"The struggle between man and pests began long before the dawn of civilization, has continued without cessation to the present time, and will continue, no doubt, as long as the human race endures. It is due to the fact that both men and certain insect species constantly want the same things at the same time...Here and there a truce has been declared, a treaty made, and even a partnership established, advantageous to both parties to the contract—as with the bees and silkworms, for example; but wherever their interests and ours are diametrically opposed, the war still goes on and neither side can claim a final victory. If they want our crops they still help themselves to them...Not only is it true that we have not really won the fight with the world of pests, but we may go farther and say that by our agricultural methods, by the extension of our commerce, and by other means connected with the development of our civilization, we often actually aid them most effectively in their competition with ourselves."

S.A. Forbes (1915).

Promoters: **Prof. dr. ir. Thomas Van Leeuwen** Department of Plants and Crops Faculty of Bioscience Engineering Ghent University

Prof. dr. Nicky Wybouw

Department of Biology Faculty of Sciences Ghent University

Dean: Prof. dr. ir. Els Van Damme Rector: Prof. dr. ir. Rik Van de Walle

Molecular basis and fitness costs of xenobiotic resistance in the polyphagous pest *Tetranychus urticae*

Christine Njiru

Thesis submitted to Ghent University in partial fulfillment of the requirements for the

degree of Doctor (PhD) in Bioscience Engineering

Dutch translation of the title of this PhD:

Moleculaire basis en fitnesskosten van xenobiotische resistentie in de polyfage plaag *Tetranychus urticae*.

To refer to this thesis:

Njiru C. (2024). Molecular basis and fitness costs of xenobiotic resistance in the polyphagous pest *Tetranychus urticae*. Ghent University, Ghent, Belgium.

ISBN-number: 978-94-6357-711-3

The author and promoters give the authorization to consult and to copy parts of this work for personal use only. Every other use is subject to copyright laws. Permission to reproduce any material contained in this work should be obtained from the author.

The promoters,

The author,

prof. dr.ir. Thomas Van Leeuwen

Christine Njiru

prof. dr. Nicky Wybouw

Members of the examination committee	
Prof. dr. ir. Thomas Van Leeuwen	Department of Plants and Crops
	Faculty of Bioscience Engineering
	Ghent University, Belgium
Prof. dr. Nicky Wybouw	Department of Biology
	Faculty of Sciences
	Ghent University. Belgium
	,,
Prof. dr. ir. Wim Verbeke	Department of Agricultural Economics
	Faculty of Bioscience engineering
	Ghent University, Belgium
Prof. dr. ir Bartel Vanholme	Department of Plant Biotechnology and Bioinformatics
	Eaculty of Sciences
	Ghent University, Belgium
Prof. dr. Kristof De Schutter	Department of Plants and Crops
	Faculty of Bioscience Engineering
	Ghent University, Belgium
dr ir Astrid Bryon	Laboratory of Virology
	Department of Plant Sciences
	Wageningen University and Research.
	The Netherlands
dr. ir. Wannes Dermauw	Department of plants and Crops
	Faculty of Bioscience Engineering
	Ghent University, Belgium
	Plant Sciences Unit
	Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Belgium

Table of contents

Chapte	er 1: General introduction	1
1.1	Crop pests: the emerging threat to food security	2
1.2	Arthropod herbivores	4
1.	2.1 Host plant utilization	5
1.	2.2 Plant-herbivore interaction	5
1.3	Chemical control and resistance of arthropod pests	8
1.	3.1 Challenges associated with chemical pest control	8
1.	3.2 Resistance development	8
1.	3.3 The spread of resistance	9
1.	3.4 Mechanisms of resistance to xenobiotics	10
	1.3.4.1 Toxicodynamic mechanisms	11
	1.3.4.2 Toxicokinetic mechanisms	14
1.4	The two spotted spider mite <i>Tetranychus urticae</i> as a model of resistance	17
1.5	Research questions and general outline of this thesis	19
Chapte	er 2: A H258Y mutation in subunit B of the succinate dehydrogenase compl	ex of the
spider	mite <i>Tetranychus urticae</i> confers resistance to cyenopyrafen and pyflubun	nide, but
	the store st	
2.1	Abstract	22 22
2.2	Materials and methods	22
2.5	3.1 Acaricides and chemicals	25
2.	3.2 T urticae strains	25
2	3.3 Toxicity bioassays	20
2.1	3.4 Detection of candidate target-site mutations	
2	3.5. Single mite DNA extraction and genotyning	26
2	3.6 Marker assisted backcrossing	20
2.3	3.7 Genetic basis of resistance in the parental and congenic lines	
2.	3.8 In vitro activity assay of complex II	
2.	3.9 <i>T. urticae</i> SDH homology modeling and ligand docking	29
2.4	Results	30
2.4	4.1 Detection of H258Y mutation in <i>sdhB</i> of JPR-R	30
2.	4.2 Establishment of near-isogenic lines	
2.4	4.3 Toxicity bioassays	30
2.4	4.4 Genetic basis of pyflubumide and cyenopyrafen resistance in JPR-R and domin	ance of
th	e two resistance phenotypes after introgression	31

2.4.5 In vitro inhibition of succinate: ubiquinone oxidoreductase	33
2.4.6 Binding modes of cyenopyrafen-OH, cyflumetofen-AB1 and pyflubumide-NH to <i>T.</i> SDH model	urticae 35
2.5 Discussion	37
2.6 Conclusion	41
Funding information	41
Supplementary information	41
Authors' contributions	42
Chapter 3: The complex II resistance mutation H258Y in succinate dehydrogenase sub B causes fitness penalties associated with mitochondrial respiratory deficiency	ounit 43
3.1 Abstract	44
3.2 Introduction	44
3.3 Materials and methods	47
3.3.1 Mite lines and husbandry	47
3.3.2 Life history traits	47
3.3.2.1 Total development time, sex ratio and immature stage survivorship	47
3.3.2.2 Oviposition and adult longevity	48
3.3.3. Data analysis	48
3.3.4 Experimental evolution of mixed populations of Y258 and H258 genotypes in an acaricide-free environment	49
3.3.4.1 Proportional sequencing	49
3.3.4.2 H258Y TaqMan assay design	49
3.3.4.3 Droplet digital PCR (ddPCR)	50
3.3.5 In vitro activity assays of the mitochondrial complexes I, II and III	50
3.4 Results	52
3.4.1 H258Y does not influence single-generation life history traits and fertility lifetable parameters in <i>T. urticae</i>	52
3.4.2 Y258 allele frequency steadily decreases in H258/Y258 <i>T. urticae</i> populations	54
3.4.3 Activity of complex II is significantly reduced in Y258 lines of <i>T. urticae</i>	55
3.5 Discussion	56
3.6 Conclusion	60
Funding information	61
Supplementary information	61
Authors' contributions	62
Chapter 4: The host plant strongly modulates acaricide resistance levels to mitochone complex II inhibitors in a multi-resistant field population of <i>Tetranychus urticae</i>	drial 63
4.1 Abstract	64

4.2 Introduction	64
4.3 Materials and methods	66
4.3.1 <i>T. urticae</i> populations	66
4.3.2 Chemicals	66
4.3.3 Toxicity and synergism assays	66
4.3.4 Detection of target-site mutations	67
4.3.5 RNA isolation	68
4.3.6 RNA sequencing, mapping and principal component analysis (PCA)	68
4.3.7 Identification of viral contaminants	69
4.3.8 Differential expression analysis and Gene ontology (GO) enrichment analysis	69
4.4 Results	70
4.4.1 VR-BE has a multi-resistant profile on bean and tomato hosts	70
4.4.2 Target-site resistance mutations of VR-BE	72
4.4.3 PBO synergizes cyflumetofen toxicity on VR-BE tomato	72
4.4.4 RNA sequencing reveals presence of viruses in VR-BE bean and GSS populations	73
4.4.5 Effect of the host plant on gene expression	74
4.5 Discussion	77
4.6 Conclusion	81
Funding information	82
Supplementary information	82
Authors' contributions	84
Chapter 5: Intradiol ring cleavage dioxygenases from herbivorous spider mites as a	new
detoxification enzyme family in animals	85
5.1 Abstract	86
5.2 Introduction	86
5.3 Materials and methods	88
5.3 1 Mite strains and tomato cultivars	88
5.3.2 Spider mite DOG nomenclature	89
5.3.3 Confirmation of horizontal gene transfer and phylogenetic analysis of tetranycho	id DOGs 89
5.3.4 <i>T. urticae DOG</i> transcript analysis in different life stages, resistant strains and stra adapted/acclimatized to different host plants	ains 90
5.3.5 <i>T. urticae</i> DOG transcript analysis in response to tomato defense	91
5.3.6 In situ hybridization	92
5.3.7 DOG silencing by dsRNA injection	93
5.3.8 Functional expression of recombinant DOGs	94

5.3.9 Ferrozine assay	96
5.3.10 Spectrophotometry	96
5.3.11 Oxygen consumption	97
5.3.12 Identification of reaction products by UPLC-MS	97
5.4 Results	98
5.4.1 The evolutionary history of spider mite DOG genes	98
5.4.2 Transcriptional responses of <i>T. urticae</i> DOGs	100
5.4.3 Spatial expression pattern of <i>DOGs</i> in <i>T. urticae</i>	102
5.4.4 Effect of DOG silencing on mite performance on a challenging host plant	103
5.4.5 Functional characterization of spider mite DOG proteins	104
5.4.6 Substrate diversity of <i>T. urticae</i> DOGs	106
5.4.7 Metabolite identification	108
5.5 Discussion	110
5.6 Conclusion	115
Funding information	115
Supplementary information	115
Authors' contributions	118
Chapter 6: General Discussion	119
6.1 General overview	120
6.2 The complexity of resistance	120
6.2.1 Resolving the genetic basis of resistance	122
6.2.2 Target site mutation versus metabolic detoxification: What is the relative i	mportance?
	123
6.2.3 Pleiotropic fitness costs associated with resistance	128
6.3. A link between host plant adaptation and resistance	130
6.4 Beyond the classics: Evolution of xenobiotic adaptation	132
6.4.1 Other potential players in xenobiotic stress response	135
6.5 Future perspectives: Transferring laboratory knowledge to the field	136
References	139
Summary	169
Samenvatting	171
Acknowledgements	174
Curriculum vitae	176

List of Abbreviations

ABC	ATP binding cassete
ACCase	acetyl CoA carboxylase
AchE	acetylcholinesterase
ADP	adenosine diphosphate
APRD	arthropod pesticide resistance database
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
BSA	bulked segregant analysis
Cas9	CRISPR associated protein 9
CCE	carboxyl/choline esterase
cDNA	complimentary DNA
CHS1	chitin synthase 1
CRISPR	clustered regularly interspaced short palindromic repeats
cv	cultivar
СҮР	cytochrome P450 monooxygenase
Cytb	mitochondrial cytochrome b
DAMPS	damage associated molecular patterns
DCPIP	2,6-Dichlorophenolindophenol
ddPCR	droplet digital polymerase chain reaction
DEF	S,S,S-tributyl phosphorotrithiolate
DEG	differentially expressed genes
DEM	diethyl maleate
DEPC	Diethyl pyrocarbonate
DIG-UTP	digoxigenin-uridine triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOG	intradiol ring cleavage dioxygenase
dsRNA	double stranded RNA
DT	development time

EDTA	ethylenediaminetetraacetic acid
FADH ₂	reduced form of Flavin adenine dinucleotide
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GluCl	glutamate-gated chloride channel
GO	gene ontology
GST	glutathione-S-transferase
HAMPS	herbivore associated molecular patterns
HGT	horizontal gene transfer
IC ₅₀	inhibition concentration for 50% activity
IPTG	isopropyl β -D-1 thiogalactopyranoside
IR	inhibition ratio
IRAC	insecticide resistance action committee
ISH	in situ hybridization
ISS	Immature stage survival
β-ΚΑΡ	Beta-ketoadipate pathway
K _{cat}	catalytic constant
К _М	Michaelis constant
LC ₅₀	lethal concentration for 50% subjects
LM	finite rate of increase
LTP	life table parameters
METI-Is	mitochondria electron transport inhibitors of complex I
MFS	major facilitator superfamily
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MoA	mode of action
MRC	mitochondria respiratory chain
m/z	mass-to-charge ratio
NADH	reduced form of nicotinamide adenine dinucleotide
NCCR	NADH-cytochrome c oxidoreductase
NE	normalized expression
OCR	oxygen consumption rate

OCT	optimal cutting temperature
ORF	open reading frame
РВО	piperonyl butoxide
PBS	phosphate buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
РРО	polyphenol oxidase
PSST	NADH-ubiquinone oxidoreductase
qPCR	quantitative polymerase chain reaction
Q-site	ubiquinone binding site
QTL	quantitative trait locus
R	resistant
RH	relative humidity
rm	intrinsic rate of increase
RNA	ribonucleic acid
RO	reproductive rate
RR	resistance ratio
S	susceptible
SDH	succinate dehydrogenase
SDR	short chain dehydrogenases
SEM	standard error of the mean
SQR	succinate:ubiquinone oxidoreductase
SR	synergism ratio
т	mean generation time
TBS	tris-buffered saline
UDP	uridine diphosphate
UGT	UDP-glycosyltransferase
UPLC-MS	ultra-performance liquid chromatography - mass spectrometry
VGSC	voltage gated sodium channel
V _{max}	maximum velocity

Chapter 1: General introduction

1.1 Crop pests: the emerging threat to food security

Crops are constantly threatened by hordes of pests including arthropods, gastropods, nematodes, pathogens and weeds (Bebber et al., 2014; Pimentel and Peshin, 2014). Pests damage the crops by feeding and transmitting pathogens, causing significant yield losses (International plant protection convention, 2021). Historic and current examples clearly show the extensive damage that can be caused by pest outbreaks. For instance, the invasion and destruction of European vineyards by grape phylloxera (Daktulosphaira vitifoliae) at the beginning of the 1860s (Tello et al., 2019), or the rapid colonization of potato fields in the Atlantic coast and western European countries by the Colorado potato beetle (Leptinotarsa decemlineata) in the twentieth century. More recently, between the years 2016 and 2022, crop destructions to a magnitude of million metric tons have been reported across sub-Saharan Africa, Arabian peninsula and the Indian subcontinent as a result of the desert locust (Schistocerca gregaria) and fall armyworm (Spodoptera frugiperda) infestations (BBC Future, 2020; FAO, 2018; Paudel Timilsena et al., 2022; Showler, 2018). These trends in crop pest distributions show that, in comparison to the pre-industrial period, crop pests and the resulting crop losses have actually largely increased under modern agronomic practices (Andow and Hidaka, 1989; Pimentel and Peshin, 2014; Reddy and Zehr, 2004; Wang et al., 2022). For instance in China, one of the world's largest producers of major cereal crops such as rice, wheat and maize, the national average rate of crop pest occurrence has increased from 53% to 218% (4-fold) during the period of 1970 to 2016, and is projected to increase even more by the end of this century (Wang et al., 2022).

Considering the technological advancements made in the agricultural sector since the 1960's Green revolution, one might wonder why crop pests continue to pose a threat to modern agriculture. There are several reasons for this. **First**, compared to the pre-industrial period, pest dispersal has been greatly facilitated during the past decades by the trade of agricultural products such as plants, planting material, food and wood (Bebber et al., 2014). Indeed, some of the most damaging insect and mite pests of greenhouse crops are connected to the ornamental industry through importation of infected plant material (Albajes et al., 1999; Wang et al., 2017). Global human travel has also facilitated the movement of pests from their native environments. Even though international phytosanitary measures are undertaken by plant protection agencies and customs officers, some pests are still able to 'piggyback' on human transit routes (Paudel Timilsena et al., 2022). Minimal damage is often observed in the native ecosystem of a pest because coevolution with its host(s) has created a relatively stable balance between the antagonistic partners (Woolhouse et al., 2002). However, introduction of new pests to new ecosystems may cause serious damage due to lack of coevolution of the pest and the host (International plant protection convention, 2021).

