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# *Myzus persicae* resistance to neonicotinoids— unravelling the contribution of different mechanisms to phenotype

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## Abstract

**Background:** Deciphering the mechanisms underlying insecticide resistance is key to devising appropriate strategies against this economically important trait. *Myzus persicae*, the green peach-potato aphid, is a major pest that has evolved resistance to many insecticide classes, including neonicotinoids. *M. persicae* resistance to neonicotinoids has previously been shown to result from two main mechanisms: metabolic resistance resulting from P450 overexpression and a targetsite mutation, R81T. However, their respective contribution to resistant phenotypes remains unclear.

**Results:** By combining extensive insecticide bioassays with and without addition of the synergist PBO, and gene copy number and expression quantification of two key P450 enzymes (*CYP6CY3* and *CYP6CY4*) in a 23 clone collection, we, (i) confirmed that metabolic resistance is correlated with P450 expression level, up to a threshold, (ii) demonstrated that the R81T mutation, in the homozygous state and in combination with P450 overexpression, leads to high levels of resistance to neonicotinoids, and, (iii) showed that there is a synergistic interaction between the P450 and R81T mechanisms, and that this interaction has the strongest impact on the strength of resistance phenotypes. However, even though the R81T mutation has a great effect on the resistance phenotype, different R81T genotypes can exhibit variation in the level of resistance, explained only partially by P450 overexpression.

**Conclusion:** To comprehend resistance phenotypes, it is important to take into account every mechanism at play, as well as the way these mechanisms interact.

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Supporting information may be found in the online version of this article.

**Keywords:** green peach aphid; neonicotinoid resistance; metabolic mechanism; target site resistance; PBO synergist

## 1 INTRODUCTION

Over the last century, the control of agricultural pests has relied primarily on the use of chemical insecticides, and their intensive use over many years has led to the evolution of widespread resistance in many orders and families of insects.<sup>1–3</sup> Resistance to insecticides has been defined as ‘the ability of insects to survive exposure to a standard dose of insecticide, owing to physiological or behavioral adaptation’.<sup>4</sup> Insecticide resistance can lead to loss of quality and yield in agricultural production, and repeated application of ineffective treatments with potentially harmful environmental impacts. Thus, there is an urgent need to design effective strategies to combat resistance underpinned by an understanding of the genetic variants involved, how these interact and their relative contribution to phenotype. Depending on the nature of resistance mechanisms, optimal resistance management strategies can differ. For example, to delay the spread of resistance caused by a mutation in the pesticide target, lowering the selection pressure is recommended, and one way of achieving this, in

theory, is to lower the application dose rate, even if this is not generally authorised.<sup>5</sup> However, in the presence of quantitative resistance, populations include a variety of resistance level phenotypes, and low dose rates can lead to the survival of individuals that would have been controlled at full dose.<sup>6</sup>

Insecticide resistance involves two main categories of mechanisms: target site resistance (TSR) resulting from mutations in the gene encoding the insecticide target protein that make it less sensitive to the toxic effect of the insecticide, and non-target site resistance (NTSR, including metabolic resistance) resulting from the enhanced production or activity of metabolic enzymes that break down or sequester the insecticide.<sup>7</sup> Other mechanisms that

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have been less well studied, namely behavioral avoidance, reduced cuticular penetration and increased excretion have also been described in resistant insect strains<sup>8</sup> (see Ref. 7 for review). TSR and metabolic resistance mechanisms are non-exclusive, and can be present at the same time within a single individual. For example, in the B-biotype of *Bemisia tabaci*, resistance to organophosphates involves both NTSR (mono-oxygenases and carboxylesterases) and TSR mechanisms (F331W mutation in the *ace-1* gene encoding acetylcholinesterase).<sup>9–11</sup> In *Aphis gossypii*, esterase overexpression and mutations in the targeted acetylcholinesterase have both been shown to confer resistance to pirimicarb,<sup>12,13</sup> whilst the upregulation of UDP-glycosyltransferases and P450s act in concert with three mutations in the nicotinic acetylcholine receptor (nAChR)  $\beta 1$  subunit to confer resistance to neonicotinoids.<sup>14–17</sup> Monitoring pest insect populations for such combinations of potential mechanisms is not easy (see Ref. 7 for review). Although they are very labor intensive, insecticide bioassays on live insects are mechanism-agnostic and thus provide insight into the combined effect of any resistance mechanisms present in the tested population on the insecticide sensitivity phenotype. Conversely, biochemical and molecular tests are much more high-throughput but rely on the identification of markers for each mechanism of interest, and can typically test for only a handful of mechanisms simultaneously (although some methods, such as KASP, can test for mutations at a relatively high number of loci concurrently). Furthermore, using molecular approaches to diagnose NTSR mechanism is compounded by the fact that this form of resistance is often polygenic, may involve epistatic interactions, and may act in combination with TSR.<sup>18–20</sup>

The green peach-potato aphid, *Myzus persicae* (Sulzer), is a pest of great economic impact, which has evolved resistance to many insecticides. It is highly polyphagous (400 species in 40 different botanical families)<sup>21</sup> and causes crop losses by direct feeding or by transmitting plant viruses. In temperate climates, *M. persicae* can reproduce both parthenogenetically and sexually, alternating between sexual reproduction on peach during late fall and many parthenogenetic generations on peach and other herbaceous species (crops and wild hosts) throughout the year.<sup>22</sup> To control populations, this pest has been intensively treated with synthetic insecticides, and over the years has developed resistance to insecticides belonging to many classes on various crops.<sup>19</sup> The spatial distribution of resistance mechanisms in *M. persicae* can be heterogeneous depending on host plant, with a genetic separation of populations from certain hosts, such as between peach and herbaceous hosts.<sup>23,24</sup> *M. persicae* has evolved TSR and NTSR mechanisms to several insecticides.<sup>19,25</sup> Resistance to pyrethroids, for instance, is caused by both NTSR (carboxylesterases E4 and FE4) and/or by TSR (L1014F and various amino acid substitutions at codon 918 of the voltage-gated sodium channel).<sup>23,24,26–30</sup>

Resistance to neonicotinoids in *M. persicae* was first reported in 2007,<sup>31</sup> and subsequently described in several countries (Greece, France, Italy, the United States, Australia, Tunisia, China).<sup>29,32–37</sup> In *M. persicae*, resistance to neonicotinoids was first reported to be associated with NTSR,<sup>38–40</sup> then with TSR.<sup>41,42</sup> Target site resistance to neonicotinoids is conferred by the R81T mutation in the  $\beta 1$  subunit of the nicotinic acetylcholine receptor (nAChR).<sup>41</sup> This mutation is associated with high-level resistance especially in the homozygous form, however, variation in phenotype within R81T genotypes point to the presence of other mechanisms.<sup>43</sup> More recently, a second mutation, V101I, in the nAChR  $\beta 1$  subunit has been described by Xu *et al.* in neonicotinoid resistant strains of *M. persicae* from China.<sup>42</sup> However, the causal role of this

mutation in resistance has not been functionally demonstrated. Metabolic resistance to neonicotinoids in *M. persicae* was first linked to the overexpression of the P450 gene *CYP6CY3*.<sup>19,38</sup> This P450 gene is amplified in neonicotinoid and nicotine resistant clones in combination with two other P450 genes, *CYP6CY4*, and *CYP6CY23*.<sup>39,44,45</sup> Both *CYP6CY3* and *CYP6CY4*, but not *CYP6CY23*, were shown to metabolize nicotine to its less toxic metabolites *in vitro*, and recombinant *CYP6CY3* has also been shown to metabolize several neonicotinoid insecticides.<sup>19,39,46</sup> Knockdown of *CYP6CY3* and *CYP6CY4* using RNA interference has provided additional evidence that they confer resistance to neonicotinoids.<sup>44</sup> Overexpression of *CYP6CY3* and *CYP6CY4* can be found in individual *M. persicae* clones in combination with the R81T TSR mechanism, however, the relative contribution of each mechanism to phenotype and how they interact (additively, synergistically, epistatically) remains unclear.

