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Acetylcholinesterase point mutations in European strains of *Tetranychus urticae* (Acari: Tetranychidae) resistant to organophosphates

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Abstract

BACKROUND: In *Tetranychus urticae* Koch, acetylcholinesterase insensitivity is often involved in organophosphate (OP) and carbamate (CARB) resistance. By combining toxicological, biochemical and molecular data from three reference laboratory and three OP selected strains (OP strains), the AChE1 mutations associated with resistance in *T. urticae* were characterised.

RESULTS: The resistance ratios of the OP strains varied from 9 to 43 for pirimiphos-methyl, from 78 to 586 for chlorpyrifos, from 8 to 333 for methomyl and from 137 to 4164 for dimethoate. The insecticide concentration needed to inhibit 50% of the AChE1 activity was, in the OP strains, at least 2.7, 55, 58 and 31 times higher for the OP pirimiphos-methyl, chlorpyrifos oxon, paraoxon and omethoate respectively, and 87 times higher for the CARB carbaryl. By comparing the AChE1 sequence, four amino acid substitutions were detected in the OP strains: (1) F331W (*Torpedo* numbering) in all the three OP strains; (2) T280A found in the three OP strains but not in all clones; (3) G328A, found in two OP strains; (4) A201S found in only one OP strain.

CONCLUSIONS: Four AChE1 mutations were found in resistant strains of *T. urticae*, and three of them, F331W, G328A and A201S, are possibly involved in resistance to OP and CARB insecticides. Among them, F331W is probably the most important and the most common in *T. urticae*. It can be easily detected by the diagnostic PCR-RLFP assay developed in this study.
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Keywords: *Tetranychus urticae*; spider mite; organophosphate resistance; acetylcholinesterase mutations; *ace-1*; resistance mechanisms

1 INTRODUCTION

Organophosphates (OPs) are among the first chemical groups used to control the two-spotted spider mite *Tetranychus urticae Koch*, an economically important agricultural pest with a wide range of host plants and geographic distribution. OP insecticides act by inhibiting acetylcholinesterase (EC 3.1.1.7, AChE), a key enzyme in the nervous system of both vertebrates and invertebrates. Since the first reported cases of OP resistance in the late 1940s,[1] *T. urticae* has developed resistance towards almost every acaricide used for its control. Within the OP family, cross-resistance is a general rule in *T. urticae*.[2–8] This can result from enhanced detoxification[6,7] and/or target-site insensitivity. The insensitivity of *T. urticae* AChE towards OPs was first reported in 1964,[9] suggesting the presence of some modifications at the active site of the enzyme.[7] Since then, AChE insensitivity to OPs has been demonstrated in various strains of *T. urticae* from the Netherlands, Germany, the USA, New Zealand, Israel, Egypt, Greece and Japan.[5,10–15]

Many insects and ticks have at least two genes, termed *ace-1* and *ace-2*, encoding for AChE1 and AChE2 respectively. These genes correspond to the *Drosophila* paralogous and orthologous genes.[16] In the dipteran order of the Cyclorrhapha (true flies), only the *ace-2* gene is present.[17] In insects that have both genes, the main synaptic function is encoded by *ace-1*.[16,18] In this gene, seven point mutations associated with resistance to OPs and CARBs have been reported, resulting in the following amino acid substitutions: G119S, A201S, F290V, G227A, S331F and

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F331W/C;[15,18–25] numbering based on *Torpedo californica* AChE nomenclature.[26] In true flies, one or a combination of mutations in the *ace-2* gene, including substitutions F78S, I129V/T, V150L, G227A/V, F290Y, G328A and G396S, conferred *in vitro* insensitivity to OP insecticides.[27–31]

Although biochemical evidence of AChE insensitivity in *T. urticae* was already revealed in 1964 by Smissaert,[9] the exploration of the molecular basis of AChE1 insensitivity in Tetranychidae started only quite recently. Among phytophagous mites, *ace-1* gene sequences encoding for the enzyme AChE1 have been determined in two tetranychid species, *T. urticae* and *T. kanzawai*.[15,32] The comparison of the AChE1 amino acid sequence from susceptible and OP-resistant *T. urticae* from Japan revealed the presence of an F331C substitution.[15] In OP-resistant *T. kanzawai* Kishida, a tryptophane was detected at position 331 (W331).[32] In the present study, a more thorough examination was made of the presence of mutations in the AChE1 of several OP- and CARB-resistant *T. urticae* strains by combining toxicological, biochemical and molecular data.

2 MATERIALS AND METHODS

2.1 Strain characteristics

This study characterised three laboratory *T. urticae* strains commonly used as reference strains (reference strains SAMB, GSS and LS-VL) and three resistant strains maintained in the laboratory under continuous OP selection (OP strains WI, MR-VL and ATHRos-Pm). SAMB and GSS had both been maintained in the laboratory without treatment since 1965. SAMB was initially collected on *Sambucus nigra* (L.) in the Netherlands and obtained from the Centre de Biologie et de Gestion des Populations, Montpellier, France. GSS was obtained from Bayer CropScience (Monheim, Germany) and was originally collected from an unknown host in Germany. LS-VL was collected in 2000 from roses in a garden near Ghent, Belgium,[33] and since then had been maintained in the laboratory without treatment.