Second, modern agricultural systems tend to simplify agroecosystems by expanding farmlands and encouraging monocultures, factors that facilitate pest outbreaks by reducing biodiversity. By the expansion of agricultural lands, marginal habitats that serve as refuge niches for beneficial species such as pollinators and predators of crop pests are destroyed (Pimentel and Peshin, 2014; Raven and Wagner, 2021). Monocultures reduce plant species richness, encouraging specialist pests to dominate and thrive. Additionally, monocultures lack crop rotation, which aggravates pest community buildup over multiple cropping seasons because the pests life cycle is not interrupted (Bullock, 1992).

Third, domestication and breeding practices to improve crop cultivars is another major factor in the modern agroecosystems that has led to an increase in pests. The process of crop domestication can be defined as selecting heritable traits that increase the economic output of a plant. The desired qualities often selected for include high yield, large size, high esthetic and nutritional quality. Unfortunately, selection for these improved qualities often comes at the expense of lower resistance to pests, as the genetic diversity of these improved varieties is reduced (Bevan et al., 2017; Chaudhary, 2013). For instance, while most hybrid tomato varieties are susceptible to the two spotted spider mite (*Tetranychus urticae*), a major pre-harvest pest of tomato, the wild relative of tomato *Solanum habrochaites* is highly resistant to this pest and other arthropods (Keskin and Kumral, 2015; Sami and Bayati, 2019). Similarly, larval performance and survival of the cotton bollworm *Helicoverpa armigera* is greatly reduced when feeding on wild relatives of chickpeas compared to the domesticated variety (Chaudhary, 2013).

Fourth, modern agroecosystems tend to intensify pesticide use in an attempt to control crop pests, a practice which encourages development of resistance. Currently, most crop pests are resistant to both natural and synthetic compounds, collectively referred to as xenobiotics. The term xenobiotics can be defined as "chemicals to which an organism is exposed that are extrinsic to the normal metabolism of that organism" (Croom, 2012). Resistance to xenobiotics form the core of this PhD, and will be discussed in details later in the chapter (see section 1.3).

Lastly, climate change is yet another major challenge to agriculture, affecting both the crops and pests. Theoretical models predict that global warming will lead to an expansion of the geographic ranges of pests, with an increased risk of introduction of alien invasive species (Skendži'c et al., 2021). For example the fall armyworm (*Spodoptera frugiperda*), has been confined to North and south America until early 2016 when outbreaks were first recorded in Benin, Togo and Nigeria (Goergen et al., 2016). Prediction models suggest that some areas such as Europe, currently classified as climatically unsuitable, may become suitable for fall armyworm invasion and establishment in the future, should global temperatures increase as projected (Paudel Timilsena et al., 2022). Increased incidences of plant

diseases vectored by pests are also expected to increase as a consequence of spread of pests to new geographic regions. Additionally, the effectiveness of biological control such as natural enemies is expected to reduce. In a study on the impacts of climate change on pest interaction with its host and natural enemy, Litskas et al., (2019) predict that by 2050, the spider mite *Tetranychus urticae* potential for outbreaks is likely to increase substantially in nine countries in Europe, Africa and Asia. Consequently, biological control failures using its key predator *Phytoseilus persimilis* would occur globally, and some regions currently ranked as top producers of tomato will become climatically unsuitable (Litskas et al., 2019).

To reduce yield loss resulting from damage by pests, studies recommend planning and formulating adaptation and mitigation strategies in the form of modified integrated pest management approaches. A general framework to quantify crop-yield loss risk is also recommended, and can be achieved by combining the crop pest occurrence data with crop damage intensity (Wang et al., 2022). Currently, accurate estimates of agricultural losses caused by pests are difficult to obtain because the damage caused depends on multiple factors such as environmental conditions, the cultivated plant species, and the control measures applied (Oliveira et al., 2014; Wang et al., 2022). Nonetheless, credible attempts have been made to assess the potential yield losses as a result of pests, with global estimates reaching 35-40% (Oerke and Dehne, 2004; Pimentel and Peshin, 2014; Popp et al., 2013). These percentages represent losses in the presence of control measures such as the use of pesticides, resistant cultivars and predators, without which yield losses would be much more worse, reaching 50-80% (Bruce, 2010; Flores-Gutierrez et al., 2020; Pimentel and Peshin, 2014).

1.2 Arthropod herbivores

The arthropod phylum is considered to harbor the most successful and diverse herbivorous animal species in terrestrial ecosystems (Labandeira, 2006; Schoonhoven et al., 2006; Wybouw et al., 2016), and consists of some of the most damaging pest species after fungi (Bebber et al., 2014). Arthropods account for approximately 18-20% of the global yield losses in agroecosystems (Arora and Sandhu, 2017; Flores-Gutierrez et al., 2020). Insects (in the subphylum Hexapoda) and mites (in the subphylum Chelicerata) account for majority of the damage caused by arthropod herbivores, with a much smaller proportion attributable to other members of arthropoda (Peshin, 2014).

Arthropod herbivores feed on the plant in several ways: (i) chewing, snipping or tearing the leaves, buds, stems, bark or fruit, (ii) piercing and sucking out the cell-content, xylem or phloem tissues (iii) boring into or tunneling through the bark, stems, twigs, fruits or seeds, or between upper and lower leaf surfaces, a process called mining (iv) feeding through gall formation or other plant distortions (Peshin, 2014; Wybouw, 2015). Arthropod clades are characterized by a defined feeding mode

(Wybouw, 2015). For instance, beetles and caterpillars (classified as insects) are chewing herbivores while true bugs (members of Hemiptera) pierce and suck the plant tissue. Plant feeding mites pierce and suck the cell content (Labandeira, 2006; Lindquist, 1998; Schoonhoven et al., 2006).

1.2.1 Host plant utilization

Plants vary in their nutritional composition, therefore herbivores are challenged with finding vegetative tissue that contains the optimal combination of carbohydrates and proteins (Le Gall and Behmer, 2014). Additionally, plants are equipped with chemical and/or physical defenses against herbivore attack (Behmer, 2008). To deal with these challenges, arthropod herbivores have evolved specific adaptations, leading to an evolutionary trend of specialization to specific host plants (Heckel, 2014) (Interactions between plants and herbivores is discussed in more detail in section 1.2.2). Arthropod herbivores can be crudely categorized as monophagous, oligophagous or polyphagous.

Monophagous arthropods (specialists) are restricted to a single or a few related plant species from a single genus. For instance the spider mite, *Amphitetranychus quercivorus*, is specialized on oak tree species (Migeon et al., 2010). Oligophagous herbivores are able to feed on a group of plants from the same family. For example the cabbage white butterfly, *Pieris rapae*, only infests cruciferous vegetables (Brassicaceae family) (Root and Kareiva, 1984). Polyphagous herbivores (generalists) are able to utilize many different unrelated plant species. Although rare, they can be found in diverse arthropod orders. For instance the dessert locust, *Schistocerca gregaria*, in the order Orthoptera; the green peach aphid, *Myzus persicae*, in the order Hemiptera; the fall armyworm, *Spodoptera frugiperda*, in the order Lepidoptera; and the two spotted spider mite, *Tetranychus urticae*, in the order Trombidiformes.

Compared to generalists, specialists tend to have a higher tolerance to their host plant allelochemicals, utilizing them for host plant selection or protection (Heckel, 2014). Since they encounter high levels of predictable toxins, specialists have often evolved efficient and specialized detoxification systems (Cornell and Hawkins, 2003). In lieu of a fine-tuned detoxification system, generalists have detoxification enzymes with a broader substrate specificity (Dermauw et al., 2013). The detoxification gene families are also often expanded, resulting in an extraordinary plasticity during herbivory (Grbić et al., 2011; Li et al., 2013; Pym et al., 2019; Snoeck et al., 2018; Wybouw et al., 2015). Moreover, neofunctionalization of gene copies and horizontal gene transfer often enhances genomic flexibility to facilitate diverse host usage by generalists (Heidel-Fischer et al., 2019; Prasad et al., 2021; Wybouw et al., 2021; Wybouw et al., 2016).

1.2.2 Plant-herbivore interaction

To defend themselves from herbivores, plants have evolved multiple strategies that deter the attacking herbivore from feeding. Plant defenses are versatile, consisting of constitutive defenses and defenses

that are only induced upon herbivore attack. Constitutive defenses include physical barriers (thorns, trichomes, waxy and/or tough leaves) or plant metabolites that are either toxic, repellent or both (Howe and Jander, 2008; Kortbeek et al., 2019). For example acylsugars, methyl ketones and terpenoids accumulate in trichomes of tomato and contribute to resistance against the spider mite Tetranychus urticae (Bleeker et al., 2012). In the antagonistic interactions between a herbivore and a plant, these constitutive defenses may render the plant unacceptable as a host plant. However, if constitutive defenses are unsuccessful in deterring the herbivore, feeding induces plant responses that can negatively impact the herbivore performance, leading to plant resistance (Rioja et al., 2017). The induced responses are triggered by the recognition of herbivore associated molecular patterns (HAMPs) or damage associate molecular patterns (DAMPs)(Frost et al., 2007; Schuman and Baldwin, 2016), and result in synthesis of phytohormones, defense products and volatiles that can induce resistance in neighboring plants (Rioja et al., 2017; Santamaria et al., 2018). Jasmonic acid is a principal and conserved regulator of induced plant defenses triggered by many insect herbivores such as aphids (Koramutla et al., 2014), thrips (Abe et al., 2009, 2008) and caterpillars (Yang et al., 2015). However, some herbivores such as T. urticae simultaneously induce both jasmonic acid and salicylic acid regulated defenses (Alba et al., 2015; Blaazer et al., 2018; Kant et al., 2004). JA and SA pathways can interact synergistically or antagonistically with each other and with growth regulating hormonal pathways such as ethylene (Pieterse et al., 2012). This hormonal cross-talk serves to customize defense responses to different attackers and to minimize wastage of resources on unnecessary defenses which don't harm the attacking herbivore (Thaler et al., 2012).

The hormonal controlled induced response by plants is referred to as pattern triggered immunity (PTI) and is successful in controlling pests that are susceptible to it. However, most herbivores have evolved mechanisms to overcome these plant defenses either by avoidance, metabolic resistance and/or host plant manipulation (suppression) (Blaazer et al., 2018). Avoidance refers to a behavioral strategy utilized by various arthropod herbivores to avoid feeding on defense compounds secreted by the plant. For example, the cabbage looper (*Trichoplusia ni*) cuts comparable trenches in plants containing latex producing cells or ducts exuding phloem sap (Dussourd, 2017). These trenches reduce exudation of plant defense compounds beyond the cuts, where the cabbage looper continues to feed (Dussourd, 2017). In addition to avoidance, metabolic resistance to plant defenses can occur in the form of metabolite modification, degradation and/or secretion (Després et al., 2007; Heckel, 2014). It is a common strategy in arthropod herbivores, especially specialists because they are adapted to a single or few closely related plants, and thus continuously encounter the same defensive compounds (Blaazer et al., 2018). Although induced expression of detoxification genes upon host plant change has been well documented in generalists such as *T. urticae* (Dermauw et al., 2013; Snoeck et al., 2018; Wybouw

et al., 2015; Zhurov et al., 2014), experimental evidence on metabolic detoxification of plant metabolites by generalists is minimal. Instead, it is hypothesized that generalists increase their fitness on different host plants by actively interfering with conserved defense signaling components (Ali and Agrawal, 2012; Kant et al., 2015). Defense suppression refers to sabotage of the plants molecular machinery (Blaazer et al., 2018), and some arthropod herbivores such as T. urticae are known to suppress their host plant defenses by secreting effector molecules (Blaazer et al., 2018; Hogenhout and Bos, 2011; Kant et al., 2015; Khan et al., 2018). How these effectors function in the host plant is still unclear, although they are thought to target plant proteins involved indirectly in the plants defense, but whose manipulation makes the plants more susceptible, otherwise referred to as susceptibility proteins (Blaazer et al., 2018). Work on aphids (Shih et al., 2022) and whiteflies (Naalden et al., 2021) uncovered the various ways in which effectors can manipulate host plant immunity. For example, Mp55, a salivary protein identified in the green peach aphid Myzus persicae reduces the production of reactive oxygen species and callose deposition when expressed in Arabidopsis (Elzinga et al., 2014). Similarly, the effector BtFer1 from the whitefly Bemisia tabaci suppresses hydrogen peroxide generated oxidative signals in tomato through its ferroxidase activity (Su et al., 2019). A summary of plant-herbivore interactions is illustrated in figure 1-1 below.



Figure 1-1: An illustration of plant-herbivore interactions. (i) The recognition of herbivore or damage associated molecular patterns (HAMPS and DAMPS) by pattern recognition receptors (PRRs) on the plant cell wall induces a pattern triggered immunity (PTI) characterized by phytohormone signaling. (ii) In response, herbivores evolved effectors to suppress PTI, resulting in effector triggered susceptibility (ETS). These effectors are produced in the salivary glands and may, depending on the feeding mode, be delivered in the plant cells via the stylet (Jonckherer et al., 2016). (iii) Plants evolved resistance genes, and the R gene products specifically recognize effectors and activate effector molecules which are nolonger recognized by the R gene product or suppress ETI. This figure is redrawn from (Kant et al., 2015, Jonckheere, 2018).

1.3 Chemical control and resistance of arthropod pests

1.3.1 Challenges associated with chemical pest control

Although other measures such as the use of biological control, cultural practices (for example crop rotation, use of resistant crop varieties, sanitation, manipulating harvest dates) or mechanical control measures such as cultivation or trapping are put in place to control arthropod pests, the major control method is based on the use of pesticides that interfere with essential biological processes such as nerve transmission, energy metabolism, lipid biosynthesis and molting. Unfortunately, due to their intensive and widespread use, resistance against most of the pesticides has emerged in many pest species worldwide (Mota-Sanchez and Wise, 2023). Worse still, the development of new effective pesticides is not 'a walk in the park'. Not only is it laborious and time consuming, but also expensive, not to mention the intensive risk assessment imposed by the regulatory framework before a chemical product can be registered in the market, and frequent withdrawal of previously registered products that no longer meet the set standards. This reduces the collection of currently available and future chemical products. It is therefore very important to safeguard the utility and efficacy of the currently available pesticides (Sparks and Nauen, 2015).

To achieve this, the insecticide Resistance Action Committee (IRAC) was established in the early 80's, with the aim of describing the different insecticides and acaricides mode of action (MoA) groups as well as updating the MoA classification (<u>https://irac-online.org/mode-of-action/classification-online/</u>). Currently, IRAC's classification scheme recognizes more than 25 different MoA groups and over 55 different chemical classes (IRAC international MoA working group, 2022; Sparks and Nauen, 2015). In addition to the IRAC database, the Arthropod Pesticide Resistance Database (APRD), is a systemic and comprehensive database of resistance reports dating back to 1914 to the present (<u>https://www.pesticideresistance.org/</u>) (Mota-Sanchez and Wise, 2023).

1.3.2 Resistance development

Multiple definitions of resistance exist (Bras et al., 2022; Pedra et al., 2004; Tabashnik et al., 2014; Zhang et al., 2012). Here, we refer to the definition proposed by WHO (1957) "resistance is the inherited ability of a strain to survive doses of a toxicant that would kill majority of individuals in a normal population of the same species." Resistance can evolve in the field, reducing pesticide efficacy in the control of a pest population. When this happens, resistance can have practical consequences for pest control. Resistance can also be confined to the lab, following selection for resistance in a laboratory setting. The genetic basis, mechanisms and extent of resistance is not necessarily the same in laboratory and field evolved resistance (Zhang et al., 2012). Cross-resistance occurs when selection

for resistance to one pesticide also leads to resistance to another pesticide to which the pest population was not earlier exposed. Multiple resistance occurs when a pest develops resistance to several compounds following exposure to pesticides of different MoA groups.

Arthropods frequently develop pesticide resistance from a combination of standing genetic variation and *de novo* mutations in either the target site genes or genes encoding metabolic resistance (Hawkins et al., 2019). It has also been suggested that pre-existing adaptations such as the ability to detoxify plant allelochemicals can prime arthropod pests to cope with pesticides, and therefore facilitate the development of pesticide resistance (Dermauw et al., 2013). Recent advances also suggest that epigenetic factors such as histone modification by DNA methylation can facilitate resistance development. For example, studies with Colorado potato beetle showed that insecticide exposure led to intergenerational patterns of DNA methylation at sites associated with cytochrome P450 detoxification genes, and these changes in DNA methylation reduced sensitivity to a different insecticide in the subsequent generations (Brevik et al., 2021).