The objective of this study was to identify the relative importance of the genetic mechanisms underlying neonicotinoid resistance in *M. persicae* using a collection of parthenogenetic clones. Studying a single resistance mechanism is classically performed by backcrossing and selecting strains possessing the genes or alleles of interest,<sup>47</sup> or, more recently, by CRISPR/Cas9 genome editing, to obtain strains differing only in their chosen resistance genotype.<sup>48,49</sup> The use of the first method is limited to organisms that can reproduce sexually and whose sexual reproduction can be induced in the laboratory. The second strategy necessitates the development of genome editing methods that can be challenging to develop for primarily asexually reproducing species.<sup>50</sup> Furthermore, when several mechanisms are involved in resistance, the challenge is greater, and such analyses have only been conducted in a limited set of species.<sup>51</sup> When neither of these two approaches is feasible, a multi-individual approach provides a solution for dissecting the association between phenotype and resistance alleles of interest. While such a population-based approach offers an alternative to these strategies, it has its constraints. In particular, bioassays must be carried out on a large number of individuals, which is labor intensive and time consuming. However, *M. persicae* is an organism that can readily produce large numbers of offspring by parthenogenesis and this allows for the easy maintenance of genetically distinct clonal lineages with different resistance mechanisms. These traits, and its long history of exposure to insecticides and resistance evolution, makes it an excellent model to study insecticide resistance mechanisms. Here, we used this system to address the following questions: (1) Do cytochrome P450 monooxygenases explain the component of neonicotinoid resistance not conferred by the R81T mutation and the observed variation in resistance level among 81<sup>RR</sup> genotypes. (2) Is there a correlation in the number of copies of the P450 genes *CYP6CY3*, *CYP6CY4* and *CYP6CY23* gene with mRNA expression and resistance level. (3) Does R81T and P450 overexpression interact additively, synergistically or epistatically?

## 2 MATERIALS AND METHODS

### 2.1 Biological material

Parthenogenetic female individuals of *M. persicae* were sampled in 2011, 2012, 2013 and 2017 in France in peach orchards or oil-seed rape fields (Table 1). One individual was collected per leaf and was reared on isolated Chinese cabbage plants in a controlled environment (20–22 °C with a 16:8 light:dark photoperiod). These rearing conditions allowed the maintenance of each aphid lineage as a clonal colony. A total of 23 clones were used (some of

**Table 1.** Sampling information, bioassays and DNA and mRNA quantification of 18 field clones and five reference clones of *M. persicae* treated with thiacloprid alone or thiacloprid + PBO

Clone ID	Field sampling information	Location†	nACHR AA81†	Thiacloprid				Thiacloprid + PBO				DNA <sup>Δ</sup>				Expression <sup>◇</sup>			
				n <sup>§</sup>	LC <sub>50</sub> (µg litre <sup>-1</sup> )	SE <sup>  </sup>	RR <sup>#</sup>	n <sup>§</sup>	LC <sub>50</sub> (µg litre <sup>-1</sup> )	SE <sup>  </sup>	RR <sup>#</sup>	Ratio	SE	Ratio	SE	Ratio	SE	Ratio	SE
17-0153-0020	Peach 2017	Eymeux, ARA	81 <sup>RR</sup>	67.57	34.43	4.53	0.5	67.13	25.54	2.44	0.5	1.02	0.04	0.93	0.03	0.26	0.08	0.59	0.08
4106A	(lab clone)	-	81 <sup>RR</sup>	119.60	68.50	3.09	1.0	110.50	54.78	3.13	1.0	1.05	0.10	1.06	0.10	1.00	0.43	1.00	0.25
13-0284-0034	Oilseed rape 2013	Savières, GES	81 <sup>RR</sup>	131.50	79.18	6.74	1.2	97.25	25.38	4.90	0.5	1.00	0.00	1.00	0.00	0.85	0.37	1.24	0.53
4916A	(lab clone)	-	81 <sup>RR</sup>	159.17	117.16	7.00	1.7	153.63	46.72	2.43	0.9	2.25	0.09	1.78	0.18	1.53	0.48	1.77	0.43
17-0153-0001	Peach 2017	Eymeux, ARA	81 <sup>RR</sup>	98.56	136.63	10.28	2.0	95.89	86.83	7.90	1.6	1.46	0.07	1.54	0.08	2.18	0.99	2.23	1.10
384C	(lab clone)	-	81 <sup>RR</sup>	94.83	148.20	6.69	2.2	91.38	45.52	2.18	0.8	3.43	0.32	1.37	0.14	4.42	2.17	5.29	2.41
11-0037-0018	Peach 2011	Fos-sur-Mer, PAC	81 <sup>RR</sup>	69.50	177.67	38.77	2.6	36.00	60.11	14.38	1.1	4.82	0.17	4.78	0.16	4.07	1.34	7.96	0.73
13-0001-0050	Peach 2013	Etoile sur Rhone, ARA	81 <sup>RR</sup>	51.67	227.02	27.20	3.3	37.67	78.47	11.44	1.4	4.34	0.19	3.07	0.17	13.86	5.43	18.37	7.60
17-0149-0006	Oilseed rape 2017	Lapeyrouse-Mornay, ARA	81 <sup>RR</sup>	73.38	229.93	30.82	3.4	73.43	45.94	5.91	0.8	2.71	0.08	2.90	0.19	4.40	2.08	3.58	1.20
Mn2	(lab clone)	-	81 <sup>RR</sup>	74.38	309.85	32.15	4.5	74.57	66.22	9.26	1.2	6.86	0.30	5.41	0.59	17.12	7.72	14.85	7.11
11-0037-0001	Peach 2011	Fos-sur-Mer, PAC	81 <sup>RR</sup>	255.04	434.70	20.16	6.3	243.06	103.67	4.48	1.9	6.66	0.16	5.79	0.20	15.61	7.57	20.22	5.96
5191A	(lab clone)	-	81 <sup>RR</sup>	160.31	468.41	15.20	6.8	151.88	86.27	3.33	1.6	5.33	0.19	4.07	0.16	8.65	3.31	13.95	3.85
12-0067-0023	Peach 2012	Lotol, ARA	81 <sup>RT</sup>	60.00	360.20	24.17	5.3	57.67	104.37	6.93	1.9	4.36	0.13	4.17	0.13	5.26	2.69	6.41	2.26
11-0037-0012	Peach 2011	Fos-sur-Mer, PAC	81 <sup>RT</sup>	62.67	401.29	65.04	5.9	58.57	158.78	27.07	2.9	6.66	0.48	7.34	0.20	19.38	6.19	31.10	9.42
12-0068-0006	Peach 2012	Roche de Glun, ARA	81 <sup>RT</sup>	57.83	550.59	111.86	8.0	58.67	111.63	20.19	2.0	5.78	0.24	5.98	0.21	8.31	2.73	16.29	6.86
13-0001-0032	Peach 2013	Etoile sur Rhone, ARA	81 <sup>RT</sup>	178.86	626.98	32.40	9.2	171.57	111.88	5.67	2.0	3.09	0.14	2.25	0.18	7.01	3.07	5.84	1.67
11-0060-0006	Peach 2011	Prades, OCC	81 <sup>RT</sup>	65.40	636.10	37.03	9.3	61.80	181.31	12.06	3.3	4.41	0.11	4.26	0.11	10.49	4.18	6.52	3.06
13-0001-0048	Peach 2013	Etoile sur Rhone, ARA	81 <sup>RT</sup>	115.43	1087.80	49.92	15.9	113.00	206.18	11.99	3.8	5.02	0.20	3.86	0.06	7.28	4.68	8.43	2.67
12-0069-0001	Peach 2012	Roche de Glun, ARA	81 <sup>RT</sup>	105.83	1174.61	50.13	17.1	100.00	226.32	9.31	4.1	6.50	0.83	2.86	0.57	11.74	1.63	13.02	5.84
17-0145-0005	Peach 2017	Saint Gilles, OCC	81 <sup>TT</sup>	74.71	5347.16	478.49	78.1	74.13	931.53	95.33	17.0	4.86	0.19	3.64	0.17	3.21	1.22	3.54	1.30
11-0039-0003	Peach 2011	St Félin d'Avall, OCC	81 <sup>TT</sup>	69.60	12262.95	3139.18	179.0	64.57	2565.35	613.88	46.8	7.99	0.88	3.26	0.60	6.75	4.99	5.49	2.83