The historical German resistant strain WI has a well-studied target-site resistance to OPs and CARBs.[5] It was obtained from Bayer CropScience where it had been maintained in the laboratory under biannual selection with the OP oxydemeton-methyl since 1954.[34] Biochemical characterisation had shown that the main resistance mechanism was an altered acetylcholinesterase with a 110-fold and 340-fold lower sensitivity to inhibition by chlorpyrifos-oxon and ethyl paraoxon respectively.[5]

The OP strains MR-VL (from Belgium) and ATHRos-Pm (from Greece) were recently collected from fields on which failure of all insecticide treatments, including OPs, had been claimed by the farmers. MR-VL was collected in 2003 from a greenhouse nursery where poplar cuttings, beans and ornamentals were grown, while ATHRos-Pm was collected in 2007 on roses in a greenhouse near Athens. Both strains had developed resistance to many acaricides currently used to control spider mites.[6,35] ATHRos-Pm was maintained in the laboratory under pirimiphos-methyl selection every two generations with concentrations causing ∼80% mortality before its characterisation after eight selections.

All mites were reared on detached bean leaves that were deposited on moist cotton wool to prevent contamination and escape. Rearing conditions were 25 ± 2 °C, 70 ± 5% RH and a 16 : 8 h light : dark photoperiod. Mites were transferred to new bean leaves weekly. Under these conditions, a new generation was obtained every 2 weeks.

2.2 Bioassays

Commercial formulations of pirimiphos-methyl 500 g L^{-1} EC (Actellic; Syngenta, UK), dimethoate 400 g L^{-1} EC (Dimethoate; Lapafarm, Greece), chlorpyrifos 480 g L^{-1} EC (Dursban; Dow Agrosciences, USA) and methomyl 200 g L^{-1} SL (Alpha, Greece) were used. The treatments were conducted on adult females placed on detached bean leaves with a precision Potter spray tower[36] (Burkard, Rickmansworth, Hertfordshire, UK), as described by Tsagkarakou *et al.*[35] LC_{50} values and 95% confidence limits were calculated by the log-probit program[37] based on Finney.[38] Resistance ratios at the LC_{50} (RR_{50}) were considered to be significantly different from 1 when their 95% confidence limits did not include this value.

2.3 Acetylcholinesterase activity and inhibition studies

For determining AChE1 activity, 1000 adult females were mass homogenised in 1 mL extraction buffer containing 10 mM Tris, pH 7.0, 1 M NaCl and 4 g L^{-1} Triton X-100. The supernatant obtained after centrifugation (10000 × *g*, 4 °C, 10 min) was used as the enzyme source. The reaction was conducted in 1 mL substrate–reagent solution containing 30–50 µg of protein and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and acetylthiocholine (AcSCh) each in a final concentration of 0.5 mM. The mean activity values were compared between strains by one-way analysis of variance (ANOVA) using SPSS statistical software version 13.0 (LSTM, UK). Residual AChE1 activities were measured with increasing concentrations of the analytical-grade inhibitors chlorpyrifos-oxon (varying between 10^{-9} and 10^{-3} M), paraoxon (varying between 10^{-9} and 10^{-3} M), carbaryl (varying between 10^{-10} and 10^{-3} M), pirimiphos-methyl (varying between 10^{-7} and 10^{-3} M) and omethoate (varying between 10^{-7} and 10^{-3} M). Briefly, the enzyme source was incubated for 10 min with the inhibitor solution at a given concentration before adding the substrate–reagent solution. Residual activity was estimated by kinetically measuring the variation in optical density at 412 nm with a spectrophotometer (M2e; Molecular Devices, UK). Blanks without homogenate or substrate were used to correct for non-enzymatic activity. Enzyme inhibition was expressed as the mean percentage of activity remaining at different inhibitor concentrations, and the inhibitor concentration inducing 50% inhibition (IC_{50}) was determined using the Microcal Origin 6.0 data analysis program.

2.4 Extraction of DNA and RNA, cDNA synthesis, cloning and sequencing

Total DNA (DNA) was extracted from mass homogenates as described by Van Leeuwen *et al.*[39] Total RNA was extracted from mass homogenates of *T. urticae* mites using TRI reagent (Sigma-Aldrich, Belgium) according to the manufacturers' instructions. The RNA was treated with DNAse RQ1 (Promega, Madison, WI) to remove genomic DNA. A quantity of 2 µg total RNA for each sample was reverse transcribed into cDNA by using the High Fidelity cDNA Synthesis Kit (Roche, Belgium) and random hexamer primers. Primers (Table 1) were designed on the basis of the published sequence of *T. urticae* AChE1 (AY188448). For cDNA amplification, a long PCR was performed using Acetrur F and Acetrur R primers (Expand Long Range Kit; Roche, Belgium), according to the manufacturer's instructions. Fragments of 2.5 kb were purified with QIAEX II (Qiagen), cloned into the pGEM-T vector (Promega) and sequenced with primer walking. To ensure allele representations, three clones were sequenced for each strain.

