-44
ID0AAK8YE17	0.83	*A. pisum*	hypothetical protein LOC100160953	5,00E-80
ID0AAK6YN06	0.82	*Apis mellifera*	similar to GTPase activating protein and VPS9 domains 1 isoform 1	2,00E-15
ID0AFF8BH01	0.82	*A. pisum*	similar to mitochondrial ribosomal protein L55 CG14283-PA	2,00E-37
ApDT-XXXIV-H7	0.81	*A. pisum*	Unknown	1.5
ID0AFF4AB10	0.81	*A. pisum*	Unknown	2.2
ApHL3LD-III-B11	0.80	*A. pisum*	Unknown	0.088
ApHL3LD-XVIII-G2	0.80	*A. pisum*	Unknown	0.24
ID0AAK2YB12	0.80	*A. pisum*	dephospho-CoA kinase	8,00E-24
ID0AAG15BB04	0.79	*A. pisum*	16S large subunit ribosomal RNA	0,00E+00
ID0AAG10AG10	0.79	*A. pisum*	similar to internalin A, putative	2,00E-100
ID0AFF13AH04	0.78	*A. mellifera*	similar to spectrin alpha chain	8,00E-90
ID0AFF13DD04	0.78	*A. pisum*	similar to CG5800 CG5800-PA	1,00E-78
ID0AAG11CA03*	0.78	*A. pisum*	NADH dehydrogenase (ubiquinone) Fe-S protein 4	2,00E-109
ID0AAK8YE09	0.76	*A. pisum*	similar to TATA-binding protein, putative	7,00E-112
ID0AAK8YB01	0.75	*A. pisum*	similar to spectrin	1,00E-72
ID0AAK1YJ21*	0.74	*A. pisum*	similar to heat shock protein hsp21.4	4,00E-79
ID0AFF3CE04	0.71	*A. pisum*	similar to insulin receptor	3,00E-18
ID0AAK2YH19*	0.69	*A. pisum*	similar to AGAP003344-PA (Acypi007529); hypothetical RNABP LOC100166675	4,00E-79
ID0AAK6YB12	0.57	*A. pisum*	Unknown	1.5

* Genes selected for relative expression quantification.

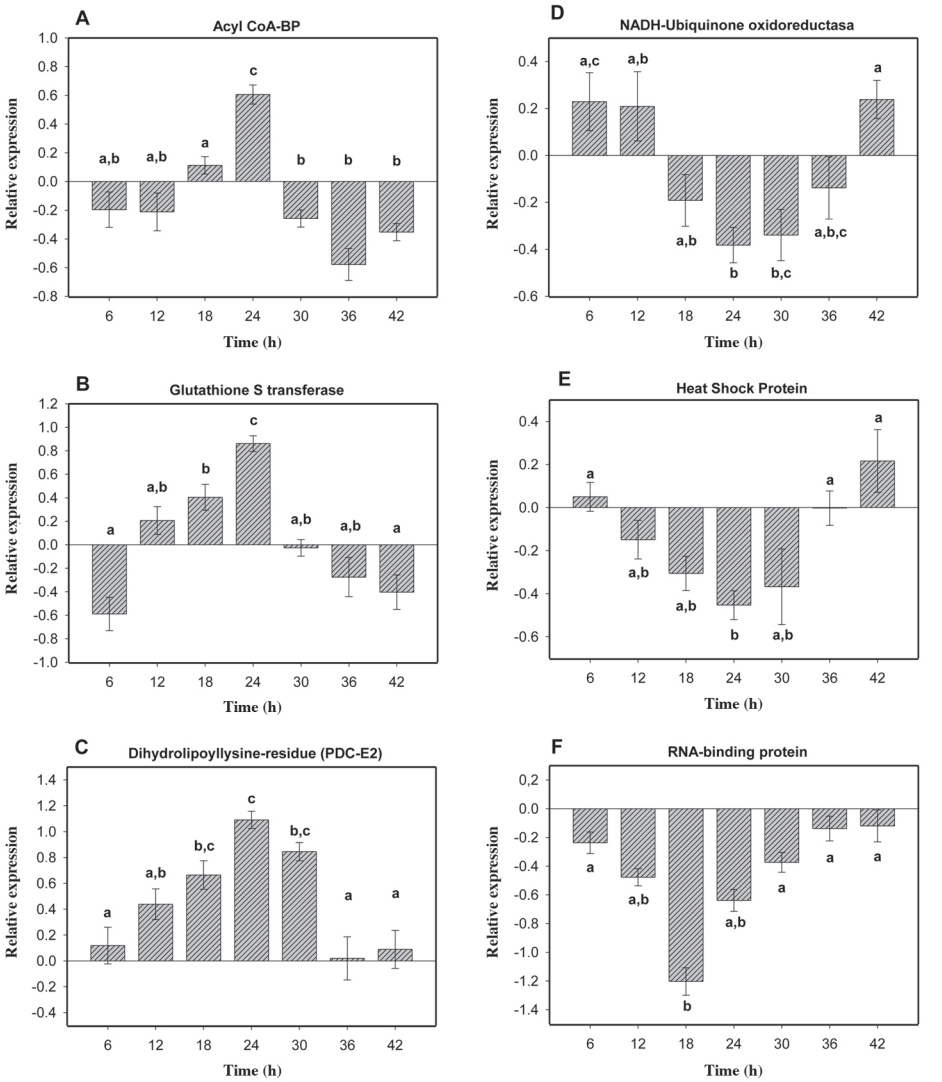
Table 3. Gene ontology (GO) annotation of differentially expressed transcripts. Enriched GO terms with their respective identities and function according to GO analysis using Blast2go software are reported.