**Table 1.** Continued

Clone ID	Field sampling information	Location†	nAChR AA81‡	Thiacloprid			Thiacloprid + PBO			DNA <sup>Δ</sup>			Expression <sup>◇</sup>						
				n <sup>§</sup>	LC <sub>50</sub> (µg litre <sup>-1</sup> )	SE <sup>  </sup>	RR <sup>#</sup>	n <sup>§</sup>	LC <sub>50</sub> (µg litre <sup>-1</sup> )	SE <sup>  </sup>	RR <sup>#</sup>	CY3 Ratio	CY3 SE	CY3 Ratio	CY3 SE	CY4 Ratio	CY4 SE		
13-0001-0045	Peach 2013	Etoile sur Rhone, ARA	81 <sup>TT</sup>	56.40	14781.53	3098.29	215.8	58.43	2857.52	662.40	52.2	5.08	0.30	1.65	0.16	7.37	1.92	11.50	2.86
13-0001-0041	Peach 2013	Etoile sur Rhone, ARA	81 <sup>TT</sup>	75.80	16520.98	2307.48	241.2	76.00	2806.31	416.50	51.2	4.02	0.18	2.93	0.12	7.90	4.15	11.30	6.14

† Location: name of the commune of the sampling site followed by an abbreviation designating the region of France. ARA, Auvergne Rhône-Alpes; GES, Grand Est; OCC, Occitanie; PAC, Provence-Alpes-Côte-d'Azur.

‡ nAChR AA81: genotype at the 81 amino-acid residue in the nAChR β1 subunit. 81<sup>RR</sup>: wild-type, 81<sup>RT</sup>: heterozygous, 81<sup>TT</sup>: homozygous resistant.

§ n: mean number of tested individuals per dose.

|| LC50: Lethal Concentration 50, the concentration of that results in death of 50% of individuals.

|| SE: standard error.

# RR: resistance ratio.

Δ DNA: copy number relative to 13-0284-0034 susceptible reference clone.

◇ Expression: gene expression relative to 13-0284-0034 susceptible reference clone.

which are the same as those used in Ref. 43). Eighteen of the clones were sampled from the field, and five were included in the bioassays as references: 4106A, a fully insecticide-sensitive reference clone; 4916A, a clone homozygous sensitive for R81T (both were provided by Diana Cox, Rothamsted Research, Harpenden); 384C, a clone sampled on French oilseed rape in 2010 homozygous susceptible for R81T; and two clones homozygous susceptible for R81T but carrying multiple copies of the *CYP6CY3* gene: Mn 2 and 5191A (=Mn4) in Refs 38,39,52. To check that clones had distinct genotypes, one adult of each clone was genotyped using the microsatellite markers and methods described in Ref. 23. Briefly, genotyping of the 14 microsatellite loci provides the allelic combinations for each clonal line. This makes it possible to identify the genotype of each clone and ensure that they are clearly distinct from each other (see Table S1 for details of the genotypes of each line).

## 2.2 Artificial feeding bioassays

Insecticide bioassays were derived from a method previously used to assay the sensitivity of *M. persicae* to neonicotinoids (thiacloprid and imidacloprid).<sup>43</sup> This method exposes the aphids to the insecticide only *via* ingestion, excluding, for example, mechanisms of reduced penetration through the cuticle.<sup>19</sup> A total of 25 to 35 adult females were placed for 24 h in a glass ring (4 cm of diameter) covered with two membrane layers of parafilm containing the artificial feeding medium AP3 (provided by BF2I (Univ. Lyon, INSA Lyon, INRAE, UMR203) and described in Ref. 53). Neonate L1 nymphs (0–24 h old) produced by the adult females were then used for the experiment. These were transferred to a fresh ring containing the AP3 medium amended with insecticide, using the commercial product soluble in water: Calypso® (Bayer CropScience, Lyon, France). A 5% solution of the formulated insecticide was prepared in AP3 medium and used to prepare serial dilutions to obtain final thiacloprid concentrations ranging from 10 to 40 960 µg.litre<sup>-1</sup> (with a factor 2 between each dose).

To investigate the role of P450s in neonicotinoid resistance, synergist bioassays were conducted using the P450 inhibitor piperonyl butoxide (PBO). For this, aphids were exposed to PBO, sprayed with a Potter tower (modified by Burgerjon),<sup>54</sup> at the rate of 4.0 mg/cm<sup>2</sup> onto the lower surface of the parafilm-covered rings containing the AP3 medium with insecticide. PBO was diluted in alcohol to a concentration of 0.01% and pure ethanol was used as the control. The 0.01% dose of PBO was previously determined to be the maximum concentration tolerated by L1 nymphs. Alcohol was allowed to evaporate completely before transferring neonates to the sprayed rings.

For each tested concentration, three temporal replicates or more, involving at least 10 L1 nymphs, were treated until a minimum of 45 L1 per dose were assayed for each modality. Results were homogeneous between replicates and were therefore pooled together. Tests were rejected if control mortality was greater than 20%. Assessments of live and dead aphids were made 48 h after exposure. For assessment, aphids were turned over on their back, those unable to turn back and move in 15 s were scored as dead. For each clone tested, each step (preparation of insecticide solutions, preparation and treatment of AP3 rings, transfer of neonates and scoring of dead/alive) was done by the same person in both conditions (with or without synergist). Alongside each test, a reference clone with known resistance level was tested as a control.

Resistance ratios (RRs) for each clone and each condition (thiacloprid alone or in association with PBO) was calculated relative

to the corresponding LC<sub>50</sub> value for the susceptible reference clone 4106A.

### 2.3 Target site mutation analyses

Each clonal lineage was genotyped for the R81T and V101I mutations by sequencing after RNA extraction from single adult aphid using the NucleoSpin 8 RNA extraction kit (Macherey Nagel, Hoerd, France) and RT-PCR using the SuperScript™ One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Thermo Fisher Scientific Inc., Massachusetts, USA) according to the manufacturer's instructions. Specific primers (Mpb1F1 and R1) were used to amplify the nicotinic acetylcholine receptor (nAChR) beta1 subunit, as described in Ref. 42. Sanger sequencing (GenoScreen, Lille, France) of PCR products was carried out with both primers.

### 2.4 Determination of P450 genomic copy number by quantitative PCR

Quantitative PCR was used to determine *CYP6CY3*, *CYP6CY4* and *CYP6CY23* gene copy numbers. We confirmed that the *CYP6CY23* gene was not involved in P450 metabolic resistance,<sup>39</sup> so results for this P450 gene are provided in supplementary material only (Table S2 and Fig. S1).

DNA from a pool of 20 adult aphids per clone was extracted using the NucleoSpin 8 or 96 Tissue Kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. The final step of DNA elution was performed in 30 µL of elution buffer. DNA quality and quantity was assessed by spectrophotometry (NanoDrop™ One – Ozyme, Saint-Cyr-l'École, France). DNAs were then diluted to 10 ng.µL<sup>-1</sup>. Three qPCR reactions were performed to amplify *CYP6CY3* and *CYP6CY4* and the housekeeping gene *para* using the primer sequences listed in Table 2.

PCR reactions were performed in a final volume of 20 µL and contained 4 µL of DNA, 10 µL of SYBR green (LightCycler® 480 SYBR Green I Master Roche). Primer concentrations were 0.10 µM for *CYP6CY3* amplification (CY3\_3F/CY3\_3R), 0.15 µM for *CYP6CY4* amplification (CY4\_F3/CY4\_R3) and 0.2 µM for *para* amplification (qMP-skdr-F/qMP-skdr-R). The efficiency of PCR for each primer pair was assessed using a serial dilution from 0.001 ng to 100 ng/µL of DNA with a dilution rate of 10 (Table 2). The primers employed were designed by Singh *et al.* 2020 avoiding previous primers that were in fact cross-hybridizing between *CYP6CY3*, *CYP6CY4* and *CYP6CY23* genes.<sup>45</sup>

Quantifications were run on a LightCycler® 480 System (Roche, Basel, Switzerland) using the temperature cycling conditions of: 5 min at 95 °C followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s. A final melt-curve step was included

post-PCR to confirm the absence of any non-specific amplification.