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Table 1. Primers used for the amplification of the *Tetranychus urticae* AChE1

Primers	Sequence (5′–3′)
Acetrur F	AAAGGGAGAAGGCAAAAGTGT
Acetrur R	TGCTCATGTTCAGTTGATCG
AcETuSeqF1	AATGCCACCTCATTTTCAGG
AcETuSeqF2	TATCACGCAACGTTTTCAGC
AcETuSeqF3	ATGAAGATCCCGAGGTTTCC
AcETuSeqF4	TGGGAGTTATTCATGGTGAGG
AcETuSeqR1	CATGAATCGATGGGCTTAGG
AcETuSeqR2	AGCTGATTCACCGAAAATGG
AcETuSeqR3	AACTGCTTCTTGGGCTAAAGG
AcETuSeqR4	CAGTGTACTCAGGCCACACG
AchEDISCF2	AAGCAATCGTGATGAGGGGCCCTATT
AchEDISCR2	CTAAAGGACTCAGATGGGGATAAAT

Plasmids were sequenced by Agowa Sequencing Service (Berlin). Sequence data were analysed using BioEdit 7.0.1.[40]

2.5 Three-dimensional modelling

The three-dimensional (3D) structure of *T. urticae* AChE1 was created by automated homology modelling, as described by Weill *et al.*[41] The structural templates used were AChE from *Torpedo californica* (PDB: 1EA5)[42] and *Drosophila melanogaster* (PDB: 1DX4).[43] The alpha-carbon skeleton of the modelled 3D structure of AChE1 was superimposed on that of the AChE of *T. californica*. RMS deviation is 1.1 Å on 532 carbon atoms (from Dali pairwise comparison webserver http://ekhidna.biocenter.helsinki.fi/dali_lite/start).

2.6 Diagnostic PCR-RFLP for W331

Two primers were designed (AcheDISCF2, AcheDISCR2) (Table 1) around position 331 that amplified a 137 bp fragment using genomic DNA as template. Amplifications from approximately 40 ng gDNA were performed in 1 × Taq reaction buffer (Invitrogen) with a final concentration of 1.5 mM $MgCl_2$ and 0.5 µM of each primer, with the following cycling conditions: 94 °C for 3 min, 35 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 40 s, followed by 72 °C for 2 min. A restriction digest using 10 µL PCR product was performed in a total volume of 30 µL using *Bgl* I according to the manufacturer's instructions (Fermentas). Approximately 20 µL was loaded onto a 3% agarose gel, and after 1.5 h at 50 V the separated restriction fragments were visualised by ethidium bromide staining.

3 RESULTS

3.1 Resistance characteristics of the *Tetranychus urticae* strains

Among the reference strains, variable levels of sensitivity were found (Table 2). LS-VL proved to be the most susceptible to all insecticides tested: LC_{50} of pirimiphos-methyl, chlorpyrifos, methomyl and dimethoate was 16, 11, 28 and 8 mg L^{-1} respectively. Therefore, this strain was used as a susceptible reference to calculate the RR_{50} of the other strains. SAMB exhibited a 31- and 29-fold resistance to chlorpyriphos and dimethoate respectively. GSS was 21- and 1411-fold more resistant to pirimiphos-methyl and dimethoate respectively.

The OP strains ATHRos-Pm, MR-VL and WI were resistant to all insecticides tested, with RR_{50} of at least 8.4 (Table 2). Among the three OP strains, the highest levels of resistance to pirimiphos-methyl (RR_{50} = 43), dimethoate (RR_{50} = 4164) and methomyl (RR_{50} = 333) were exhibited by ATHRos-Pm, and the weaker levels by WI (RR_{50} = 8.9, 137 and 8.4 to pirimiphos-methyl, dimethoate and methomyl respectively). For chlorpyrifos, the resistance ratio was higher in the WI and MR-VL strains (RR_{50} = 421 and 586 respectively).

3.2 Biochemical characterisation of the AChE1 enzyme

AChE1 activity was highest in the SAMB strain (63 ± 11 mOD min^{-1} mg^{-1} protein), followed by the MR-VL strain (37 ± 1 mOD min^{-1} mg^{-1} protein) ($P < 0.01$) (Table 3). The four other strains showed lower activities that did not differ significantly from each other.

As shown by the IC_{50} values (Table 3), AChE1 from the OP strains WI, MR-VL and ATHRos-Pm were much less sensitive to inhibition by pirimiphos-methyl, chlorpyrifos-oxon, paraoxon and carbaryl than the AChE1 from the three reference strains, and WI and MR-VL were less sensitive to inhibition by omethoate than LS-VL and SAMB (omethoate was not tested for AChE1 from GSS and ATHRos-Pm strains, and no comparison is possible). AChE1 from SAMB and LS-VL were more sensitive to inhibition by chlorpyrifos-oxon but less sensitive to inhibition by carbaryl than the AChE1 from GSS. For paraoxon, IC_{50} values for SAMB and GSS were very similar and lower than that of LS-VL. Among the OP strains, IC_{50} values were similar for the OPs and the CARBs tested.