1.3.3 The spread of resistance

The spread of resistance is dependent on factors such as the mode of inheritance, which can be monogenic or polygenic. Monogenic resistance is controlled by a single gene, and is thought to be more difficult to control in the field, especially if it is dominant. On the other hand, polygenic resistance depends on multiple genes, and may be lost when resistant individuals mate with susceptible genotypes (Roush and McKenzie, 1987). Another factor that determines the spread of resistance is the associated trait, as resistance alleles can be either recessive or dominant. When the resistant allele is recessive, resistance develops much slower because heterozygotes are still killed by pesticide application. Additionally, the spread of resistance can also be determined by population growth, as pests like T. urticae that have a short life cycle and high fecundity will require frequent pesticide application, and therefore will develop resistance much faster (Van Leeuwen et al., 2010). The initial allele frequency and the selection coefficient are also important determining factors. Here, a standing variant already occurring at a high frequency may increase faster even if it has a weaker selective advantage. In contrast, a single de novo mutation will only emerge if it has a high positive selection coefficient (Hawkins et al., 2019). Additionally, gene flow from adapted populations result in greater increases in resistance than gene flow from unadapted populations (Lewis et al., 2023). Lastly, the presence or absence of a fitness cost associated with a resistance mechanism can affect the spread of resistance. Here, the evolution and persistence of resistance is strongly impacted in a field setting if a resistant allele is associated with a fitness cost (Roush and McKenzie, 1987). The presence or absence of a fitness cost can be assessed by methods such as comparing single generation life history parameters of populations of interest (Prout, 1971; Roush and McKenzie, 1987), or rearing strains that

differ in the resistance mutation together in cages and following the alleles over time (Roush and Daly, 1990). Based on this approach, a fitness cost is said to be present when allele frequencies decrease (Hardstone et al., 2009). A more detailed assessment of fitness cost is covered in chapter three of this thesis.

1.3.4 Mechanisms of resistance to xenobiotics

Arthropod herbivores have developed strategies to cope with plant allelochemicals and synthetic pesticides. It is suggested that the targets, enzymes and transporters involved in host plant tolerance and pesticide resistance are primarily the same (Dermauw et al., 2018), although the target sites for pesticides are mostly neuroactive thus favouring fast action (Sparks and Nauen, 2015), while those of plant chemicals are diverse and can often have slower effects (Després et al., 2007; Heidel-Fischer and Vogel, 2015). Some targets are exactly the same, especially for the pesticides such as pyrethroids and nicotinoids whose chemical structures are comparable to the plant chemicals pyrethrin and nicotine (Després et al., 2007).

Adaptations to overcome toxic substances are traditionally classified based on their physiological manifestation as either: mechanisms of decreased sensitivity (toxicodynamic changes) such as target site insensitivity or mechanisms of decreased exposure (toxicokinetic changes) such as increased metabolism, transportation and excretion of the xenobiotic) (Feyereisen et al., 2015; Van Leeuwen and Dermauw, 2016). Behavioral adaptations are also recognized as a resistance mechanism in addition to toxicodynamic and toxicokinetic changes (Feyereisen et al., 2015). An illustration of the different mechanisms developed by arthropod herbivores to overcome xenobiotics is shown in figure 1-2 below.



Figure 1-2: Herbivore strategies to overcome xenobiotics. (i) The first step is a behavioral adaptation aimed at avoiding internalization of the toxic plant metabolite or pesticide. (ii) After internalization, the xenobiotic can be metabolized into a less toxic product by enzymes such as glutathione-s-transferases (GSTs) cytochrome P450 monooxygenases (CYPs), UDP-glycosyl transferases (UDPs) carboxylcholine esterases (CCEs) or intradiol ring cleavage dioxygenases (DOGs) and/or bound by transporter enzymes such as ABC transporters and excreted outside the cell. (iii) High concentrations of the xenobiotic

can also be safely stored in specialized internal compartments in a process called sequestration. (iv) Mutations in the target site of a toxin can also occur, leading to resistance. Resistance mutations have been uncovered in pesticide target sites such as; acetylcholinesterase (AchE), voltage gated sodium channel (VGSC), GABA gated chloride channel (RdI), glutamate gated chloride channel (GluCI), acetyl CoA carboxylase (Accase), chitin synthase (CHS) and mitochondria complexes. This figure is redrawn from (Wybouw, 2015).

In the context of adaptation and speciation, resistance can be classified based on the genotype. This category recognizes three types of mutations (i) mutations that change the coding sequence of a target/ detoxification gene, resulting in an altered gene product, (ii) mutations that affect the cis or trans regulatory element of a target/detoxification gene, and thus alter expression levels of these genes or (iii) whole target/detoxification gene duplication/deletion (Feyereisen et al., 2015). While a single mechanism can be attributed to resistance in some cases, resistance cases in arthropods are often complex, involving a combination of diverse mechanisms. In the next section, toxicodynamic and toxicokinetic mechanisms in insects and mites are further elaborated.

1.3.4.1 Toxicodynamic mechanisms

Toxicodynamic mechanisms mainly occur through non-synonymous point mutations at the target site that decrease the sensitivity of the target site. Target site mutations are very common in pesticide resistance but are rarely observed in the context of host plant adaptation, probably due to the multiple and unspecific mode of action of plant allelochemicals (Dermauw et al., 2018; Després et al., 2007). There are many examples of target site resistance to insecticides and acaricides of different MoA. For instance, mutations in the acetylcholinesterase (Ace) genes. Acetylcholinesterase (AchE) is a crucial enzyme that catalyzes breakdown of the neurotransmitter acetylcholine, leading to termination of synaptic transmission. Within the Acari, a single Ace gene has been reported in T. urticae (Grbić et al., 2011), while genomes of most insects have two genes (Ace1 and Ace2) except Diptera which have only one Ace gene (Ace 2) (Feyereisen et al., 2015). Already in the mid-1960s, a link was made between resistance to organophosphates (OPs) and reduced AchE sensitivity in T. urticae (Smissaert, 1964). Since then, more than a dozen mutations, either independently or in combinations, have been uncovered at different positions in the insensitive AChE from various arthropods (Feyereisen et al., 2015). For example, in Drosophilla, the four most frequent mutations (I129V, G227A, F290Y and G328A) alone or in combination, decrease sensitivity towards several OPs and carbamates (Menozzi et al., 2004). In spider mites, multiple mutations in the AchE gene of T. urticae, T. kanzawai, T. evansi and Panonychus ulmi correspond largely to mutation positions reported in insects (Bajda et al., 2015; Feyereisen et al., 2015). Besides contributing to resistance as reported in several studies (Khajehali et al., 2010; Kwon et al., 2010b), some mutations may also function as fitness modifiers, not contributing to the resistant phenotype themselves (Kwon et al., 2012). Moreover, loss of catalytic activity as result of a target site mutation can be restored via allele amplification and overexpression (Lee et al., 2015). An overview of multiple AchE mutations can be found in the ESTHER database (Lenfant et al., 2013).

Resistance to DDT and pyrethroids as a result of mutations in the voltage gated sodium channel (VGSC) is well documented. DDT and pyrethroids bind to and inhibit the closure of neuronal sodium channels, causing prolonged current which lead to repetitive nerve firing and eventually death of the pest (Vijverberg et al., 1982). The knockdown resistance caused by kdr and super-kdr mutations in the highly conserved regions of the VGSC protein were first reported in Musca domestica, and their impact has been reviewed thoroughly in (Dong et al., 2014; Rinkevich et al., 2013). To date, insensitivity to pyrethroids has been reported in at least 9 insect orders and in mites, with as much as 61 different mutations occurring in different combinations at various locations within VGSC identified (Dong et al., 2014; Feyereisen et al., 2015). Due to different aminoacid sequences in the VGSC binding pockets of insects and mites, the kdr and super-kdr mutations have not been reported in spider mite species except in T. evansi which carries the super-kdr mutation (Van Leeuwen and Dermauw, 2016). In spider mites, other mutations have been found at high frequencies and in many regions worldwide (Ilias et al., 2014; İnak et al., 2019; Kwon et al., 2010a; Simma et al., 2020; Wu et al., 2019). Marker assisted back-crossing of the T. urticae mutations F1538I + A1215D and L1024V revealed that both L1024V and F1538I+A1215D caused exceptionally strong resistance to bifenthrin, fenpropathrin and fluvalinate (Riga et al., 2017).

Compared to *Ace* and *VGSC* genes, **mutations in the GABA gated chloride channel gene (***Rdl***) are not as diverse (Feyereisen et al., 2015). The mutation A301S/G/N in the second membrane spanning domain, lining the ion channel pore has been identified in 27 species belonging to six insect orders, and has been associated with resistance to cyclodienes (endosulfan and dieldrin) (Feyereisen et al., 2015). Three orthologues of** *Rdl* **genes have been identified in the spider mite** *T. urticae***. Interestingly, none of the orthologues has Alanine at the conserved position 301 but instead, the resistance associated serine is found in the** *Tu_Rdl2* **and** *Tu_Rdl3* **genes or a histidine at** *Tu_Rdl1* **gene (Dermauw et al., 2012).**

Macrocylic lactones such as avermectins and milbemycins target the **Glutamate gated chloride channels** (**GluCls**) but also the GABA channels, although GluCls are thought to be the main target sites (Dermauw et al., 2012; Kwon et al., 2010). Compared to insects which have only one GluCl, six orthologs have been detected in the spider mite species (Van Leeuwen and Dermauw, 2016). The target site mutations G314D in GluCl1, I321T and G326E in GluCl3 have been reported in spider mites (Dermauw et al., 2012; Kwon et al., 2010; Xue et al., 2020) and functionally validated in various studies (Mermans et al., 2017; Riga et al., 2017; Xue et al., 2021). A novel substitution, V273I has recently been uncovered in *T. urticae* through QTL mapping (Villacis-Perez et al., 2022), but is yet to be functionally

validated. In *Plutella xylostella*, the substitution V263I (two amino acids away from V273I in GluCl3 of *T. urticae*) was recently validated by electrophysiology and CRISPR/Cas9, revealing that it confers high resistance to abamectin (Sun et al., 2023).

Target site mutations have also been detected in the **acetyl CoA carboxylase (ACCase)**, the target site of keto-enols. These include E645K which was associated with moderate resistance to spiromesifen in the white fly *Trialeurodes vaporariorum* (Karatolos et al., 2012) and A2083V which was also identified in a highly conserved region of the carboxyltransferase domain (CT domain) of the white fly *Bemisia tabaci* (Lueke et al., 2020) and later also in spirotetramat resistant aphids (Umina et al., 2022). Validation of A2083V using CRIPSR/Cas9 in *D. melanogaster* showed that the mutation results in high cross-resistance to the ketoenols spiromesifen and spirotetramat (Lueke et al., 2020). Two mutations A1079T and F1656L have been identified in the ACCase of the spider mite *T. urticae* (İnak et al., 2022a; Wybouw et al., 2019), but are yet to be functionally validated.

The target site of mite growth inhibitors (MGIs) such as clofentizine, hexythiazox and etoxazole was unknown until genetic mapping revealed a **mutation I1017F in the chitin synthase I gene** of *T. urticae*, thereby revealing the target site of these MGIs (Demaeght et al., 2014; Van Leeuwen et al., 2012). Functional validation revealed that the mutation alone can result in extremely high resistance levels to MGIs (Riga et al., 2017). After discovery of this mutation in mites, mutations at the same position have been found in insect species resistant to benzoylureas (lufenuron and buprofezin), for instance the mutation I1042M in *Plutella xylostella*, and functional validation of I1017F and I1042M with CRISPR/Cas9 gene editing further revealed that buprofezin and etoxazole share a MoA by directly interacting with chitin synthase (Douris et al., 2016).

Target site resistance has also been detected for compounds with a mite-specific MoA. These include **mitochondria electron transport inhibitors of complex I (METI-Is)** such as: pyridaben, tebufenpyrad and fenpyroximate which target complex I; cyflumetofen, cyenopyrafen, cyetpyrafen and pyflubumide which target complex II; bifenazate and acequinocyl which target complex III. Other compounds such as cyhexatin and fenbutatin oxide act by inhibiting mitochondria ATP synthase (complex V). The mutation H92R (*Yarrowia lipolytica* numbering) in the PSST subunit of complex I in *T. urticae* causes resistance to complex I inhibitors (Bajda et al., 2017). The mutations I260V/T in subunit B of succinate dehydrogenase (SDH, Complex II) and S56L in subunit C of SDH have been associated with cross-resistance to complex II inhibitors (Maeoka and Osakabe, 2021; Sugimoto et al., 2020). Resistance to complex III inhibitors is associated with target site mutations in the mitochondria encoded cytochrome b (*cytb*), which were uncovered following a case of maternally inherited resistance to bifenazate. Identification of the target site mutations also led to the discovery of the MoA of bifenazate (Van

Leeuwen et al., 2008). Mutations such as G132A, P262T and combinations of G126S + S141F/A133T/I136T in the conserved cd-1 and ef-helices of *cytb* have been linked to bifenazate resistance (Fotoukkiaii et al., 2019; Nieuwenhuyse et al., 2009; Van Leeuwen et al., 2012, 2008). An extensive review of the resistance mutations in spider mites can be found in De Rouck et al., (2023).

1.3.4.2 Toxicokinetic mechanisms

Toxicokinetic changes are mainly associated with differential expression of detoxification genes as a result of changes in cis or trans regulatory elements or due to gene duplication events. Three phases of detoxification are described at the physiological level. In phase I, the toxin is made more reactive and water soluble by functionalization with nucleophilic groups. In phase II, conjugation with endogenous metabolites occurs so as to increase polarity. In phase III, the metabolites are excreted (Kennedy and Tierney, 2012). The detoxification enzyme families that function in these phases are briefly discussed below.

Cytochrome P450 monooxygensases (CYPs) are mainly phase I enzymes. They make up one of the largest and functionally diverse gene family, with representatives in almost all living organisms. P450s are characterized by a heme-centered active site, and are predominantly active as monooxygenases (Dermauw et al., 2020b). In arthropods, P450 enzymes perform multiple functions such as hormone biosynthesis and metabolism of host plant chemicals and detoxification of pesticides. Six CYP clans are recognized in arthropods: CYP2, CYP3, CYP4, CYP20, CYP16 and mitochondrial CYPs (Dermauw et al., 2020b). CYP2 clan includes the most abundant spider mite CYP sequences while CYP3 and 4 clans are the most abundant in insects. Some members of CYP2 clan, especially CYP392 family responds strongly to acaricide selection and feeding to various host plants in spider mites (Van Leeuwen and Dermauw, 2016). In insects, the members of CYP3 clan, especially CYP6 family has been shown to metabolize natural and synthetic xenobiotics (Feyereisen, 2005). CYP4 clan is the least studied, and consists of highly diversified sequences. Members of this clan are inducible by xenobiotics although specialized functions have also been reported (Feyereisen, 2012). The members of the mitochondria CYP clan such as CYP302, CYP314 and CYP315 function in ecdysteroid metabolism while the functions of CYP16 and CYP20 clans is not yet known (Dermauw et al., 2020b).

Carboxyl/cholinesterases (CCEs) also mainly function as phase I enzymes, and have been shown to confer metabolic resistance via direct hydrolysis. Where the toxic compound is not efficiently metabolized, CCEs can also sequester the toxin from its target site to the fat body or haemolymph (Devonshire and Moores, 1982). In insects, CCEs are divided into 14 clades that spread over three classes based on their physiological and biochemical functions: dietary/xenobiotic detoxification (clades A-C), pheromone/hormone processing (clades D-H) and neurodevelopmental functions (clades

I-N) (Montella et al., 2012; Oakeshott et al., 2005). The neurodevelopmental class is expanded in *T. urticae*, with an additional two subclades J' and J'', and no CCEs have been identified in the classification of insect dietary CCEs (Grbić et al., 2011). In insects, resistance to organophosphates (OPs) and carbamates has been associated with increased metabolism by CCEs, which results from gene duplication or amplification (Cruse et al., 2023; Devonshire and Field, 1991; Wheelock et al., 2005). In spider mites, recombinant *T. urticae* CCE (CCEinc18) has been shown to metabolize the pyrethroid bifenthrin *in vitro* (De Beer et al., 2022a), demonstrating the importance of CCEs in pesticide detoxification.