Each qPCR experiment was performed with three technical replicates for each sample. The results of qPCR were normalized using the *para* gene (present in two genomic copies in diploid *M. persicae* genome) and ratios were calculated following the 2<sup>-ΔΔ</sup> Ct method by the Lightcycler 480 software release 1.5.1.62. Results were normalized to the gene values of the 13-0284-0034 susceptible reference clone, which has two copies of each of the *CYP6CY3* and *CYP6CY4* genes.

### 2.5 Determination of P450 gene expression by reverse transcription quantitative PCR

RT-qPCR was used to determine the expression level of *CYP6CY3* and *CYP6CY4* genes through mRNA quantification. As for genomic copy number, results for *CYP6CY23* are shown in supplementary material (Table S2). L1 aphids for this experiment were reared in the same conditions as for bioassays without insecticide (in glass rings with AP3 medium with the same photoperiod and temperature) and frozen at -80 °C. RNA from three biological replicates were extracted using the Nucleospin 8 RNA Kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Each biological replicate constituted a pool of 15 L1 aphids. 50 µL of RNA extract was treated by TURBO DNase (TURBO DNA-free™ Kit, Invitrogen) to remove traces of genomic DNA that can interfere in the quantification. Two reactions of Reverse Transcription (RT) were done for each sample to have enough cDNA for qPCR. A total of 11 µL of RNA extract was used for cDNA synthesis using Superscript IV Reverse Transcriptase and oligo hexamers (Invitrogen) according to the manufacturer's instructions.

For PCR the same primers used for genomic quantitative PCR were employed (Table 2). To accurately measure gene expression, the *L27* gene was used as a housekeeping gene in addition to the *para* gene.<sup>55</sup> *L27* qPCR efficiency was calculated using a serial dilution of cDNA from 0.001 to 100 ng.µL<sup>-1</sup> with a dilution rate of 10 (Tables 2 and S3).

Quantifications were run on a LightCycler® 480 System (Roche, Basel, Switzerland) using the same temperature cycling conditions as used for the determination of genomic copy number. Each qPCR experiment was performed with three technical replicates for each sample.

The results of qPCR were normalized using expression data for the *para* and *L27* genes and ratios were calculated for each clone using the SATqPCR tool and the geometric mean of two

**Table 2.** Sequences of primers used in this study for DNA and RNA quantification, and efficiencies of these primer pairs in qPCR

Amplified gene	Primer name	Sequence primer	Reference	qPCR efficiency
<i>CYP6CY3</i>	CY3_3F	5'-CCGTTTACCTGACAACCTAATACTG-3'	Singh <i>et al.</i> <sup>39</sup>	2.060
	CY3_3R	5'-AGGACGTTTATCTTTGTTTCGGGT-3'		
<i>CYP6CY4</i>	CY4_F3	5'-TATCAAATACCCAACGATTCACTAATA-3'	Singh <i>et al.</i> <sup>39</sup>	2.295
	CY4_R3	5'-CTTTCCGGGTCTTTATAACTTAGG-3'		
<i>para</i>	qMP SKDR-F	5'-GTGGCCACACTGAATCTTTAAT-3'	Puinean <i>et al.</i> <sup>38</sup>	2.058
	qMP SKDR-R	5'-ACAAACGTTAGGTTACCCAAGCA-3'		
<i>L27</i>	L27MyzP_F	5'-CCGAAAAGCTGTCTAATGAAGAC-3'	Mutti <i>et al.</i> <sup>55</sup>	2.255
	L27MyzP_R	5'-GGTGAAACCTGTCTACTGTTACATCTTG-3'	Kang <i>et al.</i> <sup>56</sup>	

housekeeping genes.<sup>57</sup> Results were normalized to the gene values of the 13-0284-0034 susceptible reference clone.

## 2.6 Data analysis

Dose–response data were analyzed in a non-linear regression framework using the ‘drc’ package<sup>58</sup> in R software v. 4.3.1.<sup>59</sup> A binomial distribution of errors was assumed. The survival data were fitted to the three-parameters log-normal model, which is equivalent to the classic Probit model<sup>60</sup> with an additional parameter which takes into account the ‘natural’ mortality rate observed in the control of each category. The absolute LC<sub>50</sub> and their associated 95% CI and standard errors were estimated with the ED function of the ‘drc’ package.

Means and variances of LC<sub>50</sub> and LC<sub>50</sub> with PBO values were computed using R. The equality of variance between LC<sub>50</sub> and LC<sub>50</sub> with PBO values was tested using Levene's test. Because of a significant difference between LC<sub>50</sub> and LC<sub>50</sub> with PBO variances, the correlation between LC<sub>50</sub> and LC<sub>50</sub> with PBO was evaluated on log-transformed variables using a linear model in the R framework.<sup>59</sup> The assumptions of the model were verified by plotting residuals *versus* fitted values and a QQ plot. Pairwise comparisons of log-transformed means between R81T genotypes were evaluated by computing ‘Tukey Honest Significant Differences’. Final means and standard errors were obtained by back-transformation via the delta-method using the ‘emmeans’ package.<sup>61</sup>

Dominance level was calculated as in Ref. 43, using the equation as defined in Ref. 62:  $D_{LC} = (\log LC_{R5} - \log LC_S) / (\log LC_R - \log LC_S)$ .

To investigate the link between the resistance level to thiacloprid and its genetic basis, LC<sub>50</sub> values were modeled as a function of the neutral genetic cluster membership of individuals (estimated from Ref. 23 with the same parameters, Table S4), their R81T genotype and their gene copy number and expression level of P450 genes. A Gaussian generalized linear model with a log link function, to take into account the heteroscedasticity of the response variable, was used. Fixed covariates were *Neutral genetic cluster* (categorical with two levels: ‘primary host’ and ‘secondary host’), *R81T genotype* (categorical with three levels: ‘81<sup>RR</sup>’, ‘81<sup>RT</sup>’, and ‘81<sup>TT</sup>’), *CYP6CY3 copy number* (continuous), *CYP6CY3 expression* (continuous), *CYP6CY4 copy number* (continuous) and *CYP6CY4 expression* (continuous). Because of the correlation between the four detoxification covariates (Fig. S2) and to avoid collinearity between covariates, only one was included in the model. The choice of the detoxification covariate was based on the smallest Akaike Information Criterion (AIC) produced with a complete model (three covariates ‘*Neutral genetic cluster*’, ‘*R81T genotype*’ and the selected detoxification covariate, and their interactions). A backward elimination variable selection algorithm based on AIC was used to simplify the model with the best detoxification covariate. The ‘stats’ package of the R software was used to fit the different models.<sup>59</sup> The assumptions of the model were verified by plotting residuals *versus* fitted values and a QQ plot.

In addition to modeling the effect of both TSR and NTSR and their interaction on the LC<sub>50</sub>, the independent effect of TSR was analyzed by comparing the mean LC<sub>50</sub> with PBO of the different R81T genotypes. Because of heteroscedasticity, a linear model on log transformed data was used. Pairwise comparisons of log-transformed means between R81T genotypes were evaluated by computing ‘Tukey Honest Significant Differences’. Final means and standard errors were obtained by back-transformation via the delta-method using the ‘emmeans’ package.<sup>61</sup>

Finally, the effect of detoxification covariates on the resistance phenotype was further investigated on the 81<sup>RR</sup> genotypes. Because these individuals do not carry a target site resistance allele, the proportion of the resistance linked to detoxification by P450 can be evaluated by the proportion (LC<sub>50</sub>–LC<sub>50</sub>.PBO)/LC<sub>50</sub>. This proportion was modeled as a function of one detoxification covariate (because of the correlation between these covariates) with a Michaelis–Menten model with two parameters using the ‘drc’ package.

The dataset and the code used for the different analyses, as well as for the production of the figures can be found in this online repository (<https://doi.org/10.5281/zenodo.10960421>).