3.3 Comparison of the AChE1 sequence of the reference laboratory and the OP selected strains

Using specific primers and long-PCR strategy, the cDNA sequence of a 2.5 kb fragment was determined in the six strains (GenBank accessions GQ461336 to GQ461353). This fragment contains an open reading frame encoding for an AChE1 precursor of 687 amino acid residues. Three cDNA clones were sequenced from each strain, resulting in a total of 18 clones, which revealed 88 variable nucleotide sites including inter- and intrastrain polymorphisms. Forty-four of these resulted in amino acid substitutions. By comparing these substitutions in the AChE1 primary sequence from the different strains, six mutations were identified in the OP strains that were not present in the reference strains. Only four of them were present in the mature protein (numbering based on *Torpedo californica* AChE nomenclature)[26]: (1) at position 201, an alanine residue (GCT) is replaced by a serine (TCT) (A201S) in the three clones of the OP strain ATHRos-Pm; (2) at position 280, a threonine residue (ACA) is replaced by an alanine (GCA) (T280A) in all three OP strains, except for one clone of the ATHRos-Pm strain; (3) at position 328, a glycine (GGA) is replaced by an alanine (GCA) (G328A) in the ATHRos-Pm and MR-VL strains; (4) at position 331, a phenylalanine (TTT) is replaced by a tryptophan (TGG) (F331W) in all clones of the three OP strains. Interestingly, the F331 residue is replaced by a tyrosine (TAT) (F331Y) in two clones of reference strains (one in the SAMB and one in the GSS strain). Moreover, two amino acid substitutions, a valine (GTT) to isoleucine (ATT) at position 7 (V7I) and a glycine (GGC) to serine (AGC) at position 119 (G119S), were found specifically in the reference strain SAMB (Table 4).

3.4 Three-dimensional modelling

The positions of the mutations in the structural model of *T. urticae* AChE1 are shown in Fig. 1. The active site of cholinesterases is subdivided into functional groups important for the hydrolysing

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Table 2. Toxicity data for the OP selected and the reference *Tetranychus urticae* strains treated with several OP insecticides and one carbamate insecticide

Treatment	N^a	LC_{50} (95% CI) (mg L^{-1})	Slope (± SE)	χ^{2b}	df	$RR_{50}{}^c$ (95% CI)
LS-VL						
Pirimiphos-methyl	522	16 (12–21)	2.66 (± 0.39)	11*	4	
Chlorpyrifos	435	11 (4.8–24)	0.98 (± 0.27)	14***	3	
Methomyl	435	28 (14–57)	1.20 (±0.30)	24***	4	
Dimethoate	214	8 (2–29)	0.88 (± 0.27)	12**	3	
SAMB						
Pirimiphos-methyl	254	18 (16–21)	4.50 (±0.14)	0.4	1	1.1 (0.8–1.7)
Chlorpyrifos	315	333 (173–646)	2.75 (±1.17)	16***	2	31 (15–64)
Methomyl	378	40 (9.34–167)	2.48 (± 1.36)	52***	2	1.4 (0.4–5.4)
Dimethoate	226	226 (182–282)	4.97 (± 0.14)	5	2	29 (17–47)
GSS						
Pirimiphos-methyl	577	332 (166–665)	6.56 (± 6.85)	135***	2	21 (3.9–113)
Chlorpyrifos	328	63 (19–215)	5.67 (± 7.05)	136***	2	5.8 (0.4–79)
Methomyl	279	50 (26–94)	2.11 (± 0.50)	6.7*	2	1.8 (1.0–3.2)
Dimethoate	281	11 164 (7418–16 836)	3.80 (± 1.43)	8.8*	2	1411 (686–2904)
WI						
Pirimiphos-methyl	463	142 (96–209)	4.70 (± 1.81)	37***	3	8.9 (4.4–18)
Chlorpyrifos	424	4584 (2786–7530)	3.93 (± 1.23)	28***	3	421 (196–907)
Methomyl	373	236 (151–373)	3.86 (± 1.26)	17***	3	8.4 (4.2–17)
Dimethoate	394	1502 (915–2467)	2.24 (± 0.64)	18***	3	137 (84–224)
MR-VL						
Pirimiphos-methyl	329	314 (266–374)	3.75 (± 0.41)	3.9	3	20 (14–28)
Chlorpyrifos	392	6386 (4261–9567)	4.05 (± 1.08)	21***	3	586 (302–1137)
Methomyl	419	3759 (3178–4370)	3.08 (± 0.32)	4.2	3	134 (95–189)
Dimethoate	334	5878 (2218–15 579)	3.16 (± 2.02)	59***	3	743 (223–2465)
ATHRos-Pm						
Pirimiphos-methyl	337	685 (497–944)	2.81 (± 0.73)	12**	3	43 (28–66)
Chlorpyriphos	366	895 (624–1289)	2.62 (±0.57)	9.4*	3	78 (39–156)
Methomyl	301	9338 (4421–19 649)	3.11 (± 0.95)	16***	2	333 (136–818)
Dimethoate	319	32 950 (23 977–45 249)	6.09 (± 3.28)	8.5***	1	4164 (1728–10 036)