GO ID	Function	GO terms
GO:0006412	translation	Metabolism
GO:0055114	oxidation reduction	Metabolism
GO:0040010	positive regulation of growth rate	Reproduction
GO:0009408	response to heat	Metabolism
GO:0006310	DNA recombination	Cell proliferation
GO:0007283	spermatogenesis	Reproduction
GO:0015937	coenzyme A biosynthetic process	Metabolism
GO:0030111	regulation of Wnt receptor signaling pathway	Reproduction
GO:0051301	cell division	Cell proliferation
GO:0006357	regulation of transcription from RNA polymerase II promoter	Cell proliferation
GO:0007067	mitosis	Cell proliferation
GO:0016568	chromatin modification	Cell proliferation
GO:0043461	proton-transporting ATP synthase complex assembly	Metabolism
GO:0006464	protein modification process	Metabolism
GO:0042127	regulation of cell proliferation	Cell proliferation

DISCUSSION

The *M. persicae nicotianae* genotype used in this study was initially classified as carboxylesterase R1 and *kdr* sensitive (Fuentes-Contreras et al., 2004), and the results presented here indicate this genotype is MACE susceptible due to the lack of the insensitivity mutation associated with resistance to organophosphates and carbamates (Anstead et al., 2008). Considering that MACE is the main mechanism reported to explain resistance against carbamate insecticides, the prevalence of this *M. persicae nicotianae* clone in regions where carbamates are frequently used is intriguing, particularly because this putative superclone is widely geographic distributed (Zepeda-Paulo et al., 2010). Hence, other non-mutational mechanisms of insecticide resistance should explain the time-persisting prevalence and frequency of this aphid clone on insecticide sprayed tobacco crops (Criniti et al., 2008).

Hence, the transcriptomic approach performed here explored for other putative insecticide resistance mechanisms involved in the prevalence of this clone in the field, allowing the identification of 38 transcripts



Each bar shows the mean ± SE of two biological replicates. Letters indicate significant differences among bars (*t*-test, $P < 0.05$).

Figure 1. Time-course analysis of gene expression levels following insecticide application. Each panel shows the relative gene expression level determined by real time PCR at different time points after tobacco aphids were sprayed with carbamate insecticide pure active ingredient.

differentially expressed after exposing the tobacco aphid to a carbamate insecticide (13 up-regulated and 25 down-regulated genes, including 10 unknown genes). The GO terms associated with these 38 regulated genes indicate that most of them are grouped into metabolic processes, suggesting a possible metabolic insecticide resistance pathways involved, such as xenobiotic detoxification related enzymes (Figueroa et al., 2007; Li et al., 2007).

Among those differentially expressed transcripts, we selected three up-regulated genes that are putatively involved in xenophobic responses. The Acyl-CoA- binding protein (EC 3.4.17.3), also known as a diazepam-binding inhibitor (DBI), is involved in benzodiazepine receptor modulation, acyl CoA metabolism, steroidogenesis, insulin secretion, and drug dependence (Rasmussen et al., 1994). This transcript has been shown to be up-regulated by DDT insecticide in *Drosophila* resistant genotypes (Pedra et al., 2004), which suggests a putative role in insecticide resistance for this gene also in the case of aphids. The second over-expressed RNA we selected was glutathione S transferase (GST, EC 2.5.1.18), which is a phase II enzyme of xenobiotic detoxification. In aphids, the GST contributes to tolerance to secondary metabolites from their host plants and has been implicated in resistance to carbamate, pirimicarb and cyclodiene endosulfan insecticides (Francis et al., 2005; Perera et al., 2008). The third transcript selected corresponds to adihydrolipoyllysine-residue acetyltransferase (PDC-E2) (EC 1.8.1.4), which is present in the mitochondria and plays a central role in the energetic metabolism. PDC-E2