## 3 RESULTS

Thiacloprid LC<sub>50</sub> values for the tested *M. persicae* clones ranged from 34.4 µg/L to 1.65 × 10<sup>4</sup> µg/L (Table 1, Fig. 1(a)). LC<sub>50</sub> means of each R81T genotype category were significantly different ( $F = 64.1$ ,  $P < 0.001$ ). All pairwise comparisons between R81T genotypes were highly significant (81<sup>RR</sup>–81<sup>RT</sup>, 81<sup>RR</sup>–81<sup>TT</sup> and 81<sup>RT</sup>–81<sup>TT</sup>,  $P < 0.001$  for all pairs). The estimated back transformed mean LC<sub>50</sub> values were 159 µg/L, 633 µg/L and 11.2 × 10<sup>3</sup> µg/L for the 81<sup>RR</sup>, 81<sup>RT</sup> and 81<sup>TT</sup> genotypes, respectively. The LC<sub>50</sub> of the 81<sup>RT</sup> genotype was found to be 3.98 [95% CL (1.81–8.75)] times higher than for the 81<sup>RR</sup> genotype. The 81<sup>TT</sup> genotype displayed a 70.8 [95% CL (27.2–184)] times and 17.8 [95% CL (6.30–50.2)] times higher LC<sub>50</sub> than the 81<sup>RR</sup> and 81<sup>RT</sup> genotypes, respectively.

A strong correlation ( $t = 19.2$ ,  $P < 0.001$ ,  $R^2_{adj} = 0.94$ ) between log-transformed LC<sub>50</sub> values and LC<sub>50</sub> values with PBO was observed (Fig. 1(a)). The use of the synergist PBO in combination with thiacloprid reduced ostensibly the LC<sub>50</sub>s for all clones (mean LC<sub>50</sub>s decrease from 2.44 × 10<sup>3</sup> to 4.77 × 10<sup>2</sup> µg/L). A reduction in resistance was observed within all three R81T genotypes means: from 2.03 × 10<sup>2</sup> to 60.5 µg/L for 81<sup>RR</sup>, from 6.91 × 10<sup>3</sup> to 1.57 × 10<sup>2</sup> in 81<sup>RT</sup> and from 1.22 × 10<sup>4</sup> to 2.29 × 10<sup>3</sup> in 81<sup>TT</sup> (Fig. 1(b)). The addition of PBO also reduced LC<sub>50</sub> variances of all genotypes together by 30-fold (from 2.44 × 10<sup>7</sup> for thiacloprid alone to 8.40 × 10<sup>5</sup> with PBO,  $F = 13.9$ ,  $P < 0.001$ ), as well as within each genotype (from 1.95 × 10<sup>4</sup> to 6.08 × 10<sup>2</sup>, from 1.02 × 10<sup>5</sup> to 2.45 × 10<sup>3</sup> and from 2.41 × 10<sup>7</sup> to 8.37 × 10<sup>5</sup>, for 81<sup>RR</sup>, 81<sup>RT</sup> and 81<sup>TT</sup>, respectively).

In the case of the mode of inheritance of R81T, the mutant allele ‘T’ was found to be semi-recessive, both when calculated on data obtained with thiacloprid ( $D_{LC} = 0.3$ ) and with thiacloprid + PBO ( $D_{LC} = 0.26$ ), as  $D_{LC}$  is comprised between 0 (full recessivity) and 0.5 (codominance).

Genotyping clones for the R81T and V101I mutations revealed only R81T mutations in 11 of the 23 clones tested – seven of these clones were heterozygous and four homozygous for the R81T mutation (Table 1). The V101I mutation, recently identified in China<sup>42</sup> was not detected in our sampling.

*CYP6CY3* and *CYP6CY4* copy number, expressed as a ratio to a susceptible reference clone (13-0284-0034) that presents the minimum number of copies (2), ranged from 1 to almost 8 for *CYP6CY3* and from 0.9 to 7.3 for *CYP6CY4* (Table 1, Fig. 2) among the tested clones.

The expression level of each gene, expressed as RNA ratios against the reference 13-0284-0034 clone, varied for *CYP6CY3* from 0.26 to 19.38 and for *CYP6CY4* from 0.59 to 31.1 (Table 1, Fig. 2).

A significant correlation between relative copy number and relative expression was observed for both *CYP6CY4* ( $R^2 = 0.78$ ), and *CYP6CY3* ( $R^2 = 0.75$ ). A significant correlation was also found between relative expression for *CYP6CY3* and relative expression for *CYP6CY4* ( $R^2 = 0.91$ ) and to a lesser extent between the relative copy number of *CYP6CY3* and *CYP6CY4* ( $R^2 = 0.75$ ).

Based on the AIC of complete models, the *CYP6CY3* expression covariate was selected as the best detoxification variable to take into account in the model. Both the expression of *CYP6CY3* and *CYP6CY4* were linked to  $LC_{50}$ , however, they cannot both be included in the model, as their expressions are correlated. As it is not possible to distinguish the effect of one from the effects of the other, the expression of *CYP6CY3* was used as a proxy for the combined effects of *CYP6CY3* and *CYP6CY4*. After backward elimination of variables, the final model included *R81T genotype*, *CYP6CY3 expression* and their interaction (Table S5). The *Neutral genetic cluster* did not have a notable impact on  $LC_{50}$  values. The *R81T genotype* had a significant effect on the  $LC_{50}$  (Chi-square = 27.5,  $P < 0.001$ ) as well as the interaction between *R81T genotype* and *CYP6CY3 expression* (Chi-square = 61.1,  $P < 0.001$ ). Holding *CYP6CY3* expression constant, the  $81^{RT}$  genotype increased the  $LC_{50}$  by 5.97 times compared to the  $81^{RR}$  genotype [95% CL (1.59–22.3)] while the  $81^{TT}$  genotype increased it by 19.2 times [95% CL (5.86–62.8)].

To investigate further the effect of the *R81T genotype* covariate, we compared the  $LC_{50}$ s obtained from thiacloprid bioassays with and without PBO of the different genotypes. The addition of PBO allows the effects of P450 detoxification enzymes (both direct effect and potential interaction with *R81T genotype*) to be removed. For the sake of simplicity, we assume that the addition of PBO allows the effects of P450 detoxification enzymes (both direct effect and potential interaction with *R81T genotype*) to be removed for all clones. This approximation is a common practice in this type of experimentation, even though PBO does not necessarily have the same effect on all clones.<sup>40</sup> The *R81T genotype* was confirmed to have a highly significant effect on the  $LC_{50}$ s with PBO ( $F = 105$ ,  $P < 0.001$ ). All pairwise comparisons between *R81T* genotypes were highly significant ( $81^{RR}$ – $81^{RT}$ ,  $81^{RR}$ – $81^{TT}$  and  $81^{RT}$ – $81^{TT}$ ,  $P < 0.001$  for all pairs). The estimated back transformed mean  $LC_{50}$  values with PBO were 55.4  $\mu\text{g/L}$ , 150  $\mu\text{g/L}$  and  $2.09 \times 10^3$   $\mu\text{g/L}$  for  $81^{RR}$ ,  $81^{RT}$  and  $81^{TT}$  genotypes, respectively. The  $LC_{50}$  with PBO was found to be 2.72 [95% CL (1.61–4.57)] times higher for the  $81^{RT}$  genotype than for the  $81^{RR}$  genotype. The  $81^{TT}$  genotype displayed a 37.7 [95% CL (20.0–71.1)] times and 13.9 [95% CL (6.99–27.6)] times higher  $LC_{50}$  with PBO than the  $81^{RR}$  and  $81^{RT}$  genotypes respectively (Fig. 3).

Thanks to the absence of a target resistance allele in the  $81^{RR}$  genotype clones, we were able to study in greater detail the relationship between *CYP6CY3 expression* level and the proportion of resistance due to this detoxification mechanism alone without the interference of target site resistance. The  $LC_{50}$ s for the  $81^{RR}$  genotype stayed low or moderate (between 34.4  $\mu\text{g/L}$  and  $3.10 \times 10^2$   $\mu\text{g/L}$ ), even though their *CYP6CY3* expression ratios display great variation from 0.26 to 17.12. The proportion of the  $LC_{50}$ s linked to detoxification, estimated as the proportion  $(LC_{50} - LC_{50-PBO})/LC_{50}$ , ranged from 0.20 to 0.82. Interestingly, this proportion seems to increase for low *CYP6CY3* expression levels and reach a plateau. By fitting a two parameter Michaelis–Menten model, the upper limit of the proportion of the  $LC_{50}$  linked to detoxification for infinitely large *CYP6CY3* expression level was estimated to be 0.793 (SE =  $\pm 0.086$ ), and a *CYP6CY3* expression level yielding a response halfway between 0 and the upper limit

was estimated to be 0.864 (SE =  $\pm 0.477$ ) (Fig. 4). The results of the models for the other detoxification covariates were qualitatively very similar (data not shown).