[a] *N*, number of mites tested.
[b] χ^2, chi-square testing linearity: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
[c] RR, resistance ratio = LC/LC_{LS-VL}.

process: the catalytic triad (S200, E327 and H440), the peripheral anionic site (D72, Y121, W279 and Y334), the choline-binding site (W84, Y130, Y330 and F331), the acyl-binding pocket (F288 and F290) and the oxyanion hole (G118, G119 and A201).[42,44–47]

Among the two substitutions specifically found in the susceptible strain SAMB, the first one, V7I, is located in the extremity of the N-terminal of the enzyme structure and may not influence the catalytic properties of AChE1. The second substitution, G119S, is located in the active site that belongs to the oxyanion hole and affects an important residue close to the catalytic serine residue (S200); thus, it may modify the catalytic properties of the enzyme.

The T280A mutation was found in all OP strains, and the structural model reveals its position on the surface of the enzyme near the gorge entrance. However, the T280 residue is not involved in the catalytic process and was never shown to confer AChE insensitivity (neither AChE1 nor AChE2). Hence, this mutation might be neutral. In contrast, the F331W mutation that is also found in all OP strains is at a residue that is conserved among AChE1 from both vertebrates and invertebrates, and is a component of the choline-binding site involved in the catalytic process. Thus, it is probably responsible, at least partially, for the modification of the catalytic properties of *T. urticae* AChE1 in the three OP strains. In addition to the F331W, a G328A substitution was found in ATHRos-Pm and in MR-VL strains. This position is not located in the active site but is in the same α-helix as the 331 position. Therefore, this substitution may indirectly influence the catalytic process. In the ATHRos-Pm strain only, besides the above three substitutions (T280A, G328A and F331W), the A201S substitution was also detected. This substitution is close to the catalytic serine (S200) and therefore may modify some of the biochemical properties of the enzyme.

3.5 Diagnostic PCR-RFLP for W331

The authors have developed a PCR-RFLP assay, based on primer-induced mutagenesis, that directly revealed the presence of W at codon 331. Three (CAA to GGC in MR-VL and ATHRos-Pm) or two (GAA to GGC in LS-VL, GSS, SAMB and WI) mutations were

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Table 3. Specific activities and IC_{50} of various OP and CX inhibitors on AChE1 extracted from OP selected and reference *Tetranychus urticae* strains

Strain	AChE activity (mOD min^{-1} mg^{-1})	IC_{50} (M)				
		Pirimiphos-methyl	Chlorpyrifos-oxon	Paraoxon	Carbaryl	Omethoate
LS-VL	22(±1.5) a	$3.7 \pm 0.7 \times 10^{-4}$	$7.0 \pm 2 \times 10^{-8}$	$4.3 \pm 1.2 \times 10^{-6}$	$6.0 \pm 1.8 \times 10^{-6}$	$7.4 \pm 2.7 \times 10^{-6}$
SAMB	63(±11) b	$2.7 \pm 0.4 \times 10^{-4}$	$2.5 \pm 0.3 \times 10^{-8}$	$1.0 \pm 0.4 \times 10^{-7}$	$8.7 \pm 4.3 \times 10^{-6}$	$5.2 \pm 3.4 \times 10^{-6}$
GSS	24(±4) a	$3.8 \pm 1.2 \times 10^{-4}$	$1.8 \pm 0.2 \times 10^{-7}$	$1.2 \pm 0.1 \times 10^{-7}$	$8.2 \pm 3.8 \times 10^{-7}$	
WI	23(±2) a	$>10^{-3}$	$1.8 \pm 0.6 \times 10^{-6}$	$8.0 \pm 3 \times 10^{-4}$	$7.6 \pm 4.2 \times 10^{-5}$	$2.3 \pm 1.5 \times 10^{-4}$
MR-VL	37(±1) c	$>10^{-3}$	$1.1 \pm 0.9 \times 10^{-6}$	$4.3 \pm 1.8 \times 10^{-4}$	$4.4 \pm 2.2 \times 10^{-4}$	$8.7 \pm 5.2 \times 10^{-4}$
ATHRos-Pm	26(±6) a	$>10^{-3}$	$1.0 \pm 0.5 \times 10^{-6}$	$2.5 \pm 0.3 \times 10^{-4}$	$6.2 \pm 3.2 \times 10^{-4}$	

Table 4. Amino acid polymorphism between reference laboratory and OP selected strains of *Tetranychus urticae*. Numbers refer to the position in mature AChE1 of *Torpedo californica*.[26] Numbers in brackets refer to the position in *T. urticae* AChE1 precursor