Glutathione S-transferases (GSTs) can either function as phase I enzyme or in most cases, in phase II where they act on secondary products generated by other phase I detoxification enzymes (P450s and CCEs). As phase II enzymes in xenobiotic metabolism, GSTs catalyze the conjugation of reduced glutathione to electrophilic substrates, thus increasing their solubility (Mannervik, 1985; Pavlidi et al., 2018). Through their peroxidase activity, GSTs also play a role in protection against oxidative stress caused by insecticides (Pavlidi et al., 2018). Insect cytosolic GSTs are divided into six classes: delta, epsilon, omega, theta, sigma and zeta, with delta and epsilon GSTs frequently associated with resistance to organophosphates and organochlorines (Enayati et al., 2005; Feyereisen et al., 2015). In spider mites, 31 GST genes have been identified in *T. urticae*, 19 in the European red mite *Panonychus ulmi* and 37 in the Citrus red mite *Panonychus citri*, all grouped into four families: delta, mu, omega and zeta (Bajda et al., 2015). The involvement of GSTs in resistance has been evaluated using genetic methods (RNA interference, ectopic expression) or functional expression and analytical techniques (reviewed in Pavlidi et al., 2018).

UDP-glycosyltransferases (UGTs) are phase II enzymes that catalyze the conjugation of small lipophilic molecules to sugars, generating more hydrophilic compounds that can be easily excreted (Bock, 2016; Jancova et al., 2010). Over 300 UGTs have been identified in insects, with phylogenetic analysis revealing order-specific gene diversification and inter-species conservation (Ahn et al., 2012). Only members of UGT50 family are expressed in nearly all insects, and have been suggested to play multiple roles such as: detoxification, olfaction, endobiotic modulation and sequestration (Ahn et al., 2012; Bock, 2016). Chelicerates seem to have lost UGTs early in evolution, but *T. urticae* regained them after horizontal gene transfer from bacteria (Ahn et al., 2014). Moreover, the UGT gene family is specifically expanded in *T. urticae*, and strong transcriptomic responses have been found in host plant transfer and acaricide resistance (Ahn et al., 2014; Papapostolou et al., 2021; Xue et al., 2020; Zhurov et al., 2014). Recent studies have linked arthropod UGTs to detoxification of xenobiotics. For instance, eight recombinant UGTs from *T. urticae* were shown to glycosylate both plant secondary metabolites and

acaricides (Snoeck et al., 2019b). Similarly, Xue et al., (2020) recently showed that recombinant UGT11 from *T. urticae* could glycosylate the acaricides abamectin and milbemectin.

In addition to the classical detoxification gene families discussed above, membrane transporters play a crucial role in tolerance to xenobitics. The ATP binding cassete (ABC) transporters are integral membrane proteins of a large ABC superfamily. They utilize energy derived from ATP to transport a diverse array of substrates across lipid membranes (Merzendorfer, 2014). ABC transporters are divided into eight subfamilies (A to H), based on sequence similarity of the nucleotide binding domain which binds ATP (Merzendorfer, 2014). Subfamilies E and F contain soluble proteins that regulate the biosynthesis of proteins, and are highly conserved across insect species. The rest of the subfamilies A-D and G-H consist of integral membrane transport molecules and are functionally diverse, which reflects adaptive evolution to specific environmental conditions (Merzendorfer, 2014). Although ABC transporters have been extensively studied in bacteria and vertebrates for their role in drug resistance (Falguières, 2022; Luckenbach et al., 2014; Orelle et al., 2019), knowledge on their physiological functions in arthropods is still limited. In insects, alterations in the subfamily C genes are associated with resistance to Cry toxins from Bacillus thuringensis by reducing the binding affinity of these toxins to the brush boarder membrane vesicles in different lepidopteran species (Chen et al., 2018; Pardo-López et al., 2013; Xiao et al., 2014). Their involvement in xenobiotic metabolism in spider mites has been inferred from differential expression in acaricide resistant T. urticae populations (Dermauw et al., 2013; Papapostolou et al., 2021).

Major facilitator superfamily (MFS), another example of membrane transporters have also shown rapid and more profound transcriptional changes upon exposure to xenobiotics (Dermauw et al., 2013; Van Leeuwen and Dermauw, 2016). MFS are single polypeptide carriers that work in a symport-antiport system. Their role in transport of xenobiotics has been mainly studied in bacteria and fungi (Chen et al., 2017; Saier et al., 1999; Wan et al., 2021; Xu et al., 2014), hence further research is needed to elucidate the role of arthropod MFS transporters in metabolic detoxification.

16



1.4 The two spotted spider mite Tetranychus urticae as a model of resistance

Figure 1-3: The two-spotted spider mite Tetranychus urticae © Sander De Rouck

T. urticae is an example of an extreme generalist, with the ability to feed on over 1100 plant species belonging to more than 250 distinct plant families, including many major agricultural crops (Jeppson et al., 1975; Migeon et al., 2010). T. urticae can easily be recognized by two characteristic lateral black spots caused by accumulation of food waste in its digestive tract (Figure 1-3). When feeding, T. urticae uses its stylet to penetrate the leaf either in between epidermal pavement cells or through the stomatal opening without damaging the epidermal cellular layer, and sucks out the cell content within the leaf mesophyll (Bensoussan et al., 2016). This leads to necrosis and eventually leaf abscission. T. urticae is also known for its high potential to develop pesticide resistance. Currently, the pest ranks among the top 10 pesticide-resistant arthropods, with more than 500 resistance cases to 96 compounds reported (https://www.pesticideresistance.org/, Mota-Sanchez and Wise, 2023). The success of T. urticae as a polyphagous pest with the ability to rapidly develop resistance are partly attributable to characteristics such as: (i) arrhenotokous parthenogenesis reproduction. Being a haplodiploid species, virgin T. urticae females only produce haploid males while fertilized females produce both diploid females and haploid male offsprings with a 3:1 female to male sex ratio (Krainacker and Carey, 1990). This means that any mutations in the genome will manifest in the phenotype of the haploid males regardless of its dominance status, and in cases of resistance mutations, pesticide selection can quickly increase the frequency of the mutation. (ii) High fecundity and short life cycle. A single T. urticae female can lay up to 10 eggs a day on average (Macke et al., 2011), and a fertilized female can produce over 50 female off-springs in a life cycle that lasts between 8-12 days (Jeppson et al., 1975). This results in an exponential population growth and host plant overexploitation, therefore requiring frequent acaricide application to get rid of the mites. Persistent

acaricide use can promote resistance development (Croft and Van De Baan, 1988). However, it is important to note that fecundity and duration of the lifecycle can vary strongly depending on environmental factors (temperature, humidity, light and the level of predation) and inherent factors (genetic diversity, population density and fertilization) (Gotoh et al., 1993; Jeppson et al., 1975). (iii) Ability to overwinter. To survive unfavourable conditions, T. urticae goes into diapause, a period characterized by developmental arrest, suppression of metabolism, altered behaviour, increased stress tolerance and increased energy reserves (Bryon et al., 2013). Overwintering facilitates local persistence of a pest all year round. With the current global climate change crisis, warmer temperatures will lead to a higher survival rate as most pest species that go into diapause do not freeze to death. Thus, the expansion of the conditions that allow a pest to persist locally can be an important driver of pesticide resistance, as has been observed with the diamond back moth, Plutella xylostella (Ma et al., 2021). (iv) Quick adaptability to a new host plant. T. urticae is able to rapidly adapt to a new or less favourable host plant without correlated fitness costs compared to its ancestral host (Agrawal, 2000). Besides, long-term adaptation on a single host-plant does not markedly reduce T. urticae's genetic variation or its capability to subsequently adapt to a different host plant (Fry, 1989; Magalhães et al., 2007). Additionally, experimental evolution studies have shown that T. urticae can suppress plant defenses to enhance its host colonization (Alba et al., 2015; Blaazer et al., 2018). It has been suggested that, due to their ability to cope with diverse plant defense chemicals encountered during feeding, polyphagous herbivores such as T. urticae are pre-adapted to develop pesticide resistance (Alyokhin and Chen, 2017; Dermauw et al., 2013).

In addition to the characteristics described above, annotation of the *T. urticae* genome has revealed lineage-specific expansions of gene families that could aid in detoxification of xenobiotics (Grbić et al., 2011). These include cysteine peptidases, chemosensory receptors (Grbić et al., 2011), and genes coding for effector molecules and salivary proteins that aid in coping with plant defenses (Jonckheere et al., 2016; Villarroel et al., 2016), in addition to the detoxification gene families and transporters discussed earlier in section 1.3.4.2 (UGTs, CYPs, CCEs, GSTs and ABC transporters). Additionally, to expand its metabolic repertoire, *T. urticae* has incorporated multiple microbial genes in its genome (For an overview see Wybouw et al., 2018). These laterally acquired genes (also referred to as horizontal gene transfer (HGT) genes) have been linked to xenobiotic adaptation, pigmentation, diapause and predicted biochemical functions in vitamin B5 production, carbohydrate, lipid and folate metabolism (Wybouw et al., 2018). For instance, a laterally acquired gene from bacteria that codes for the enzyme beta-cyanoalanine synthase has been discovered in *T. urticae* (Wybouw et al., 2014). Recombinant expression of this enzyme in *E. coli* revealed that it could detoxify hydrogen cyanide, a toxic plant defense compound generated from hydrolysis of cyanogenic glucosides, to beta-

cyanoalanine (Wybouw et al., 2014). *T. urticae* is thus able to thrive on plants defended by cyanogenic glucosides. Other HGT genes such as UGTs (discussed earlier in section 1.3.4.2) and intradiol ring cleavage dioxygenases (DOGs) have been discovered in the genome of *T. urticae* (Grbić et al., 2011). Transcriptomic data suggests that DOGs are involved in metabolic detoxification of plant defense compounds and acaricides, as they have been found upregulated in acaricide resistant strains and upon host transfer (Dermauw et al., 2013). DOGs cleave between adjacent hydroxyl groups (ortho cleavage) of catecholic groups found in aromatic compounds. Schlachter et al., (2019) recently showed that a recombinant *T. urticae* DOG could split the aromatic ring of the model substrate catechol. Whether DOGs can metabolize complex aromatic ring structures found in plant defense compounds and acaricides is a subject of further research, and was investigated in this PhD.

1.5 Research questions and general outline of this thesis

Three major research questions were addressed in the various chapters of this thesis:

- 1) Which mechanisms underlie resistance to various acaricides used in the control of T. urticae?
 - a) What is the role of a novel target site mutation uncovered in a *T. urticae* population highly resistant to pyflubumide?
 - b) Which mechanisms underlie resistance to various pesticides in a multi-resistant population of *T. urticae*? Does the host plant have an influence on pesticide resistance?
- 2) How can we investigate whether a fitness cost is associated with a resistance mechanism?
- 3) What is the role of the laterally acquired intradiol ring cleavage dioxygenase gene family in spider mites?

In **chapter 2**, we characterize a novel amino acid substitution, H258Y, discovered in a laboratory strain of *T. urticae* that was highly resistant to pyflubumide. The mutation is located on a highly conserved histidine residue in subunit B of succinate dehydrogenase (SDH). Substitutions involving this residue in fungi are associated with resistance to multiple carboxamide fungicides. Using marker assisted back-crossing, *in vitro* mitochondrial assays, homology modeling and ligand docking, the relative contribution of H258Y to resistance and interaction of the mutation with three SDH inhibitors is investigated.

In **chapter 3**, various approaches are combined to investigate whether H258Y mutation (described in chapter 2) results in any fitness costs of the resistant populations.

In **chapter 4**, resistance mechanisms in a resistant field population of *T. urticae* are investigated. The population, VR-BE, was collected from a tomato greenhouse in Belgium where the grower had reported very low efficacy after treatment with various acaricides, resulting in crop failure. In the

laboratory, we maintained VR-BE on its original tomato host and the standard laboratory host, bean. Toxicity bioassays were carried out on the populations on both hosts so as to determine the resistance levels to acaricides from various mode of action groups. We also screened for target site mutations. Further, synergism bioassays were carried out to determine if metabolic detoxification is responsible for differences in toxicity observed between the bean and tomato population. Gene expression analysis was also carried out to identify the differentially expressed genes on both hosts.

In **chapter 5**, a multi-disciplinary approach is used to functionally characterize laterally acquired *T*. *urticae* intradiol ring cleavage dioxygenases (*DOG*) genes. First, we carried out a phylogenetic reconstruction to understand the evolutionary history of spider mite *DOGs*. Then, we investigated the interaction between *DOGs* and jasmonic acid defense in plants. We then determined the localization of *DOG* gene product using *in situ* hybridization and the effect of silencing some key DOGs on mite survival and fecundity on a challenging host. Lastly, the ability of DOGs to cleave plant metabolites was investigated using recombinant DOG enzymes.

In **chapter 6**, the findings from the four experimental chapters are integrated and discussed in the light of advances made in understanding complex resistance mechanisms, exploring the fitness costs associated with resistance and the connection between host plant adaptation and pesticide resistance. Potential resistance management strategies are also explored. Chapter 2: A H258Y mutation in subunit B of the succinate dehydrogenase complex of the spider mite *Tetranychus urticae* confers resistance to cyenopyrafen and pyflubumide, but likely reinforces cyflumetofen binding and toxicity

This chapter has been partially redrafted from:

Christine Njiru, Corinna Saalwaechter, Oliver Gutbrod, Sven Geibel, Nicky Wybouw and Thomas Van Leeuwen. (2022) A H258Y mutation in subunit B of the succinate dehydrogenase complex of the spider mite *Tetranychus urticae* confers resistance to cyenopyrafen and pyflubumide, but likely reinforces cyflumetofen binding and toxicity. Insect Biochemistry and Molecular Biology, 144: 103761

2.1 Abstract

Succinate dehydrogenase (SDH) inhibitors such as cyflumetofen, cyenopyrafen and pyflubumide, are selective acaricides that control plant-feeding spider mite pests. Resistance development to SDH inhibitors has been investigated in a limited number of populations of the spider mite Tetranychus urticae and is associated with cytochrome P450 based detoxification and target-site mutations such as 1260 T/V in subunit B and S56L in subunit C of SDH. Here, we report the discovery of a H258Y substitution in subunit B of SDH in a highly pyflubumide resistant population of T. urticae. As this highly conserved residue corresponds to one of the ubiquinone binding residues in fungi and bacteria, we hypothesized that H258Y could have a strong impact on SDH inhibitors toxicity. Marker assisted introgression and toxicity bioassays revealed that H258Y caused high cross resistance between cyenopyrafen and pyflubumide, but increased cyflumetofen toxicity. Resistance associated with H258Y was determined as dominant for cyenopyrafen, but recessive for pyflubumide. In vitro SDH assays with extracted H258 mitochondria showed that cyenopyrafen and the active metabolites of pyflubumide and cyflumetofen, interacted strongly with complex II. However, a clear shift in IC_{50s} was observed for cyenopyrafen and the metabolite of pyflubumide when Y258 mitochondria were investigated. In contrast, the mutation slightly increased affinity of the cyflumetofen metabolite, likely explaining its increased toxicity for the mite lines carrying the substitution. Homology modeling and ligand docking further revealed that, although the three acaricides share a common binding motif in the Q-site of SDH, H258Y eliminated an important hydrogen bond required for cyenopyrafen and pyflubumide binding. In addition, the hydrogen bond between cyenopyrafen and Y117 in subunit D was also lost upon mutation. In contrast, cyflumetofen affinity was enhanced due to an additional hydrogen bond to W215 and hydrophobic interactions with the introduced Y258 in subunit B. Altogether, our findings not only highlight the importance of the highly conserved histidine residue in the binding of SDH inhibitors, but also reveal that a resistance mutation can provide both positive and negative crossresistance within the same acaricide mode of action group.