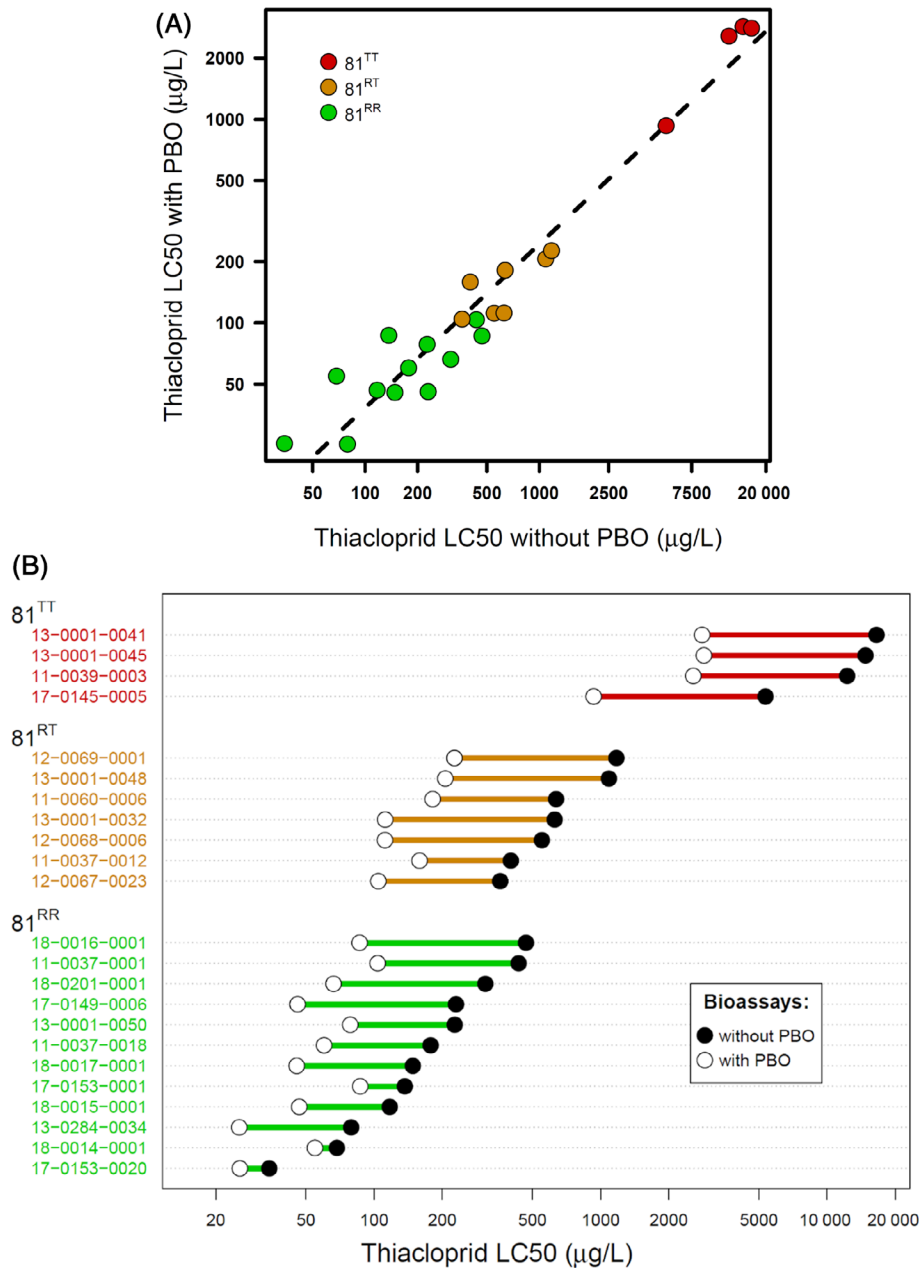
A similar modeling approach for the  $81^{RT}$  and  $81^{TT}$  genotypes was not possible because of the low number of clones and the absence of clones with low *CYP6CY3* expression level for these genotype categories (ranging from 6.16 to 22.67 and from 3.75 to 9.24 for  $81^{RT}$  and  $81^{TT}$ , respectively). Nonetheless, when representing these clones on the regression plot, all but one fall in the 95% confidence interval of the model, where it plateaus (Fig. 4).

## 4 DISCUSSION

Understanding the relative contribution of different mechanisms to insecticide resistance, and how these mechanisms interact, is essential to accurately predict the level of resistance associated with particular genotypes and to understand the evolutionary dynamics of resistance that can be observed in the field.<sup>63</sup> However, the interaction between multiple insecticide resistance loci has been the subject of a relatively limited number of studies, particularly for species of agronomic interest. In this study, we addressed this knowledge gap by investigating the interplay between two mechanisms in *M. persicae* that are associated with resistance to the neonicotinoid insecticide thiacloprid using both bioassays and biomolecular approaches.

We demonstrate a significant link between P450 expression level and thiacloprid  $LC_{50}$  values for a variety of *M. persicae* clones. Specifically, based on 12 clones carrying no *R81T* targetsite resistance allele, we have found a significant relationship between *CYP6CY3* expression and the proportion of resistance linked to metabolic resistance (Fig. 4). Expression of *CYP6CY3* and of *CYP6CY4* were found to be correlated ( $R^2 = 0.91$ ) in our study (Fig. S2), which is expected, as they are co-located on the same tandemly duplicated region of the *M. persicae* genome, suggesting that they may be coregulated.<sup>39</sup> This finding corroborates previous work, which employed a smaller number of *M. persicae* clones/genotypes.<sup>39,52</sup> Interestingly, the proportion of resistance linked to metabolic resistance was found to reach a threshold of 73%: above an expression level of five, whatever the *R81T* genotype, increasing the expression level of *CYP6CY3* does not increase the resistance level. High expression levels do not appear to increase neonicotinoid resistance, but its advantage might lie elsewhere, for example in protection against plant secondary metabolites such as nicotine in the aphid gut and bacteriocyte.<sup>39,64</sup> In our study, the *CYP6CY3* and *CYP6CY4* mechanisms, in the absence of the *R81T* mutation, confer a 6.8 resistance factor – a low to moderate level of resistance. This is similar to the level of *M. persicae* resistance against imidacloprid under field conditions described by Foster et al.,<sup>65</sup> which was not sufficient to compromise efficacy when applied at the recommended field rate. However, in a more recent study, while *CYP6CY3* overexpression was associated with only modest resistance levels in the laboratory, this mechanism was shown to affect the efficacy of neonicotinoid seed treatments on canola (*Brassica napus*) in semi-field trials.<sup>66</sup>

Interestingly, for both the combined NTSR and TSR model and the NTSR model, expression level proved to be a better predictor of thiacloprid  $LC_{50}$  values than gene copy number. This is likely due to the fact that other mutations have been shown to influence the expression of *CYP6CY3* that are independent of gene copy number, namely the presence of an expanded AC(n) microsatellite repeat in the promoter of this gene.<sup>52</sup>