Strains-clones (Genbank accession)	Position					
	7 (115)	119 (228)	201 (309)	280 (391)	328 (436)	331 (439)
LS-VL-1 (GQ461336)	V (GTT)	G (GGC)	A (GCT)	T (ACA)	G (GGA)	F (TTT)
LS-VL-2 (GQ461337)	V	G	A	T	G	F
LS-VL-3 (GQ461338)	V	G	A	T	G	F
SAMB-1 (GQ461342)	I (ATT)	S (AGC)	A	T	G	Y (TAT)
SAMB-2 (GQ461343)	I	S	A	T	G	F
SAMB-3 (GQ461344)	I	S	A	T	G	F
GSS-1 (GQ461348)	V	G	A	T	G	F
GSS-2 (GQ461349)	V	G	A	T	G	Y
GSS-3 (GQ461350)	V	G	A	T	G	F
WI-1 (GQ4613451)	V	G	A	A(GCA)	G	W (TGG)
WI-2 (GQ461352)	V	G	A	A	G	W
WI-3 (GQ461353)	V	G	A	A	G	W
ATHRos-Pm-1 (GQ461345)	V	G	S (TCT)	A	A (GCA)	W
ATHRos-Pm-2 (GQ461346)	V	G	S	A	A	W
ATHRos-Pm-3 (GQ461347)	V	G	S	T	A	W
MR-VL-1 (GQ461339)	V	G	A	A	A	W
MR-VL-2 (GQ461340)	V	G	A	A	A	W
MR-VL-3 (GQ461341)	V	G	A	A	A	W

introduced by the forward primer by site-directed mutagenesis, resulting in the generation of a *Bgl* I restriction site (5′-GCCNNNNNGGC) specifically in the PCR product of the resistant allele carrying W331. Digestion of the PCR products (137 bp) with *Bgl* I consequently allowed for discrimination between the resistance-associated W331 and the F or Y 331 alleles (Fig. 2). The PCR-RFLP assay with male (*haploid*) *T. urticae* displays two different patterns: one with two fragments 112 bp and 25 bp in 331 W males and one with one fragment (137 bp), the original PCR product, in F 331 or Y 331 males (Fig. 2). When pooled DNA from F331 and W331 males was used as template, a third pattern with three fragments (137 bp, 112 bp and 25 bp) was obtained, suggesting that the assay can be used to detect heterozygous females (Fig. 2). The fragment of 25 bp is not detected, as it is too small to be visualised by electrophoresis in a routine agarose gel. Note that sequence conservation and the presence of other resistant alleles should be confirmed by sequencing in any future studies of *T. urticae* from other geographic origins before this diagnostic test is used, as it will not identify the C331 allele previously suggested to be associated with OP resistance in a Japanese *T. urticae* strain.[15]

4 DISCUSSION

Since the development of OP resistance in *T. urticae* in the late 1960s, the use of these compounds to control spider mites has largely been abandoned in many parts of the world. However, OPs and CARBs, which account for more than 35% of the total global insecticide sales,[48] are still among the most widely used insecticides to control a broad range of arthropod pests of agricultural importance. This keeps spider mites like *T. urticae* under continuous OP selection pressure in many open environment and greenhouse crops, and OP-resistant spider mite populations can resurge after OP use. This is why OP resistance in *T. urticae* remains a not to be neglected economic factor. In addition, it was not long ago shown how OPs can antagonise the activity of the recently developed acaricide bifenazate, especially in strains resistant to organophosphates. This is due to an inhibition of general esterase activity by OPs, which is needed to metabolise bifenazate to its active component.[49,50]

In an attempt to investigate the molecular basis of OP resistance and its relationship to the observed phenotype, a study was made of the characteristics of resistance in three reference laboratory strains (LS-VL, SAMB and GSS) and three strains of *T. urticae* that

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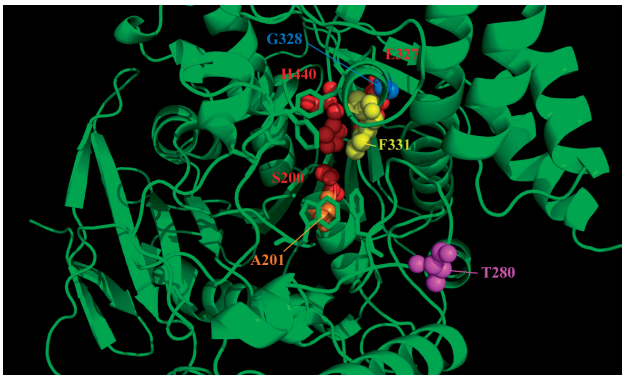

Figure 1. Three-dimensional structure model of *Tetranychus urticae* AChE1. The backbone of the enzyme structure is rendered as green ribbon with secondary structure. The catalytic triad (S200, E327 and H440) appears as Van der Waals red spheres. The different amino acid substitutions are shown as Van der Waals coloured spheres. The view points to the entrance of the catalytic gorge.