2.2 Introduction

The two-spotted spider mite *Tetranychus urticae* is an important agricultural crop pest, well known for its polyphagous lifestyle and ability to feed on economically important crops. Conventional chemical formulations of acaricides remain the major control measure applied in many important crops, although biological control strategies are also being developed and implemented. However, *T. urticae* rapidly develops resistance, greatly reducing the efficacy of acaricide application (Dermauw et al., 2013; Van Leeuwen et al., 2010; Wybouw et al., 2019). Resistance can result from changes in the molecular target of the pesticide (toxicodynamic resistance), or from qualitative or quantitative

changes in the activity of major detoxification enzymes (including cytochrome P450s (CYPs), carboxyl choline esterases, glutathione-s-transferases and UDP-glycosyltransferases) that alter penetration, metabolism, sequestration and excretion of the pesticide (toxicokinetic resistance) (Kwon et al. 2015; Feyereisen et al. 2015; Van Leeuwen and Dermauw 2016; Balabanidou et al. 2018). The development of an efficient resistance management regime is therefore crucial, warranting regular introduction of acaricides with novel modes of action and in-depth studies of cross-resistance risks.

Mitochondrial respiration generates energy in the form of ATP. In this process, electrons flow through four transmembrane complexes (I, II, III and IV) to the final electron acceptor oxygen (Giovani et al. 2011). Stored energy in highly reduced substrates is liberated and used to pump protons from the mitochondrial matrix to the intermembrane space, creating a proton gradient that drives ATP synthase (complex V) to convert ADP to ATP (Hoeks et al., 2012). Complex II, otherwise known as succinate dehydrogenase complex (hereafter referred to as SDH) consists of 4 subunits (A, B, C and D), through which electron transfer from FADH₂ to ubiquinone occurs (Figure 2-1). Interference with mitochondrial respiration by inhibition of electron transport in one or more of the complexes is an effective mode of action of acaricides to control spider mite pests like T. urticae (Van Leeuwen et al. 2015). The first developed mitochondrial electron transport inhibitors (METI-Is) such as quinazolines, pyrimidinamines, pyrazoles and pyridazinones interfere with complex I functioning (Hollingworth and Ahammadsahib, 1995), while acequinocyl and bifenazate inhibit complex III (Nieuwenhuyse et al., 2009; Van Leeuwen et al., 2008). Recently, compounds specifically targeting complex II (hereafter referred to as SDH inhibitors) have also been developed and commercialized as acarcides. However, this mode of action has already been extensively used worldwide for the control of various agricultural pathogens, for instance carboxamide fungicides to control plant pathogenic fungi (reviewed in Fraaije et al. 2012). Compelling evidence from fungal organisms shows that SDH inhibitors target the highly conserved ubiquinone binding site (Q-site) formed by residues from subunits B, C and D of the SDH complex. SDH inhibitors interrupt mitochondrial respiration by blocking electron transfer from the iron-sulphur cluster found in subunit B to ubiquinone at regions overlapping with the ubiquinone site (Sun et al. 2005; Horsefield et al. 2006; Huang et al. 2006).

The acaricidal SDH inhibitors cyflumetofen, cyenopyrafen and pyflubumide (IRAC group 25) are used for selective spider mite control in agricultural crops (Nakahira 2011; Hayashi et al. 2013; Nakano et al. 2015) (Figure 2-1). Cyflumetofen and cyenopyrafen are beta-ketonitrile acaricides, while pyflubumide is structurally similar to carboxamide fungicides (Khalighi et al. 2014; Sugimoto et al. 2020). All three compounds are pro-acaricides, of which the active metabolites (cyenopyrafen-OH, cyflumetofen–AB1 and pyflubumide-NH) selectively and potently inhibit complex II activity (Nakahira 2011; Hayashi et al. 2013; Nakano et al. 2015; Furuya et al. 2017). LC₅₀ concentrations of the active
metabolite differ by more than 100-fold between spider mites and key insect species, showing high selectivity (Furuya et al., 2017; Hayashi et al., 2013; Nakahira, 2011). *In vitro* determined target site affinities (IC₅₀s) of cyenopyrafen between *T. urticae* and predatory mites were also markedly distinct and amount to about 10-50 fold difference (Nakahira, 2011). With cyflumetofen and pyflubumide metabolites, the IC₅₀s differed by more than 300 and 400 fold respectively between *T. urticae* and other organisms (insects and mammals) (Hayashi et al., 2013; Nakano et al., 2015). Together, this suggest that at least a part of the observed biological selectivity is due to different sensitivity of the target-site to this class of compounds.



Figure 2-1: Panel A shows the chemical structures of the three pro-acaricides. All structures were drawn using chemdraw v.19. Panel B shows a schematic of complex II, highlighting the major electron transfer reactions that occur in each subunit. Subunit B is coloured in green and the predicted position of the H258Y mutation is indicated with a red star. Panel C shows a partial protein sequence alignment of subunit B of *T. urticae* (London reference genome *tetur01g15710*) and other organisms (GenBank accession numbers for *G. gallus, H. sapiens, B. Taurus, D. melanogaster, U. maydis, Z. triciti, S. cerevisiae* and *E. coli* are NP_001264968.1, NP_002991.2, NP_001035573.1, NP_477101.1, XP_011386878.1, AER08702.1, NP_013059.1 and EFY1451087.1 respectively). Amino acids indicated in dark green shading show the conserved residues. The H258Y mutation detected in pyflubumide resistant strain JPR-R is shown in red. Mutations reported in fungal SDH inhibitors resistance (Fraaije et al., 2012) are indicated with an arrow and previously reported mutations in *T. urticae* (Sugimoto et al., 2020) are shown with a black rectangle.

The frequent and widespread use of fungicidal SDH inhibitors has led to resistance development in many field populations of pathogens (Avenot and Michailides, 2010). Studies have shown that multiple resistance mutations in fungal complex II subunits cause resistance to SDH inhibitor fungicides. The amino acid substitutions P225T/L/F, N230I or H272Y/R/L in subunit B of *Botrytis cinerea* SDH, all have been associated with resistance to carboxamide fungicides (Lalève et al., 2014). Although acaricidal SDH inhibitors belong to the most recently developed mode of action group for arthropod control, their frequent use in some regions has also led to resistance in *T. urticae* populations (Maeoka and

Osakabe, 2021; Sugimoto et al., 2020; Xu et al., 2018; Zhang et al., 2021). Coincidentaly, some multiresistant European populations of *T. urticae* showed cross-resistance to cyflumetofen and cyenopyrafen even before the products were used in the field (Khalighi et al., 2014). Molecular analyses and functional expression studies have suggested that metabolic resistance mechanisms via detoxification enzymes might be involved in resistance to cyflumetofen, cyenopyrafen and pyflubumide, with CYPs playing a major role (Khalighi et al. 2014; Khalighi et al. 2015; Fotoukkiaii et al. 2020). Fotoukkiaii et al., (2021) gathered evidence that points towards the involvement of *cis* regulatory variation of *CYP392A16* in pyflubumide resistance. Further *in vitro* validation showed that recombinant CYP392A16 metabolizes the active compound of pyflubumide (pyflubumide-NH metabolite), to a derivative that is not toxic to spider mites (Fotoukkiaii et al., 2021). Recently, resistance to SDH inhibitors has also been associated with target site resistance mechanisms, namely amino acid substitutions in the SDH subunits of *T. urticae* (Sugimoto et al. 2020; Maeoka and Osakabe 2021). The I260T mutation in subunit B confers resistance to cyflumetofen, while the co-occurrence of I260V in subunit B and S56L in subunit C were shown to cause high level cross-resistance between cyenopyrafen and pyflubumide (Sugimoto et al. 2020; Maeoka and Osakabe 2021).

In this study, we discovered a novel amino acid substitution (H258Y) in *sdhB* from T. urticae and functionally validated its role in resistance to SDH inhibitors. Using marker assisted back-crossing, the H258Y mutation was introduced into a susceptible genetic background, creating a set of congenic lines. The contribution to resistance was assessed by toxicity bioassays and the dominance of H258Y determined by reciprocal crosses. We subsequently carried out *in vitro* complex II activity assays to quantify acaricide mediated inhibition on extracted mitochondria with and without the H258Y mutation. Finally, homology modeling and ligand docking provided a deeper understanding on how the three acaricides interact with SDH, and how the resistance mutation influences these interactions differently.

2.3 Materials and methods

2.3.1 Acaricides and chemicals

The acaricides used in toxicity bioassays were commercial formulations of pyflubumide (Dani-kong 20 SC, Nihon Nohyaku Co., Ltd, Japan), cyflumetofen (Dani-saraba 20 SC, Otsuka AgriTechno Co., Ltd, Japan) and cyenopyrafen (Starmite 30 SC, Nissan Chemical Co., Ltd, Japan). Atpenin A5 of analytical purity was purchased from Enzo Life Sciences (USA), (EZ)-cyenopyrafen and cyflumetofen were purchased from Fluka (Germany). Cyflumetofen-AB1 metabolite was purchased from HPC Standards (Germany), whereas pyflubumide and pyflubumide metabolite NNI-0711-NH (hereafter referred to as pyflubumide-NH metabolite) were synthesized at Bayer AG (Germany).

2.3.2 T. urticae strains

The pyflubumide resistant strain JPR-R1 (hereafter referred to as JPR-R) was selected for high levels of resistance to pyflubumide from the JPR strain (Fotoukkiaii et al. 2020). JPR-R was maintained on potted bean plants sprayed with 100 mg/L pyflubumide. The susceptible strain London is a reference laboratory strain (Grbić et al. 2011). All strains were kept under laboratory conditions (25 °C, 60% relative humidity and 16:8 h light:dark photoperiod) and maintained on *Phaseolus vulgaris* cv. 'Prelude'.

2.3.3 Toxicity bioassays

Toxicity bioassays were performed on adult females using the standard method described by Van Leeuwen et al., (2004). Briefly, 20-30 young (2-5 days old) adult females were placed on the upper side of a 9 cm² kidney bean leaf discs prepared on wet cotton wool. Using a spray tower (Burkard Scientific, UK), plates were sprayed with 800 µl of at least 5-8 serial dilutions of each acaricide and a control (distilled water) at 1 bar pressure to obtain a homogenous spray film (2 mg deposit/cm²). Four replicates were used for each acaricide dose. Mortality was scored after 24 hours. When 5000 mg/L acaricide did not cause 50% mortality, higher concentrations were not tested. LC₅₀ values, slopes, resistance ratios (RR) and 95% confidence limits were calculated by probit analysis using Polo Plus 2.0 software. Mortality was corrected using Abbott's formula (Abbott, 1925).

2.3.4 Detection of candidate target-site mutations

Two years after the genetic and functional characterization of pyflubumide resistance in JPR-R (Fotoukkiaii et al. 2020; Fotoukkiaii et al. 2021), we re-evaluated the *sdh* genotypes and screened for target-site substitutions in the *sdhB*, *sdhC* and *sdhD* subunits, previously implicated in inhibitor binding. Genomic DNA was extracted from approximately 200 adult female mites of JPR-R. Quality and quantity of DNA were checked via a spectrophotometer (DeNovix DS-11 (DeNovix, USA). PCR amplification of the *sdhB* (*tetur01g15710*), *sdhC* (*tetur30g00210*) and *sdhD* (*tetur20g00790*) regions was carried out as described in Fotoukkiaii et al. (2020). The primer pairs are outlined in supplementary Table S2-1. PCR products were purified using E.Z.N.A Cycle pure kit (Omega Bio-tek, USA) and sequenced by LGC Genomics (Germany). To identify mutations, nucleotide sequences were visualized in SnapGene Viewer version 5.1.6 and aligned to London reference genome using BioEdit version 7.2.

2.3.5 Single mite DNA extraction and genotyping

To genotype single mites for H258Y mutation, single adult females were homogenized in 20 μ I STE buffer (10 mM Tris-HCL, 100 mM NaCl, 1 mM EDTA, pH 8) and 2 μ I (10mg mL⁻¹) proteinase K (Sigma-Aldrich, Belgium). Homogenates were incubated at 60 °C for 30 min followed by proteinase K

inactivation at 95 °C for 5 min. DNA from single mites was screened for the H258Y mutation by standard PCR and sequencing. The PCR components were; 5 μ l 10x PCR buffer, 1.5 μ l of 50 mM MgCl₂, 1 μ l (0.2 mM each) of 10 mM dNTP mix, 1 μ l (0.2 μ M) of 10 μ M forward and reverse primers (5'-ATCGATTCAGCCTTGGCTAC and 5'-CTTAGACCAGCCTGCGAGAA respectively), 1 μ l single mite DNA extract, 0.2 μ l platinum Taq DNA polymerase (Thermo Fisher Scientific, USA) (2 U/reaction) and nuclease free water (Integrated DNA Technologies, USA) to a total volume of 50 μ l. The thermocycler conditions were: initial denaturation at 94 °C for 2 min, 40 cycles of 94 °C for 30 s,55 °C for 30 s, 72 °C for 30 s and a final extension of 72 °C for 2 min. Amplification was confirmed by running 5 μ l PCR product on 2% agarose gel at 100 V for 30 min. Gels were examined for the presence of a band using ethidium bromide staining and visualized in a gel imager. PCR products were purified using E.Z.N.A [®] Cycle pure kit (Omega Bio-tek, USA) and sequenced at LGC Genomics (Germany). Sequencing data was visualized using SnapGene Viewer version 5.1.6.

2.3.6 Marker assisted backcrossing

To assess the relative contribution of H258Y to resistance to SDH inhibitors, a marker assisted backcrossing approach was used to generate six near-isogenic sister lines (three with the H258Y substitution and three respective controls). Prior to the backcrossing, *sdhB* genotypes were confirmed in the resistant JPR-R and the susceptible London strains. DNA extracted from a pool of 200 mites was used to screen for H258Y mutation by PCR and sequencing as described in section 2.3.5 above. After confirming that the susceptible strain did not carry the mutation, backcrossing was carried out as described in Bajda et al., (2017). Briefly, a haploid male of the resistant JPR-R strain was crossed with a diploid virgin female (teleiochrysalid) of the susceptible London strain. Heterozygous virgin F₁ females were backcrossed to London males and F₂ females heterozygous at the *sdhB* locus were identified by PCR and sequencing (see description in 2.3.5 above). The process was repeated for eight sequential generations. In the last generation, heterozygous virgin females were crossed to their first born sons to generate either H258/H258 (S) or Y258/Y258 (R) genotypes. All paired near-isogenic lines were allowed to expand into larger populations on unsprayed bean leaves.

2.3.7 Genetic basis of resistance in the parental and congenic lines

The mode of inheritance for pyflubumide and cyenopyrafen resistance was determined in the JPR-R strain by performing reciprocal crosses with the susceptible London strain. The mode of inheritance of pyflubumide and cyenopyrafen resistance was also determined by reciprocal crosses between the H258/H258 (S) and the Y258/Y258 (R) genotypes of the three introgressed lines. For each cross, 60 teleiochrysalid females and 30 adult males were placed on leaf discs and allowed to mate after which the females were transferred to new leaf discs every two days throughout their oviposition period.

Toxicity bioassays for F_1 generation ($\delta S \times \sigma R$ and $\delta R \times \sigma S$ crosses) were carried out as previously described (see 2.3.3). The degree of dominance was calculated using Stone's formula (Stone, 1968). To obtain F_2 females, 80 F_1 teleiochrysalid females of $\delta S \times \sigma R$ cross were backcrossed to either R or S males. Due to the differences in the mode of inheritance of pyflubumide and cyenopyrafen resistance (recessive and dominant, respectively), the F_1 teleiochrysalid females were crossed to R males for tests with pyflubumide or to S males for tests with cyenopyrafen. Toxicity bioassays for F_2 females were also carried out as previously described (see 2.3.3), using 8-13 acaricide concentrations. The expected dose-response relationships for F_2 females for monogenic resistance was calculated using the formula C = 0.5 W (F1) + 0.5 W (R or S) where C is the expected mortality at a given dose and W is the observed mortality of the parents at that concentration (Georghiou, 1969). The hypothesis of a monogenic mode of inheritance was tested with X² goodness of fit test.