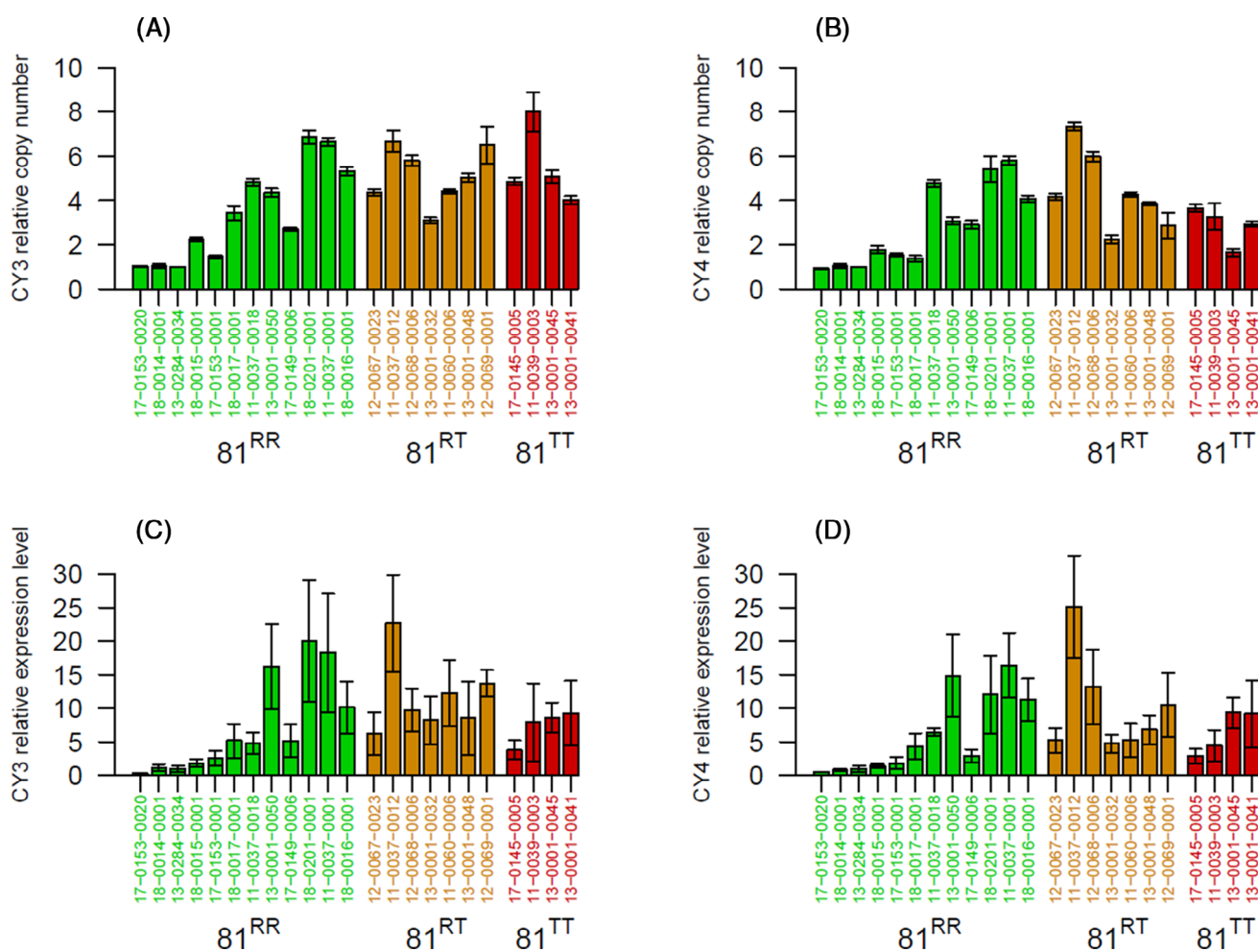


**Figure 1.** (A) Correlation between thiacloprid  $LC_{50}$  values with and without the P450 inhibitor piperonyl butoxide (PBO) (displayed using a logarithmic scale). Each dot represents a clone, in green  $81^{RR}$  genotypes, in orange  $81^{RT}$  genotypes and in red  $81^{TT}$  genotypes. (B) Dumbbell plot showing reduction of  $LC_{50}$  mean with the addition of PBO, by  $81T$  genotype and by growing order of  $LC_{50}$  without PBO. For each clone, a black dot represents the  $LC_{50}$  without PBO, a white dot the  $LC_{50}$  with PBO. Lines are in green for  $81^{RR}$  genotypes, in orange  $81^{RT}$  genotypes and in red  $81^{TT}$  genotypes.

In the case of the  $R81T$  mutation, our data indicate that the mutant allele 'T' is semi-recessive: dominance level ( $D_{LC}$ ) for allele 'T' comprised between 0 (full recessivity) and 0.5 (codominance), both when calculated on data obtained with thiacloprid ( $D_{LC} = 0.3$ ) and with thiacloprid + PBO ( $D_{LC} = 0.26$ ). These findings are consistent with those of a previous study.<sup>43</sup> Individuals homozygous for 'T' display high levels of resistance, when treated with thiacloprid + PBO their resistance factors are between 17 and 52-fold. These results are broadly in accordance with results obtained by Homem *et al.*<sup>67</sup> on CRISPR CAS9 modified *Drosophila melanogaster* possessing the  $R81T$  mutation in the homozygous form (without *CYP6CY3* amplification), which exhibited 6.5 and 32.6-fold resistance to the neonicotinoids acetamiprid and

imidacloprid respectively. In our study, *M. persicae* with this mutation in the heterozygous form had a moderate level of resistance, with resistance factors in thiacloprid assays with PBO ranging from 1.9 to 4.1. Such levels of resistance have been proven insufficient to resist field-dose applications of neonicotinoids.<sup>35,41</sup> Theoretically, individuals with one 'T' allele would survive only if retaining the association with P450 detoxification through *CYP6CY3* amplification, which increases resistance levels.

Together,  $R81T$  target site resistance in the homozygous state and metabolic resistance due to overexpression of *CYP6CY3* and *CYP6CY4*, confer high levels of thiacloprid resistance in *M. persicae*. There is a synergy between NTSR and TSR, indicated by: (1) the variation of the reduction of mean thiacloprid + PBO

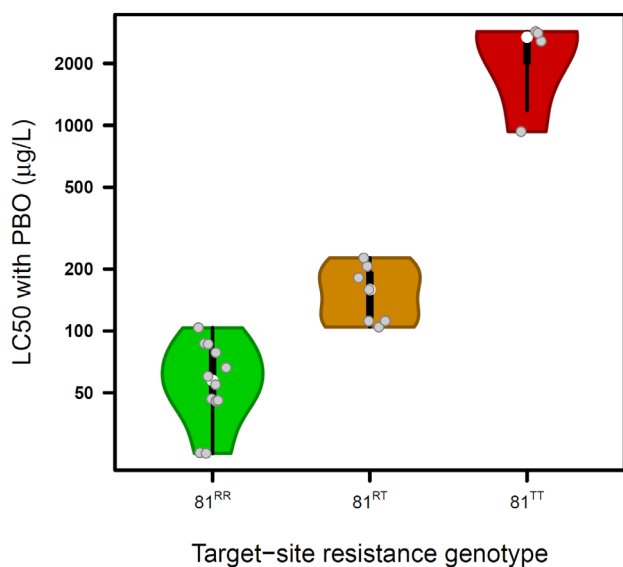


**Figure 2.** (A, B) Relative copy number (ratio with reference 13-0284-0034) for P450 resistance genes: (A) *CYP6CY3*, (B) *CYP6CY4*. Each bar represents a clone and each error bar the standard error, in green 81<sup>RR</sup> genotypes, in orange 81<sup>RT</sup> genotypes and in red 81<sup>TT</sup> genotypes. (C, D) Relative expression level (ratio to the susceptible reference clone 13-0284-0034) for each gene: (C) *CYP6CY3*, (D) *CYP6CY4*. Each bar represents a clone and each error bar the standard error, in green 81<sup>RR</sup> genotypes, in orange 81<sup>RT</sup> genotypes and in red 81<sup>TT</sup> genotypes. Clones are sorted from left to right by LC<sub>50</sub> values within each R81T genotype.