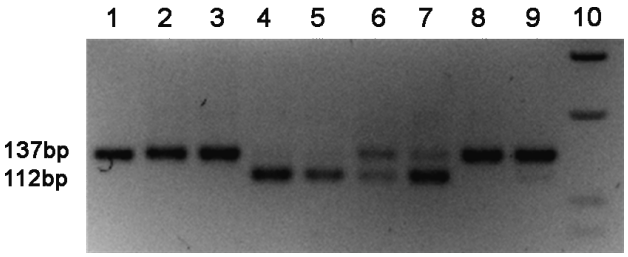

Figure 2. Diagnostic PCR-RFLP for 331 W after primer-induced mutagenesis. Lane 1: uncut PCR product. Lane 2: PCR performed on plasmid DNA containing GSS 331 Y clone. Lane 3: PCR performed on plasmid DNA containing LS-VL 331F clone. Lane 4: PCR performed on plasmid DNA containing WI 331 W clone. Lane 5: PCR performed on DNA extracted from a single male of strain MR-VL (containing 331 W). Lane 6, PCR performed on pooled plasmid DNA containing GSS and WI clones. Lane 7: PCR performed on pooled gDNA from two males from GSS and MR-VL strains. Lane 8: PCR product on pooled mites from GSS. Lane 9: PCR products on pooled mites from LS-VL. Lane 10: MR DNA ladder (Invitrogen, Belgium). The PCR-RFLP assay on heterozygote females (F, Y331/W331) is expected to display the pattern of lanes 6 and 7, whereas the patterns of lanes 1, 2, 3, 8 and 9 are expected from homozygote (F or Y331) females, and the patterns of lanes 4 and 5 from homozygote (W331) females.

were continuously kept under OP selection: a strain that historically developed OP resistance in the 1960s (WI) and two recently collected field strains (ATHRos-Pm from Greece and MR-VL from Belgium) that exhibited OP resistance. Toxicity bioassays on the OP strains revealed varying but high levels of resistance to most of the insecticides tested (Table 2). The WI strain proved to be more resistant to chlorpyrifos, whereas the recently collected strains MR-VL and ATHRos-Pm were particularly resistant to dimethoate. Compared to the reference strains, the biochemical experiments confirmed the presence of a more insensitive AChE1 in all OP strains (Table 3). The high variation found between LC_{50}s of OP strains was not reflected in a high variation between IC_{50}s. Thus, an insensitive AChE1 is probably not the only mechanism involved in the observed resistant phenotype, and other mechanisms such as metabolic mediated resistance (carboxylesterase, gluthathione-*S*-transferase or P450 monooxygenase activity) are likely also to be involved.

Target-site insensitivity as a mechanism of OP resistance in *T. urticae* was the first case ever reported, and provided a proof of principle for resistance development in arthropods in the early 1960s.[7,9] Here, the amino acid sequence of AChE1 was determined in three reference strains SAMB, GSS and LS-VL and three OP-resistant strains WI, MR-VL and ATHRos-Pm, and four amino acid substitutions were identified in the mature AChE1 that are present in one or more resistant strains and absent in the susceptible ones.

Two substitutions were consistently present in all three OP strains (F331W and T280A). The position of the T280A substitution in the AChE1 structural model indicates that this mutation does not seem to be responsible for the modification of the biochemical properties of AChE1 (Fig. 1). This is further supported by the fact that this residue is not conserved among species and that both resistant and susceptible strains of *T. urticae* from Japan have a threonine at position 280.[15] In contrast, the phenylalanine 331 residue is involved in substrate guidance and in the hydrolysing process by interacting with the catalytic histidine (H440).[51] A substitution at this position could lead to the modification of the catalytic properties of AChE1 (i.e. catalytic efficiency and/or inhibitor sensitivity). In *T. urticae* from Japan, an F331C substitution was found to be associated with OP resistance.[16] In *T. kanzawai*, a species closely related to *T. urticae*, a tryptophane at position 331 has been detected in resistant strains.[31] The F331W substitution has also been shown to be responsible for AChE1 insensitivity to OP insecticides in the mosquito *Culex tritaeniorhynchus* Giles by functional characterisation after site-directed mutagenesis and *in vitro* expression.[25,52] In addition, in the aphid species *Aphis gossypii* (Glov.) and *Myzus persicae* (Sulzer), a S331F was shown to confer insensitivity to AChE1, especially to the CARB pirimicarb.[53,54] More recently, the same substitution was associated with OP resistance in the B- and Q-biotypes of *Bemisia tabaci* Genn. from Israel and Greece.[55,56] In conclusion, the 331 position in AChE1 has been associated with OP resistance in many species, and the functional expression has clearly illustrated the effect of the substitutions on AChE1 sensitivity. Hence, the F331W substitution is most probably the major factor in many OP-resistant spider mite strains. This is further emphasised by the fact that the WI strain, which developed resistance in the 1960s, and the recently collected strains from geographically distant regions (MR-VL and ATHPros-Pm) carry this same F331W substitution.

An allele with an F331Y substitution was sequenced in one clone of reference strains (SAMB and GSS strains). The F331Y substitution may play a role in carbaryl sensitivity, as AChE1 of the GSS strain is more sensitive to carbaryl than AChE1 of the SAMB strain. Genotyping of single mites revealed that in GSS the F331Y is predominant, whereas in the SAMB strain the F331 is predominant (Tsagkarakou A and Van Leeuwen T, unpublished data). However, it is not known whether or not this substitution influences the catalytic parameters of AChE1 in these two strains.