2.3.8 In vitro activity assay of complex II

To measure the differences in binding curves between the three SDH inhibitors and two SDHB protein variants, biochemical assays were carried out using mitochondria purified from the resistant strain JPR-R and the susceptible London strain. Mitochondria were extracted following a modification of the protocol described by Schägger and von Jagow., (1991). Briefly, 2,000 adult female spider mites were flash frozen in liquid nitrogen and homogenized in 2 mL phosphate free MOPS-sucrose buffer pH 8.0 (440 mM sucrose, 20 mM MOPS, 1 mM EDTA and protease inhibitor) for 1 min on ice at 800 rpm in a Dounce homogenizer. The suspension was centrifuged twice at 1000 g and 4 °C to remove cell debris. The supernatant was centrifuged twice at 15000 g for 30 min to sediment mitochondria with enzymatically active membrane protein complexes. Protein concentrations were determined by BCA protein assay with BSA standards. Aliquots of mitochondrial pellets were stored at -80 °C. For complex Il activity assay, Atpenin A5, EZ-cyenopyrafen, cyflumetofen and its metabolite AB-1, pyflubumide and its metabolite pyflubumide-NH were dissolved in DMSO to 10 mM and 100 μ M stocks. Subsequent 1:5 dilution series in DMSO (v/v) with seven concentrations were used to determine inhibitory effects on complex II activity, with each inhibitor represented in two independent dilutions spread out in a randomized layout in clear 384 well microtiter plates (Greiner Bio-one, Austria). In vitro activity of succinate: ubiquinone oxidoreductase (SQR) was assessed using a modification of the protocol by Spinazzi et al., (2012). Testing of mitochondrial pellets from JPR-R and London always took place in parallel on each plate. In summary, mitochondrial pellets were thawed on ice and diluted in enzyme buffer (20 mM succinate, 100 μM EDTA, 1 μM myxothiazol, 300 μM NaCN, 1 mg mL⁻¹ BSA in 25 mM potassium phosphate buffer pH 7.5) to a final concentration of 25 μ g mL⁻¹. 40 μ L (1 μ g well⁻¹) were transferred in clear 384 well microtiter plates and incubated for five minutes at room temperature. Then, 5 μ L of pre-diluted compounds (1: 8,33 (v/v) in 25 mM potassium phosphate buffer, pH 7.4 with

1% DMSO (v/v)) was added to each well followed by ten minutes of incubation at room temperature to activate complex II. Reactions were initiated by adding 15 μ L of the soluble electron accepting agents (50 μ M decyl-ubiquinone,150 μ M DCPIP in potassium phosphate buffer, pH 7.4). Absorption changes ($\lambda = 600$ nm, 25 flashes s⁻¹, 30s intervals for 30 min) were measured in a M1000 Pro microplate photometer (Tecan Group, Switzerland) and wells without mitochondria served as negative controls. Saturation of complex II-mediated DCPIP reduction was reached after 25 min. Absorption decrease between three and seventeen minutes was linear ($R^2 > 0.9$) and considered in the final analyses. Inhibitor concentrations causing 50% inhibition (IC_{50}) were calculated in GraphPad Prism version 9.1.2 (Graphpad Software, Inc., USA). Raw data was expressed as specific activities (nanomoles DCPIP reduced per mg protein per minute) with the molar extinction coefficient of DCPIP ($\epsilon = 19.1$ mM⁻¹ cm⁻¹). Data were normalized to wells only containing solvent (< 1.7% DMSO (v/v)) and therefore referring to 100% activity. 100% inhibition was set as 0 nmol mg⁻¹ min⁻¹ specific activity. A total of 168 technical replicates from three independent biological replicates each were averaged.

2.3.9 T. urticae SDH homology modeling and ligand docking

All modeling steps were undertaken using the Maestro program suite (Schrödinger Release 2021-3: Maestro, Schrödinger, LLC, New York) (Jumper et al., 2021). Sequences of SDH subunits B, C and D from *T. urticae* were accessed from ORCAE database (Sterck et al., 2012). The corresponding gene identities for the subunits are *tetur01g15710*, *tetur30g00210* and *tetur20g00790*, respectively. After BLAST homology search, knowledge-based ligand docking was performed using the split succinate dehydrogenase subunit 3D-models from *Gallus gallus* with bound thiapronil (PDB ID: 6MYR; Huang et al. 2021) as a scaffold. The single chain homology models (individual identities of 74%, 37% and 42% for SDH subunits B, C and D after alignment) were merged to create the projected, thiapronil binding *T. urticae* holoenzyme. Subsequently, heme and iron sulfur clusters were introduced in the homology model from the original 3D structure of PDB ID: 6MYR. Maestro's Protein Preparation wizard was used to correct for atom types (heme, iron-sulfur-cluster, and co-substrates) and the addition of hydrogen atoms. Finally, a structural refinement was performed within the protein preparation wizard of Maestro using the force field OPLS4.

Structure models of the active metabolites cyenopyrafen-OH, cyflumetofen-AB1 and pyflubumide-NH were constructed in Maestro via the 2D-Sketcher option and superimposed on thiapronil according to their common functional groups (nitrile and carbonyl or hydroxyl pharmacophores) and rigidly docked into the catalytic ubiquinone binding site. Another refinement with OPLS4 and solvation model VSGB was initiated to ensure realistic binding conditions. 15 adjacent amino acid residues of SDH subunits B, C and D in vicinity of 4 Å were identified and examined for steric interactions with the respective acaricides. The H258Y substitution found in subunit B of JPR-R SDH was introduced *in silico*.

2.4 Results

2.4.1 Detection of H258Y mutation in sdhB of JPR-R

Sanger sequencing did not uncover candidate resistance mutations in *sdhC* and *sdhD*. However, a H258Y (*T. urticae* numbering) mutation in *sdhB* was fixed in JPR-R and absent in the London reference strain (sequence alignments are provided in supplementary Files S2-1a,b, c and d). This histidine residue is highly conserved across the tree of life, and is predicted to be at the ubiquinone binding site within the cysteine-rich cluster III in fungal organisms (Figure 2-1; Horsefield et al. 2006; Sierotzki and Scalliet 2013). Therefore, we suspect that H258Y could play an important role in *T. urticae* resistance to SDH inhibitors.

2.4.2 Establishment of near-isogenic lines

H258Y arose in the JPR-R genetic background that possesses multiple genetic factors that contribute to pyflubumide resistance Fotoukkiaii et al., (2021). In this study, we assessed the contribution of the H258Y mutation in pyflubumide, cyenopyrafen and cyflumetofen resistance by uncoupling it from other resistance variants of JPR-R. This was accomplished by marker assisted backcrossing into a genetic background that was susceptible to all SDH inhibitors. Three independent back-crossing experiments were carried out between JPR-R and the susceptible London strain. After eight rounds of backcrossing and a final cross between the heterozygous backcrossed females and their sons, three lines that were homozygous for the H258Y mutation (R1,R2 and R3; Y258/Y258 genotype); and three congenic control wild-type lines (S1, S2 and S3; H258/H258 genotype) were obtained.

2.4.3 Toxicity bioassays

The toxicity of pyflubumide, cyenopyrafen and cyflumetofen in the parental strains (London and JPR-R) and the introgressed lines (R1,R2,R3,S1,S2,S3) is presented in Table 2-1. The Y258/Y258 genotype (R) lines exhibited high levels of resistance to pyflubumide (LC₅₀ values between 400 and 700 mg/L and resistance ratios (RR) ranging between 120 to 198 fold compared to the control lines). In addition, the Y258/Y258 genotypes displayed very high resistance to cyenopyrafen (LC₅₀s >5000mg/L and RRs >500 fold compared to the control lines). Strikingly, the Y258/Y258 genotypes were susceptible to cyflumetofen (LC₅₀s <20mg/L and RRs <0.5 fold), a strong indication that the H258Y mutation does not contribute to cyflumetofen resistance. All the control lines (H258/H258 genotype) were susceptible to all three acaricides. Overall, our results strongly suggest that the H258Y substitution contributes to high cross-resistance levels between cyenopyrafen and pyflubumide, but not cyflumetofen.

Table 2-1: Probit mortality data of cyenopyrafen, cyflumetofen and pyflubumide. The LC_{50} values presented in the table are in mg/L. The resistance ratio (RR) is calculated as the ratio of LC_{50} values between resistant and susceptible lines. i.e JPR-R vs London for the parental lines, R1 vs S1, R2 vs S2 and R3 vs S3 for the congenic lines. LC_{50} values >5000 means that 50%

mortality was not achieved at this acaricide dose and concentrations higher than 5000mg/L were not tested. All values are rounded off to two significant figures.

Line	e Cyenopyrafen			Cyflumetofen			Pyflubumide		
	LC ₅₀ (95%CI)	Slope (±SE)	RR	LC ₅₀ (95% CI)	Slope (±SE)	RR	LC ₅₀ (95% CI)	Slope (±SE)	RR
JPR-R	>5000	0.59 (±0.36)	>700	7.0 (5.1-8.6)	3.8 (±0.32)	14	>5000	2.4 (±0.42)	>830
London	7.1 (5.0-9.4)	3.3 (±0.45)	2700	5.1 (3.7-6.7)	3.4 (±0.34)	1.4	6.0 (5.3-6.9)	3.3 (±0.22)	- 000
R1	>5000	0.41 (±0.27)	>470	4.3 (3.7-4.8)	4.4 (±0.34)	0.22	430 (330-550)	1.3 (±0.10)	120
S1	11 (8.0-12)	5.5 (±0.69)	>470	13 (11-15)	4.0 (± 0.35)	0.55	3.4 (2.8-4.1)	3.3 (±0.23)	150
R2	>5000	0.32 (±0.19)	>520	2.9 (2.3-3.2)	7.6 (±1.3)	0.20	810 (650-1010)	1.6 (±0.17)	190
S2	11 (9.3-12)	9.7 (±1.1)		14 (11-17)	6.9 (±0.72)		4.3 (2.6-6.5)	2.3 (±0.18)	
R3	>5000	0.66 (±0.24)	>E20	3.0 (2.2-3.5)	5.3 (±0.71)	0.34	740 (510-950)	2.0 (±0.22)	200
S 3	9.6 (7.9-11)	5.6 (±0.55)	~520	8.9 (6.9-11)	3.1 (±0.26)		3.7 (3.1-4.4)	3.6 (±0.34)	

2.4.4 Genetic basis of pyflubumide and cyenopyrafen resistance in JPR-R and dominance of the two resistance phenotypes after introgression

First, we reciprocally crossed JPR-R and London to look at the mode of inheritance of pyflubumide and cyenopyrafen resistance in the parental lines. Cyenopyrafen resistance inherited as a dominant, monogenic trait ($X^2 = 12.62$, df = 8, $\alpha = 0.05$), while pyflubumide resistance inherited as incomplete recessive, polygenic trait ($X^2 = 99.30$, df = 7, $\alpha = 0.05$) (Figure 2-2).



Figure 2-2: The genetic basis of cyenopyrafen and pyflubumide resistance in JPR-R. Dose-response relationships of cyenopyrafen and pyflubumide toxicity on JPR-R (diamond shapes), London (circles), reciprocal crosses (triangles) and back-cross (squares). The figure shows that cyenopyrafen has a dominant mode of inheritance while pyflubumide has an incomplete recessive mode of inheritance. The observed dose-response curve of F_2 females does not differ significantly from the calculated curve under the hypothesis of a recessive monogenic mode of inheritance ($X^2 = 12.62$, df=8, $\alpha=0.05$), indicating that cyenopyrafen resistance is monogenic in JPR-R. As for pyflubumide, the dose-response curve differed significantly from

the calculated curve under the hypothesis of a recessive monogenic mode of inheritance, indicating that pyflubumide resistance is polygenic (X^2 = 99.30, df=7, α = 0.05).

Next, reciprocal crosses were carried out with congenic lines to investigate the dominance of the H258Y mutation in conferring pyflubumide and cyenopyrafen resistance. Toxicity bioassays on F₁ females revealed that there were no maternal effects for both resistance phenotypes. Pyflubumide resistance inherited incompletely recessive, while cyenopyrafen resistance inherited as an incomplete dominant trait (Figure 2-3). The dose response curves of cyenopyrafen toxicity of the backcrossed population (F₂ female mites) was not significantly different from the expected curve for monogenic inheritance (Line 1: $X^2 = 10.69$, df = 10, Line 2: $X^2 = 6.81$, df = 9, Line 3: $X^2 = 9.32$, df = 8, $\alpha = 0.05$), indicating that cyenopyrafen resistance was monogenic, as to be expected after introgression of the *sdhB* locus. Surprisingly, pyflubumide resistance still inherited as a polygenic trait in all introgressed lines (Line 1: $X^2 = 80.62$, df = 6, Line 2: $X^2 = 66.53$, df = 10, Line 3: $X^2 = 85.92$, df = 9, $\alpha = 0.05$) (Figure 2-3).



Figure 2-3: Dominance of the H258Y mutation in conferring pyflubumide and cyenopyrafen resistance. Dose-response relationships of pyflubumide (top graphs) and cyenopyrafen (bottom graphs) toxicity on the three congenic line pairs (Line 1-3) (RR; diamond shapes, SS; circles), reciprocal crosses (triangles) and back-cross (squares). Cyenopyrafen resistance was monogenic in all the three congenic line pairs as expected after introgression (Line 1: $X^2 = 10.69$, df = 10, Line 2: $X^2 = 6.81$, df = 9, Line 3: $X^2 = 9.32$, df = 8, $\alpha = 0.05$) while pyflubumide resistance inherited as a polygenic trait in all introgressed lines (Line 1: $X^2 = 80.62$, df = 6, Line 2: $X^2 = 66.53$, df = 10, Line 3: $X^2 = 85.92$, df = 9, $\alpha = 0.05$).

2.4.5 In vitro inhibition of succinate: ubiquinone oxidoreductase

To investigate the inhibitory effect of the three acaricides on their molecular target, the mitochondrial complex II, *in vitro* activity assays were carried out using extracted mitochondria. An attempt to measure the activity of succinate dehydrogenase (SDH) by electron transfer to the water soluble dye thiazolyl blue tetrazolium bromide (MTT) was unsuccessful, as the intrinsic ubiquinone pool reduced the electron acceptor even before the addition of MTT. As such, activity assay on succinate: ubiquinone oxidoreductase (SQR) was measured instead. SQR activity was determined as the transfer of electrons from succinate to ubiquinone leading to reduction of ubiquinone (Q) to ubiquinol (QH₂) (Figure 2-1). Formation of ubiquinol is measured by spectrophotometric reduction of DCPIP at 600 nm and is influenced by SDH or SQR inhibitors. The IC₅₀ values depicting the acaricide concentration required to cause 50% inhibition of SQR activity are shown in Table 2-2. Cyflumetofen, pyflubumide and cyenopyrafen exhibited an inhibitory effect on SQR of the susceptible London strain (IC₅₀s of 568.8 nM, 558.0 nM and 153.2 nM respectively, compared to 90.8 nM for the control Atpenin A5).

Table 2-2: In vitro complex II assay on mitochondria extracted from susceptible (London) and resistant (JPR-R) strains of *T. urticae*. Calculated inhibition concentration (IC_{50} values in nM with 95% confidence limits (shown in brackets) and slopes \pm standard error of the mean. n =168 per compound. The inhibition ratios (IR) were calculated as ratio of the IC_{50} values between JPR-R and London strain for each compound. All values are rounded off to two significant figures.