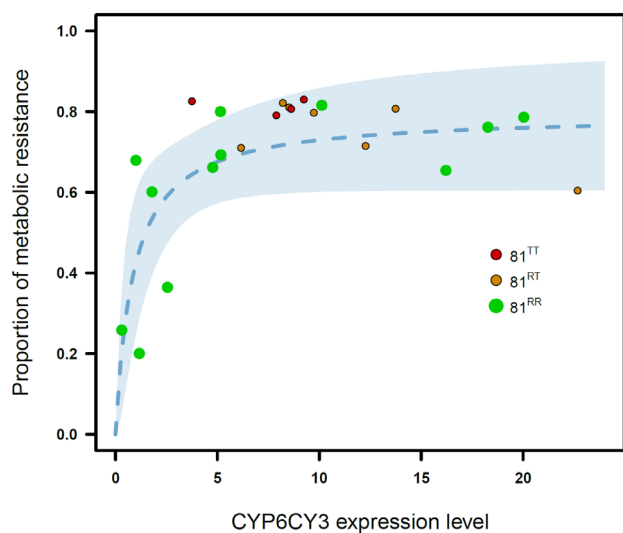
LC<sub>50</sub> values in between R81T genotypes compared to thiacloprid only LC<sub>50</sub> values (Fig. 1(b)), (2) the significant interaction between the covariates 'R81T genotype' and 'CYP6CY3 expression level' in Gaussian GLM models. This is similar to a multiplicative interaction between mechanisms, as described in Ref. 68 for DDT and pyrethroid resistance. Recent work using functional genomic approaches has also revealed synergism between metabolic and target site mechanisms conferring resistance to pyrethroids<sup>49</sup> in a model species. The contribution of synergism between P450 overexpression and target site mutation to thiacloprid resistant phenotypes is predominant compared to other factors in our model (Fig. 3). As hypothesized by Samantsidis *et al.*<sup>49</sup> for *D. melanogaster*, the reduced affinity between the active substance and the mutated target site might provide a greater amount of time to metabolize the active substance before irreversible toxicity. The target site mutation might also allow sufficient time for the induction of P450 enzyme expression.<sup>64</sup> Finally, the capacity of an individual carrying the mutant target site to withstand a higher concentration of the active substance might also allow a greater molar activity of the detoxification enzyme, approaching the concentration for which there is maximum degradation speed

(because the activity of an enzyme is dependent on the concentration of its substrate).

Surprisingly, the contribution of P450 metabolic resistance to the thiacloprid resistance phenotype in *M. persicae* was found to be highest in 81<sup>TT</sup> genotypes, where one might expect target site mechanism to play a predominant role. This is consistent with the hypothesis of the emergence of R81T target site resistance on a genetic background of P450 metabolic resistance.<sup>41,45</sup> This hypothesis is supported by the fact that individuals carrying R81T have, to date, always been found with this mutation in association with *CYP6CY3* overexpression (in our study, in Singh *et al.*'s dataset,<sup>39</sup> where the genomes of 127 clonal aphid lines were sequenced, and in other research).<sup>19,45</sup> This could be explained by the fact that while P450 metabolic resistance is insufficient to compromise efficacy when applied at the recommended field rate,<sup>65</sup> in agricultural environments, where spatial and temporal differences in the insecticide concentrations present in crop plants can occur, it could promote survival under some contexts. This in turn could facilitate the subsequent emergence of individuals carrying additional resistance mechanisms such as R81T.<sup>45</sup> Clones carrying the 81<sup>TT</sup> genotype in *M. persicae* were not found at high frequency in a recent analysis of 127 clonal aphid lines



**Figure 3.** Comparison of  $LC_{50}$  values with piperonyl butoxide (PBO) (displayed using a logarithmic scale) between R81T genotypes. Each grey dot represents a clone. The mean  $LC_{50}$  values with PBO are significantly different between R81T genotypes ( $81^{RR}$ - $81^{RT}$ ,  $81^{RR}$ - $81^{TT}$  and  $81^{RT}$ - $81^{TT}$ ,  $P < 0.001$  for all pairs).



**Figure 4.** Proportion of metabolic resistance ( $LC_{50} - LC_{50} + PBO$ )/ $LC_{50}$  as a function of expression level of the  $CYP6CY3$  gene. Only the  $81^{RR}$  genotypes were used for the Michaelis–Menten regression analysis. The blue envelope represents the 95% confidence interval of the model. The  $81^{RT}$  and  $81^{TT}$  genotypes were additionally plotted for illustration.

collected worldwide,<sup>24</sup> even in areas where the mutant 'T' allele is present, consistent with a fitness cost in the absence of insecticide. This is supported by the report of a high fitness cost associated with the 81T allele in the homozygous state in CRISPR CAS9 modified *Drosophila melanogaster*.<sup>67</sup> The association of R81T with  $CYP6CY3$  and  $CYP6CY4$ , even though on different chromosomes,<sup>24</sup> could be the result of this fitness cost for  $81^{TT}$  genotypes: by recombination due to sexual reproduction,  $81^{TT}$  individuals would appear, but would not thrive and be maintained.  $81^{TT}$  individuals would be counter-selected in the absence of insecticide and

individuals carrying  $81^{RT} + CYP6CY3/CY4$  overexpression would be favored. This is probably what can be observed on peach, where the 'T' allele has been predominantly found, with a strong genetic differentiation between primary/secondary hosts.<sup>23,24</sup> Interestingly, the highest expression levels of both  $CYP6CY3$  and of  $CYP6CY4$  are found in some  $81^{RR}$  and  $81^{RT}$  genotypes, but not in  $81^{TT}$  individuals. This could be linked to  $CYP6CY3$  copies outside of the tandemly duplicated genomic region that occur at varying frequency in different aphid lineages and particularly in clones without the 'T' allele.<sup>39</sup> This recent appearance of new  $CYP6CY3$  copies associated with the activity of transposable elements could be a further illustration of the specific role of transposons in adaptation to stress such as that imposed by pesticides.<sup>64,69–71</sup>

The focus of this study was on *M. persicae*'s resistance to one neonicotinoid in particular, thiacloprid, and caution is required in generalizing the results to other insecticides of the same family. However, a strong cross-resistance between thiacloprid and imidacloprid has been demonstrated,<sup>43</sup> mostly on the same clones as the ones used in this study, and cross-resistance between different IRAC Group 4 chemicals has been acknowledged between the closely related compounds imidacloprid, thiacloprid, thiamethoxam, acetamiprid, clothianidin, dinotefuran and nitenpyram,<sup>35</sup> suggesting our results may be applicable, to some extent, to these compounds.

We have discussed how P450 metabolic resistance and R81T targetsite mutation contribute to neonicotinoid resistance in *M. persicae*. However, we observed variability in  $LC_{50}$  values for thiacloprid in combination with PBO within each R81T genotype (Fig. 3): the two mechanisms and their interaction do not completely explain the variability of the phenotypes we observe, implying there may be other factors at play. One of these could be modulated regulation of the nicotinic acetylcholine receptor nAChR  $\beta 1$  subunit, which is the target for neonicotinoids, as reported in *Musca domestica*, *Nilaparvata lugens*, and *Leptinotarsa decemlineata* Say.<sup>15</sup> Beckingham et al.<sup>72</sup> also found down-regulation of neonicotinoid-sensitive receptors in a strain selected in the laboratory with neonicotinoid pressure. In our study, we have considered P450 metabolic resistance solely as the overexpression of  $CYP6CY3$  and  $CYP6CY4$ . Besides this quantitative aspect, qualitative changes have also been reported, such as mutations in the P450 sequence, provoking enhanced P450 activity, as described in Ref. 73 in the resistance of beet armyworm to avermectin. The activity of P450s can also be modulated by NADPH-cytochrome P450 reductase (CPR), because in the mono-oxygenation reaction, CPR is indispensable, as it functions as a unique electron transporter for almost all microsomal P450s. It is possible that the over-expression of CPR in insects could effectively enhance P450 metabolism of insecticides.<sup>74</sup>

Other detoxification enzymes play a minor role in the ability of certain *M. persicae* to feed on tobacco plants producing nicotine<sup>75</sup> and may also be involved in neonicotinoid resistance, such as GSTs and UDP-glucosyltransferases, that were identified as being differentially transcribed between neonicotinoid resistant and susceptible clones in previous transcriptomic analysis.<sup>38,41</sup>

## 5 CONCLUSION

This work has advanced understanding of the relative contribution of metabolic and target site mechanisms to neonicotinoid resistance in *M. persicae*. The first methods developed for the characterization of insecticide resistance in pest populations were bioassays. Molecular tools now allow us to better appreciate the

diversity and complexity of the underlying resistance mechanisms. We show that metabolic and TSR resistance mechanisms act in synergy in *M. persicae*. The challenge remains to understand not only the interactions between the different mechanisms, but also the evolutionary trajectories that lead to what we observe in the field.

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Zenodo at <https://doi.org/10.5281/zenodo.10960421>.

## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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