In the recently collected ATHRos-Pm and MR-VL strains, the G328A substitution was found in addition to F331W and T280A. The position 328 in the *T. urticae* AChE1 model suggests that this substitution may indirectly influence the catalytic process by affecting position 331 (located in the same α-helix). However, when AChE1 sensitivity of the MR-VL and ATHRos-Pm strains is compared with that of the WI strain, the additional G328A substitution does not seem to alter the sensitivity to the tested insecticides, except for carbaryl. This substitution has also been found in the AChE2 of *Ceratitis capitata* Wied.,[31] *Drosophila melanogaster* Meig.[30] and *Musca domestica* L.[28] Site-directed mutagenesis followed by *in vitro* expression of *D. melanogaster* and *M. domestica* AChE2 showed that this mutation conferred insensitivity to chlorpyrifos-oxon, omethoate, malaoxon, methamidophos, paraoxon, paraoxon-

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methyl and pirimicarb, but increased the sensitivity to coumaphos and carbaryl.[28,30]

In the ATHRos-Pm strain, a fourth substitution (A201S) was found in addition to T280A, G328A and F331W. The proximity of this position to the catalytic serine (S200) may lead to some modifications in AChE1 biochemical properties. However, when IC_{50} values were compared between MR-VL and ATHRos-Pm strains (differing only in the presence of the A201S), no effect on AChE1 sensitivity towards the tested inhibitors was detected. This mutation was also found in other arthropod species, such as a resistant clone of *A. gossypii*[19,53] and a resistant strain of *P. xylostella*.[22,57] In *A. gossypii* the A201S substitution was always found in tandem with S331F, and in *P. xylostella* in tandem with G227A. In both cases it was suggested that its presence increases resistance levels, playing a supplementary role to the main mutations.[22,53,57] In *A. gossypii* it was shown that AChE1 from insects carrying both mutations possess a better substrate affinity than when carrying the S331F mutation alone.[53] The role of A201S mutation in resistance was characterised by site-directed mutagenesis in AChE1 of *C. tritaeniorhynchus*,[58] but no functional expression of recombinant A201S AChE1 of *A. gossypii* or *P. xylostella* has been performed until now.

Among the two substitutions specifically found in the reference SAMB strain (V7I and G119S), only G119S is located in the active site. This substitution is known to be responsible for AChE1 insensitivity[18] or reduced AChE1 activity in mosquitoes.[59,60] The 119 position is close to the catalytic serine (S200) and the G-to-S substitution would reduce the accessibility to inhibitors and substrates by steric hindrance. Recombinant *Culex pipiens* G119S AChE1 was similarly or more resistant than the recombinant F331W AChE1 to most of the OPs and CARBs tested, including chlorpyrifos-oxon and paraoxon.[24] SAMB exhibited moderate levels of resistance towards chlorpyrifos and dimethoate, and the involvement of an AChE mutation in resistance cannot be ruled out. However, the biochemical data do not support the involvement of G119S mutation in resistance towards the insecticides tested in this study, which is in line with previous reports on Tetranychidae.[15,32] Moreover, the AChE specific activity of the SAMB strain is higher than that of all other strains. However, before any sound conclusions can be made about the role of this mutation in *T. urticae* OP resistance, the frequency of this mutation should be determined by genotyping single mites of different strains, combined with a functional analysis of this mutation.

Although it is presumed that the F331W substitution reported in this study is essential in determining the insensitivity of AChE1 towards OP and CARB, the A201S and G328A mutations might have been selected by the application of other unknown OPs in the population's history. These substitutions may confer an advantage to other inhibitors. Different mutated AChE1 could cause a strong insensitivity to specific insecticides, as a good relationship between the highest insensitivities detected and the insecticide(s) used locally in mosquito control programs has been found.[61] Thus, it is possible that, in *T. urticae* also, different mutations are selected and maintained in the populations, each one conferring advantage against a particular OP or CARB insecticide. Alternatively, substitutions A201S and G328A might be selected to reduce a possible fitness cost associated with the F331W substitution in *T. urticae*. Valuable information on the importance of the different substitutions in developing and maintaining an OP-resistant phenotype could be obtained by following their dynamics with molecular diagnostic tests under different OP spraying protocols aimed at controlling other arthropod pests in the field. The development of such a diagnostic test was illustrated for F331W mutation, as it was consistently present in recently resistant spider mite strains from different geographical regions, as well as in a strain that developed resistance in the late 1960s.

Different mutations and their combinations can result in varying levels of resistance to many OP and CARB insecticides.[27] Some of them have essentially an additive effect, both in terms of reducing the sensitivity of the enzyme to inhibition by the insecticide and in terms of increasing the stability of the enzyme and its ability to turn over acetylcholine.[62] Functional analysis should be performed to test the influence of each mutation (alone or in combination) in OP and CARB insensitivity and in the specific activity of the recombinant mutated AChE1 in *T. urticae*.

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