Compound	Parameters	London strain	JPR-R strain	IR
Atpenin A5	IC ₅₀	91(86-96)	740 (650-840)	8.2
	Slope	1.2 ± 0.03	1.2 ± 0.09	
(EZ)-cyenopyrafen	IC ₅₀	150 (135 -170)	1900 (1600 -2400)	13
	Slope	1.1 ± 0.07	0.85 ± 0.07	
Cvflumetofen	IC ₅₀	570 (530-620)	300 (280 - 330)	0.53
,	Slope	1.1 ± 0.04	1.2 ± 0.06	
Cvflumetofen AB-1	IC ₅₀	17 (16 - 19)	8.3 (7.5-9.2)	0.48
metabolite	Slope	1.1 ± 0.05	1.3 ± 0.1	
Pvflubumide	IC ₅₀	560 (500- 620)	18000 (13000-26000)	33
	Slope	1.2 ± 0.07	0.86 ± 0.10	
Pvflubumide-NH	IC ₅₀	94 (87 - 100)	2100 (1800 - 2600)	23
metabolite	Slope	1.1 ± 0.04	0.73 ± 0.05	

Inhibition of SQR activity dramatically increased with the active metabolites of cyflumetofen and pyflubumide and was 10-50 times more potent than their respective pro-acaricides (with IC_{50} s as low as 17.4 and 93.8 nM respectively). For the pro-acaricde cyenopyrafen, the active metabolite was not available. The cyflumetofen metabolite AB-1 showed the strongest inhibition both on the London strain SQR (IC_{50} of 17.4nM) and the JPR-R strain SQR (IC_{50} of 8.3 nM; Figure 2-4). However, cyenopyrafen and the pyflubumide-NH metabolite did not exhibit a potent inhibitory effect in

mitochondria with the Y258 genotype (IC_{50} S 1913.2 and 2136.8 nM respectively; Figure 2-4), resulting in IRs of 22.8 fold for the pyflubumide metabolite and 12.5 fold for cyenopyrafen. In conclusion, the H258Y mutation dramatically decreases the inhibition of SDH/SQR activity by cyenopyrafen, pyflubumide and its metabolite, but does not negatively impact affinity of cyflumetofen, nor that of its active metabolite.



Figure 2-4: Complex II Inhibition curves. The percentage inhibition of succinate: ubiquinone oxidoreductase (SQR) in the susceptible London strain and the resistant JPR-R strain using the ubiquinoe analogue Atpenin A5, (EZ)-cyenopyrafen and the metabolite forms of pyflubumide (pyflubumide-NH) and cyflumetofen (cyflumetofen-AB1) acaricides. Error bars represent standard deviations calculated from averages of three biological independent replicates and 168 technical replicates per compound. N = 12 for each concentration. 100% specific activity was 356 \pm 19nmol/mg/min with H258 mitochondria.

2.4.6 Binding modes of cyenopyrafen-OH, cyflumetofen-AB1 and pyflubumide-NH to *T. urticae* SDH model

Following the structural similarities emphasized in Huang et al. (2021) for the carboxamide inhibitors carboxin, flutolanil and thiapronil, we hypothesize that the active metabolites of cyenopyrafen, cyflumetofen and pyflubumide bind with their functionally similar anchor groups to the SDH catalytic site in a comparable manner. Although flutolanil (carboxamide like) and thiapronil (nitril) do not belong to an identical chemical class, their interaction with the Q-site is very similar as revealed in Huang et al. (2021). As such, we selected the co-crystal structure of chicken *Gallus gallus* SDH with thiapronil as a template (supplementary Figure S2-1) to model both the protein structure of *T. urticae* and the binding mode of the metabolite forms of cyenopyrafen, cyflumetofen and pyflubumide. The 3D *in silico* modeling of *T. urticae* SDH revealed that all three metabolites share a common binding motif in the Q-site of complex II (Figure 2-5), conveyed by their nitrile and carbonyl or hydroxyl pharmacophores nearly identical to that of thiapronil in *G. gallus* SDH. Non-polar aromatic tails of medium lengths carrying 4-tert-butylphenyl (present in cyenopyrafen-OH and cyflumetofen-AB1) or *N*-[4-(1,1,1,3,3,3-hexafluoro-2-methoxypropan-2-yl)-3-(2-methylpropyl)phenyl] (present in pyflubumide) reach into a highly hydrophobic pocket between subunits B, C and D (Figure 2-5). These lipophilic ring moieties most likely contribute to the overall binding affinity of the respective acaricides by entropic effects.



Figure 2-5: Ligands docked in *T. urticae* SDH homology model with 6MYR as scaffold. In panel A, thiapronil (violet, position given in 6MYR) and cyflumetofen-AB1 (black) in stick optic share the same binding mode with their hydrophobic phenyl head groups forcing the polar imidazole ring (grey, custom heteroatoms) of H258 to twist away. In panel B, the pyrazole acaricides cyenopyrafen (magenta) and pyflubumide (yellow) can interact with H258, evident by ligand docking and the apparent turn of the histidine residue towards the respective active metabolites. To achieve a better overview, binding site occluding residues of SDHB (green ribbons) are not shown in this model. Ribbon and surface of chain C and D are depicted in orange and turquoise, respectively.

The main interactions of the three compounds with wildtype and H258Y substituted SDH are shown in Figure 2-6. In H258 *T. urticae* SDH, the hydroxyl groups of cyflumetofen-AB1 and cyenopyrafen-OH showed a hydrogen bond to D:Y117 or additionally to B:W215 (W173 in *G. gallus*), respectively (Figure

2-6, panel A and B). The keto group of pyflubumide was jointly hydrogen-bound by the strictly conserved residues B:W215 and D:Y117 as well (panel C). Additionally, the polar pyrazole "headgroup" of cyenopyrafen and pyflubumide-NH H-bonds another conserved residue B:H258 (H216 in *G. gallus*) in the polar region of the binding site of the wildtype SDH. This was not applicable for cyflumetofen-AB1 due to its lack of the required H-bond acceptor function as it carries a phenylic moiety with an electron withdrawing 2-trifluoromethyl group. In Y258 SDH, the replacement of a histidine with a tyrosine residue increased hydrophobicity of the binding pocket, reinforcing π - π -stacking of cyflumetofen-AB1 with the aromatic moiety of B:W215 and sterically enabling π - π -stacking with the introduced Y258 residue (panel D). Furthermore, a bifurcative H-bond of the hydroxyl group with B:W215 and D:Y117 is facilitated, thus strengthening cyflumetofen-AB1 binding. On the contrary, the replacement of histidine with a tyrosine side chain prevents additional H-bonding with the pyrazole-type inhibitors cyenopyrafen-OH and pyflubumide-NH, and both ligands experience a polarity mismatch (panel E and F). The tyrosine residue can neither bridge the angle nor the distance to these two ligands.



Figure 2-6: Ligand interaction with the *T. urticae* **SDH homology model.** Cyflumetofen-AB1 (**A**), cyenopyrafen-OH (**B**) and pyflubumide-NH (**C**) docked into the binding site of wildtype *T. urticae* SDH homology model. Only pyflubumide-NH and cyenopyrafen-OH can H-bond B:H258. Cyflumetofen-AB1 (D), cyenopyrafen-OH (E) and pyflubumide-NH (F) docked into the binding pocket of H258Y substituted *T. urticae* SDH homology model. H-bond of cyenopyrafen-OH and pyflubumide-NH to residue B:Y258 is not possible while cyflumetofen-AB1 can interact with the introduced tyrosine via π-π-stacking. For cyenopyrafen-OH, also a hydrogen bond to D:Y117 and hydrophobic interactions (e.g., π-π-stacking) with B:W214 is hindered. Coincidentally, with the mutation from histidine to tyrosine (H258Y), π-π-stacking of cyflumetofen-AB1 with B:W215 is reinforced and H-bonds to B:W215 and D:Y117 are strengthened. Relevant residues in proximity of 2.5 Å and their harboring subunits are given in one letter codes. Pink arrows symbolize H-bonds while green lines between the ligand and the amino acid residues represent π-π-stacking. Positively charged residue R74 appears dark blue. Hydrophobic and polar amino acids are green and blue, respectively. Their contribution to the binding site's environment is indicated with a colored line around the ligand.

2.5 Discussion

We report the discovery of a new mutation (H258Y) in subunit B of SDH in the spider mite T. urticae. This mutation was detected in a pyflubumide resistant population that was previously analyzed (Fotoukkiaii et al. 2020; Fotoukkiaii et al. 2021). This earlier work revealed that pyflubumide resistance inherits as a complex polygenic trait and subsequent QTL mapping uncovered three major-effect genomic loci involved in resistance, none of which were associated with complex II as the target-site. We therefore re-analyzed the DNA and RNA datasets of the segregating offspring, and confirm here that indeed, at the time of QTL cross, the mutation was not present in the parental JPR-R and derived experimental populations. Since this original genetic study, JPR-R has been maintained on pyflubumide treated bean plants (100 mg/L) for several years. It is likely that this continuous selection pressure resulted in the evolution of additional resistance mechanisms, such as the target-site resistance reported here. We therefore likely captured a de novo mutation in laboratory settings. As this mutation arose in a population already highly resistant under relatively mild selection pressure, it might alleviate associated fitness costs of metabolic resistance reported in Fotoukkiaii et al. (2020), and therefore spread rapidly through the population. The presence of absence of a fitness costs associated with this mutation could be assessed via following allele frequencies in mixed populations or life table analysis of introgression lines (Bajda et al., 2018) and is subject of further study.

The main aim of this study was to functionally characterize the H258Y target-site mutation in subunit B of SDH. We confirm that the JPR-R population is highly resistant to pyflubumide, and uncovered a high cross-resistance to cyenopyrafen, but not cyflumetofen, even though all three compounds inhibit SDH. We reveal here that cyenopyrafen resistance inherits as a monogenic, dominant trait, an observation that contrasts with the inheritance of pyflubumide resistance as a polygenic trait in the same JPR-R population (Fotoukkiaii et al., 2021) which we also confirmed in this study. To further elucidate the phenotypic strength and dominance of H258Y, we introgressed the mutation into the London susceptible background by marker assisted back-crossing. Compared to the resistance profile of JPR-R, we show that in the introgressed lines resistance dropped markedly for pyflubumide, but not cyenopyrafen. This indicates that the mutation alone confers very high levels of resistance for cyenopyrafen, which is also corroborated by the monogenic inheritance in JPR-R. In contrast, although the mutation provides strong resistance to pyflubumide, with LC₅₀s between 400-800 mg/L, this is far less when compared to JPRR where LC_{50} exceeds 5000 mg/L. As outlined above, this is perfectly in line with Fotoukkiaii et al. (2021), where multiple QTLs were associated with pyflubumide resistance, including a CYP392A16 that was shown to detoxify the active pyflubumide metabolite. At the time of genetic mapping, the LC₅₀ for pyflubumide was reported to be 1400 mg/L and it is now feasible to hypothesize that the additional target-site mutation is probably responsible for increased resistance

levels reported here. The genetic crosses with introgression lines confirmed the dominant mode of inheritance for cyenopyrafen, but surprisingly revealed the pyflubumide resistance associated with H258Y inherits recessively. It is surprising to find that a single target-site mutation leads to different dominance levels of resistance to compounds with suspected very similar binding sites, and comparable IC₅₀s. One of the possible explanations could be the biological availability at the target-site in vivo, as the pharmacokinetics of both compounds might differ. Introgression is expected to uncouple genetic factors of resistance, and the expectation is that this results in a monogenic mode of inheritance as was the case for cyenopyrafen. However, crossing experiments suggest a polygenic mode of inheritance for pyflubumide resistance (Figure 2-3). Given the high recombination rate in T. urticae, apparent from many genetic studies (Demaeght et al., 2014; Van Leeuwen et al., 2012; Wybouw et al., 2019) and the previous successful introgression experiments (Bajda et al., 2017; Riga et al., 2017), it is hard to explain this result. Of course, the effect of a single locus in resistance can be overestimated if multiple loci are closely linked and fail to uncouple during repeated back-crossing (Hospital, 2001). However, resistance should still inherit as an apparent monogenic trait when F_2 progeny is phenotyped, as uncoupling will not have occurred for the great majority of F₂ females. This clearly points to potential caveats in the current introgression set-up, that might be resolved if an inbred susceptible line would be used for introgression. As many allelic variants in the London population still segregate, this might result in nuclear-mitochondrial interactions that are not fully understood. We show here that a single mutation is responsible for high levels of resistance to cyenopyrafen and pyflubumide, while previous studies have reported the need for a combination of both sdhB and sdhC mutations (I260V and S56L) (Maeoka and Osakabe, 2021). Surprisingly, the I260V substitution alone was sufficient to cause very high resistance to cyflumetofen, while H258Y uncovered in this study, located two residues away, does not affect cyflumetofen toxicity, but instead causes resistance to cyenopyrafen and pyflubumide.

Given the phenotypic effect of the SDH target-site mutations, we used *in vitro* assays with extracted Y258 and H258 mitochondria to re-confirm the mode of action of this class as inhibitors of succinate: ubiquinone oxidoreductase activity (Hayashi et al., 2013; Nakano et al., 2015; Zhou et al., 2020). Of all compounds tested, the cyflumetofen metabolite-AB1 displayed the strongest inhibition of activity, and this was not significantly different between Y258 and H258 mitochondrial preparations indicating that the substitution did not impact binding. In contrast, we found strong effects on IC₅₀ for cyenopyrafen and the pyflubumide active metabolite (13- and 23-fold reduced affinities respectively between Y258 and H258 mitochondria preparations), functionally validating the role of H258Y mutation in resistance to these SDH inhibitors. Although we did not have the active metabolite of cyenopyrafen available for testing, the shift in IC₅₀ suggests that the metabolite is at least partially formed spontaneously during

the assay. In this light, for pyflubumide and cyflumetofen we could test compounds and metabolites separately and see much higher inhibition of the metabolite (Table 2-2), but whether the parental compound physically interacts with the binding site, or is partially converted to the metabolite, remains unclear.

The mutation occurs on a histidine residue that is highly conserved across the tree of life (Figure 2-1c) and is two residues away from the previously reported mutation I260T conferring high levels of resistance to cyflumetofen in T. urticae (Sugimoto et al., 2020). The H258 residue in T. urticae corresponds to H207 in Escherichia coli and H267 in Zymoseptoria triciti and Mycosphaerella graminicola (Horsefield et al. 2006; Avenot and Michailides 2010; Sierotzki and Scalliet 2013). In fungi, this histidine is located in the ubiquinone binding pocket (Q-site), where it interacts with the core moiety of fungal carboxamides via hydrogen bonding at the bottom of the cavity (Sierotzki and Scalliet 2013). Mutations involving this residue confer high resistance levels to SDHI fungicides in the fungus Mycosphaerella graminicola and include H267F/L/N/Y (Fraaije et al., 2012). Previous studies have suggested that both pyflubumide and the fungal carboxamides bind to the Q-site (Nakano et al., 2015). Additionally, pyflubumide is structurally similar to one of the fungal carboxamides named carboxin while the β-ketonitriles cyenopyrafen and cyflumetofen contain hydroxyl oxygen and structural features preferred for H-bond formation by Q-site inhibitors such as flutolanil and thiapronil (Furuya et al. 2017; Fotoukkiaii et al. 2020; Huang et al. 2021). We hypothesized that a switch from the highly conserved histidine to tyrosine at position 258 in subunit B T. urticae SDH may very likely affect the interactions between complex II and the active metabolites of cyenopyrafen, cyflumetofen and pyflubumide. Like most SDHI fungicides, the three acaricides are shown to act on complex II by inhibiting the activity of SQR (Hayashi et al., 2013; Nakano et al., 2015; Zhou et al., 2020). Our inhibition assays with extracted mitochondria showed that in the presence of the mutation, SQR lost sensitivity to cyenopyrafen and pyflubumide NH-metabolite, with inhibition concentrations significantly higher than the 25 nM reported for the pyflubumide NH metabolite (Nakano et al. 2015) (Table 2-2, Figure 2-4). The sensitivity to cyflumetofen AB-1 metabolite was enhanced in the presence of the mutation, with inhibition concentration falling in line with previous studies that have reported an inhibition concentration of 6.55 nM in susceptible strains (Hayashi et al. 2013). This suggests that the active metabolites of the three acaricides probably differ in their mode of interaction with the active site, and enhances the notion that H258Y mutation influences the manner in which these metabolites bind to the active site at complex II.