links the glycolysis to the citric acid cycle, resulting in the production of several molecules such as NADH, which plays a key role during the mitochondrial respiratory chain for ATP production. Deficits in PDC-E2 result in a lactic acidosis due to metabolization of pyruvate into lactic acid (Yu et al., 2008). Carbamate insecticide applications usually result in an uncontrollable muscular contraction in target insects, which significantly increases the energetic recruitments, causing a high pyruvate concentration and producing a "bottle neck" between glycolysis and citric acid cycle that trigger a severe lactic acidosis (Fukuto, 1990). The increased levels of PDC-E2 transcripts observed in our experiments suggests a molecular strategy tending to avoid this "bottle neck" between these metabolic processes, and thereby to prevent the lactic acidosis induced by carbamate insecticide in the MACE-susceptible tobacco aphid clone studied.

Regarding the three down-regulated genes selected for qPCR validation, the UBIQ (EC 1.6.5.3) participates in the electron transport complex of the mitochondrial respiratory chain complex I that use NADH as substrate. This is an essential molecule for several physiological processes as muscular contraction playing a key role during the ATP synthesis (Friedrich et al., 2000), and the down-regulation observed after insecticide exposure could be result in uncontrollable muscular contractions prevention by reducing the ATP availability. Other selected down-regulated gene was a heat shock protein (HSP). The HSPs belong to a family of proteins participating in numerous cellular processes (i.e., protein sorting, signal transduction, gene expression regulation by post-transcriptional control of RNAs, DNA replication, and protein synthesis) (Johnstone and Lasko, 2001). These proteins are usually produced in response to a variety of biotic and abiotic stresses (Picard, 2002), as well as in response to insecticides, showing inter specific variations in the expression patterns; in some cases the HSPs are increased under carbamate exposure in the brown planthopper (*Nilaparvata lugens*) (Sharma et al., 2004), or show an invariable expression when the cabbage armyworm (*Mamestra brassicae*) is exposed to permethrin, chlorfluazuron, chlorfenapyr, prothiofos, methomyl, and thiocyclam (Sonoda and Tsumuki, 2007), thus suggesting a non-specific response of HSPs to insecticides. Finally, the RNABP were found also to be down-regulated. This gene belongs to a family of genes coding for proteins that play key roles during gene expression regulation by post-transcriptional control of RNAs (Wickens et al., 2000). Thus, the down regulation of RNABP transcripts exhibited after aphids exposure to carbamate insecticide suggest a decreased gene expression regulation at RNA post-transcriptional level, which could favor a gene expression regulation at other levels of regulation (e.g., DNA transcription, protein translation and protein posttranslational mechanisms) (Wickens et al., 2000).

The different results obtained in the microarray and qRT-PCR assays (e.g., RNABP and Acyl coA at 12 h) are possibly due to the fact that a commercial insecticide was used in the microarray experiments, while the insecticide pure active ingredient was used in the qPCR assays. Additionally, the sensitivity of microarray and qPCR assays is different and can also explain those differences, since the microarray is a qualitative while qPCR is a quantitative technique.

The results reported here are in accordance to the insecticide residual effect time observed in the field (12-24 h) (Estay, 2006), because it matches with the time-course of aphid's gene expression modification in response to insecticide. This suggests that aphids could moderate the effects of certain insecticides like carbamates by using the cholesterol and pyruvate metabolism to prevent lactic acidosis, while using a GST detoxification system to detoxify the organism, thus decreasing the gene expression when the presence of the active insecticide is lower. In the opposite side, HSP and RNABP remain down-regulated after insecticide application, apparently for deviating energy use for other metabolic pathways. The down-regulation of NADH ubiquinone dehydrogenase could be preventing uncontrollable muscular contractions produced by carbamate insecticide in a sensitive aphid clone by reducing the ATP availability, recovering their expression levels when the presence of the active insecticide is lower.

The results obtained in this work, suggest the involvement of resistance mechanisms other than those previously reported for *M. persicae* (Silva et al., 2012), thus complementing our picture of the genes underlying insecticide resistance in aphids. Further functional studies including proteomics, RNAi and biochemical studies will help going deeper into the characterization of the insecticide resistance mechanisms that participate in aphids, which should explain the persistence of certain aphid clones not carrying insensitivity mutations under strong insecticide applications in the field.

CONCLUSIONS

The findings reported here show the presence of transcriptomic mechanisms involved in the aphid insecticide resistance by suppression of the insecticides metabolic effects, which could explain the wide geographic distribution exhibited by this superclone in The Americas, including areas where the insecticides are frequently used. This should lead to new hypotheses in order to understand the molecular mechanism underlying the aphid insecticide resistance.

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