To better understand how the three acaricides differentially interact with complex II, homology modeling of *T. urticae* SDH and ligand docking studies using metabolite forms of the three acaricides (cyenopyrafen-OH, cyflumetofen-AB1 and pyflubumide-NH) was carried out, revealing that the three

acaricides share a common binding motif at the Q-site and that they interact with the binding site in a different but comparable manner (Figure 2-5). Our findings are in partial agreement with a previous study which suggested that the binding sites of metabolite forms of cyenopyrafen and pyflubumide may be different but close to each other (Nakano et al., 2015). Our modeling data suggests that the carbonyl oxygen of pyflubumide-NH may form a bifurcative H-bond with W215 in subunit B and Y117 in subunit D. Likewise, the hydroxyl oxygen of cyenopyrafen-OH interacts with both W215 in subunit B and Y117 in subunit D, while the hydroxyl oxygen of cyflumetofen-AB1 only H-bonds to Y117 in subunit D. Cyenopyrafen-OH and pyflubumide-NH also forms an extra H-bond with the conserved H258 residue in subunit B. As such, we can conclude that metabolite forms of the three acaricides interact with the same target site but in different manners. Previous docking studies with SDHI fungicides reveal the involvement of similar amino acid residues in the formation of hydrogen bonds and hydrophobic π -interactions at the Q-site. W224, H267 in subunit B and Y130 in subunit D (M. graminicola numbering, corresponding to W215, H258 and Y117 in T. urticae) have been identified as some of the major active site residues involved in these interactions (Fraaije et al., 2012; Inaoka et al., 2015; Xiong et al., 2017, 2015). Confirming the important role of tryptophan, tyrosine and histidine residues in ligand-receptor interactions, docking studies with the catalytic Q-site of E.coli have also shown that the side chains of W164 in subunit B (corresponding to W215 in T. urticae) and H207 in subunit B (corresponding to H258 in *T. urticae*) are suitably positioned to provide hydrogen bonding partners to carbonyl oxygen and methoxy group of ubiquinone (Horsefield et al., 2006; Ruprecht et al., 2009). Interestingly, inhibition assays with extracted mitochondria from the resistant strain JPR-R carrying the H258Y mutation also showed reduced sensitivity of SQR to Atpenin A5, a natural analogue to ubiquinone. As sensitivity to cyenopyrafen and pyflubumide was also reduced in this strain, it is highly likely that the binding motif shared by these two acaricides is actually the ubiquinone binding pocket (Q-site) as previously proposed for fungal carboxamides (Sierotzi and Scalliot 2013).

In summary, this is a remarkable case of high selectivity of a target-site resistance mutation within a set of compounds with the same mode of action. This is supported by a previous study by Huang et al. (2021) who showed that variability of specific residues in the proximity of 4 Å significantly affected ligand binding of several Q-site inhibitors. In our study, the variation of a single histidine residue to tyrosine at the Q-site offsets cyenopyrafen and pyflubumide binding. A strong bond to these two pro-acaricides cannot be formed with tyrosine in the binding pocket, therefore they cannot efficiently inhibit complex II activity in the resistant strain JPR-R that carries this mutation, resulting in cross-resistance between the two acaricides. The mutation to tyrosine however increases hydrophobicity in the binding pocket. As a result, the hydrophobic interactions (e.g., π - π -stacking) with cyflumetofen are reinforced, which generally improves the binding of this acaricide. This might explain the observed

increase in SQR activity with cyflumetofen in the resistant JPR-R strain, as more cyflumetofen is presumably bound to the active site. Concomitantly, the higher susceptibility of H258Y for cyflumetofen-AB1 highlights chemical plasticity of the Q-site, as this exchange from a polar into a hydrophobic residue favors entropy driven accumulation of this acaricide.

2.6 Conclusion

In this study, we uncovered the new mutation H258Y, positioned at a highly conserved histidine residue in mitochondrial SDH of the spider mite *T. urticae*. Introgression of the mutation into a susceptible mite strain validated that H258Y results in cross-resistance between cyenopyrafen and pyflubumide but revealed different dominance levels. Further, *in vitro* activity assays and docking studies validated the resistance mutation and provided a deeper understanding of the complex interactions between complex II and different SDH inhibitors in spider mite pests.

Funding information

This work was supported by the Research Council (ERC) under the European Union's Horizon 2020 research and innovation program, grant 772026-POLYADAPT to T.V.L. and 773902–SUPERPEST to T.V.L. N.W. was supported by BOF fellowship (Ghent University, 01P03420) throughout this project.

Supplementary information

All supplementary data can be found at https://doi.org/10.1016/j.ibmb.2022.103761.

Table S2-1: Primers used to amplify and sequence sdhB, sdhC and sdhD subunits.

T. urticae gene ID	Gene Name	Primer Name	Sequence, 5'–3'
tetur01g15710	SDHB	sdhB_F	AGTTGCTTTCCTTGGCTTCA
		sdhB_R	ACCAGTTACTTGGGGGCTTT
tetur30g00210	SDHC	sdhC_F	AAATCATGTTATTTCCACGTTTGA
		sdhC_R	GCAATTGGTTACGGGTAGTTTAGTAT
tetur20g00790	SDHD	sdhD_F	CCATGAACCGAGTTTTGTCA
		sdhD_R	CGATGACTTTTCCGTAATTCCT



Figure S2-1: Overview of Gallus gallus SDH (6MYR). In panel A, the 3D structure model. Subunits A (yellow with transparent surface), B (green), C (orange) and D (blue). Catalytic site containing FAD (calottes in panel A, ball-and-stick in panel B) and oxaloacetate (calottes in panel A and separated from FAD in panel B) harbored in SDH subunit A, three distinct iron-sulfurclusters (2Fe2S, 4Fe4S, 3Fe4S) along SDH subunit B (orange and yellow calottes), heme (gray carbons with custom heteroatoms) as well as thiapronil (white surface in panel A, magenta and custom heteroatoms in panel B) are shown.

File S2-1: Sequence alignment of the *T. urticae* SDH subunits B,C and D. Using London as the reference, no mutations were uncovered in subunits C and D. Subunit B contained a non-synonymous H258Y substitution that was fixed in a pyflubumide resistant strain JPR-R. A view of the substitution in the amino acid sequence of subunit B from the JPR-R strain is shown in file S2-1a while the DNA alignments of the three subunits are shown in files S2-1b, c and d. The DNA alignments should be opened with programs that can read fasta files e.g. SnapGene, BioEdit or MEGA softwares.

Authors' contributions

TVL and NW conceived the study. CN, NW and TVL designed the experiments. CN and CS performed the experiments. CN, CS and OG analysed data. CN wrote the manuscript with significant input from NW, SG and TVL. All authors read and approved the final manuscript.

Chapter 3: The complex II resistance mutation H258Y in succinate dehydrogenase subunit B causes fitness penalties associated with mitochondrial respiratory deficiency

This chapter has been partially redrafted from:

Christine Njiru, Corinna Saalwaechter, Konstantinos Mavridis, John Vontas, Sven Geibel, Nicky Wybouw and Thomas Van Leeuwen. (2023) The complex II resistance mutation H258Y in succinate dehydrogenase subunit B causes fitness penalties associated with mitochondrial respiratory deficiency. Pest Management Science, 79: 4403-4413.

3.1 Abstract

The acaricides cyflumetofen, cyenopyrafen and pyflubumide inhibit the mitochondrial electron transport chain at complex II (succinate dehydrogenase complex, SDH). A target site mutation H258Y was recently discovered in a resistant strain of the spider mite pest Tetranychus urticae. H258Y causes strong cross-resistance between cyenopyrafen and pyflubumide, but not cyflumetofen. In fungal pests, fitness costs associated with substitutions at the corresponding H258 position that confer resistance to fungicidal SDH inhibitors have not been uncovered. Here, we used H258 and Y258 near-isogenic lines of T. urticae to quantify potential pleiotropic fitness effects on mite physiology. The H258Y mutation was not associated with consistent significant changes of single generation life history traits and fertility life table parameters. In contrast, proportional Sanger sequencing and droplet digital PCR showed that the frequency of the resistant Y258 allele decreased when replicated 50:50 Y258:H258 experimentally evolving populations were maintained in an acaricide-free environment for approximately 12 generations. Using in vitro assays with mitochondrial extracts from resistant (Y258) and susceptible (H258) lines, we identified a significantly reduced SDH activity (48% lower activity) and a slightly enhanced combined complex I and III activity (18% higher activity) in the Y258 lines. Our findings suggest that the H258Y mutation is associated with a high fitness cost in the spider mite T. urticae. Importantly, while it is the most common approach, it is clear that only comparing life history traits and life table fecundity does not allow to reliably estimate fitness costs of target site mutations in natural pest populations.

3.2 Introduction

Pesticide resistance presents a major challenge in the control of arthropod pests in agriculture. Resistance mainly results from increased activity of detoxification enzymes that transport, metabolize and excrete pesticides more rapidly, and include well studied enzyme families such as cytochrome P450s (CYPs), carboxyl/cholinesterases (CCEs), glutathione-S-transferases (GSTs), UDP-glycosyl transferases (UGTs) and ABC-transporters, although more recently also novel gene families such as intradiol ring cleavage dioxygenases (DOGs), lipocalins, short chain dehydrogenases (SDRs) and the major facilitator superfamily (MFS) have been implicated in metabolic resistance (Kurlovs et al., 2022; Van Leeuwen and Dermauw, 2016). As a second main mechanism, alterations of the molecular target of the pesticide by nonsynonymous point mutations often underlie pesticide resistance in arthropods, including spider mites (Feyereisen et al., 2015; Kwon et al., 2015; Van Leeuwen and Dermauw, 2016).

Resistance is said to be associated with a fitness cost when the development of resistance is accompanied by an energetic cost. For instance, when overexpression of a resistance allele results in reallocation of energy and resources at the expense of other metabolic and developmental processes

(Kliot and Ghanim, 2012). Resistance development can also result in significant disadvantages that diminish the fitness of the resistant individuals as compared to their susceptible counterparts. For example when a target site mutation results in (near) loss of function (Kliot and Ghanim, 2012). It is clear that if a resistant allele is associated with a fitness cost, the evolution and persistence of resistance is strongly impacted in a field setting, which is of crucial importance for resistance management (Roush and McKenzie, 1987). It is believed that resistance alleles are therefore rarely fixed in a natural environment. Resistance alleles can however be maintained in pest populations if these fitness costs are negligible or compensated by additional mutations which restore fitness to its original level (Davies et al., 1996; Labbé et al., 2009, 2007; McKenzie and Clarke, 1988). Creating a successful and sustainable pest management regime requires examination of the fitness of resistant pests. This will not only help to understand the dynamics of the resistant alleles but also to obtain information for the optimal timing of pesticide rotations (Plapp et al., 1990).

Pleiotropic fitness costs of a resistance mutation in the absence of the pesticide can be experimentally verified via a range of methods. In a first method, single generation life history parameters such as the development time, fecundity, fertility and mating competitiveness are estimated and compared between populations or strains of interest (Prout, 1971; Roush and McKenzie, 1987). In a second method, strains that differ in the resistance mutation are reared together in cages and alleles are followed over time (Roush and Daly, 1990). In this approach, when allele frequencies decrease, increase or remain constant over time, they are said to be costly, beneficial or neutral, respectively (Hardstone et al., 2009). For both methods, it is important to use genetically related strains in order to limit the causal effect to the mutation under investigation (Bourguet et al., 2004). Excluding a few studies, the use of genetically unrelated strains is a common design flaw in previous experiments that evaluate pesticide resistance related fitness costs in arthropod pests (ffrench-Constant and Bass, 2017; Kliot and Ghanim, 2012). The claim that the observed presence or absence of a fitness cost is due to a resistance mutation becomes unreliable in different genetic backgrounds. The unrelated resistant and susceptible strains may differ not only in most other genes than those involved in resistance but also in their adaptive life history traits (Bourguet et al., 2004). Although often challenging, (near-)isogenic lines that only differ for the resistance mutation need to be generated for a correct estimation of fitness costs. A precise way to generate genetically related strains in vivo is by introducing the mutation of interest in a defined susceptible background using CRISPR-Cas9 (Douris et al., 2016; Zimmer et al., 2016). In species where genome editing is not yet applicable, this can be accomplished by repeated back-crossing of the resistance mutation into a susceptible genomic background over multiple generations (Bajda et al., 2017; Bourguet et al., 2004; Brito et al., 2013; ffrench-Constant and Bass, 2017).

The spider mite Tetranychus urticae is a major agricultural pest that quickly develops resistance to pesticides of varying modes of action. The resistance traits are often complex, involving a combination of multiple factors (De Beer et al., 2022b, 2022a; Fotoukkiaii et al., 2021; Snoeck et al., 2019a; Sugimoto et al., 2020; Villacis-Perez et al., 2022). In most resistance cases, both metabolic detoxification genes and target-site mutations have been identified in the resistant phenotypes (Kwon et al., 2015; Sugimoto et al., 2020; Van Leeuwen et al., 2010; Van Leeuwen and Dermauw, 2016). By carrying out repeated backcrossing, a number of target site mutations have been uncoupled from other mechanisms and their relative contribution to resistance assessed (Bajda et al., 2017; De Beer et al., 2022b; Fotoukkiaii et al., 2019; Maeoka and Osakabe, 2021; Chapter 2; Riga et al., 2017). In particular, mutations in the various subunits of SDH, voltage gated sodium channel, chitin synthase 1 and cytochrome b have been associated with high levels of acaricide resistance while mutations in the glutamate-gated chloride channels do not lead to high acaricide resistance levels (Maeoka and Osakabe, 2021; Chapter 2; Riga et al., 2017). Further, fitness costs studies in isogenic lines have revealed that the chitin synthase 1 mutation I1017F and the co-occurring G314D and G326E mutations in the glutamate-gated chloride channels alter some of the fitness parameters (Bajda et al., 2018). However, Bajda et al., (2018) did not detect any fitness costs associated with the cytochrome b mutation P262T and L1024V mutation in the voltage-gated sodium channel.

Acaricidal SDH inhibitors such as cyflumetofen, cyenopyrafen, pyflubumide and cyetpyrafen belong to the most recently developed mode of action group (IRAC Group 25) (Furuya et al., 2017; Hayashi et al., 2013; IRAC international MoA working group, 2022; Li et al., 2016; Nakahira, 2011; Nakano et al., 2015). Resistance and cross-resistance cases have already been reported in regions where these products are widely used, with most cases reported in T. urticae populations identifying metabolic resistance as the major resistance mechanism (İnak et al., 2022b; Sun et al., 2022; Tian et al., 2022; Xu et al., 2018; Zhang et al., 2021). Metabolic resistance to SDH inhibitors in spider mites is mainly mediated by CYP detoxification and leads to low to moderate resistance levels (Fotoukkiaii et al., 2020; Khalighi et al., 2015, 2014; Sugimoto and Osakabe, 2014). For example, CYP392A16 was shown to metabolize pyflubumide to a non-toxic metabolite (Fotoukkiaii et al., 2021). Sugimoto et al., (2020) were the first to discover target-site resistance mutations I260T/V in subunit B and S56L in subunit C of SDH. In the current study, we focus on evaluating the fitness costs associated with a recently uncovered H258Y mutation in subunit B that causes high cross-resistance against cyenopyrafen and pyflubumide (Chapter 2). The mutation was discovered in a field strain that was selected for resistance in the laboratory with pyflubumide (Fotoukkiaii et al., 2020). The H258Y mutation occurs on a highly conserved histidine residue found in subunit B and mutations involving this residue in fungi have been associated with high levels of resistance to multiple carboxamide fungicides with no associated fitness

costs (Avenot and Michailides, 2010, 2007; Kim and Xiao, 2011; Sierotzki and Scalliet, 2013; Zhang et al., 2007).

We used the replicated near-isogenic lines developed in chapter 2 to compare nine single generation life history traits of the resistant Y258/Y258 genotype to the wildtype H258/H258 genotype. Additionally, the cumulative effect of these parameters on population dynamics was inferred from five life table parameters namely: reproductive rate (R0), intrinsic rate of increase (rm), mean generation time (T), finite rate of increase (LM) and doubling time (DT). Using replicated populations in an experimental evolution setup, the frequency of the resistant Y258 allele was monitored over a period of six months in a pesticide free environment. *In vitro* assays with extracted mitochondria were carried out to determine whether the mitochondrial electron transport activity is affected by the H258Y mutation.

3.3 Materials and methods

3.3.1 Mite lines and husbandry

Six independent near-isogenic lines of *T. urticae* were generated via marker-assisted introgression in a previous study (Chapter 2). Briefly, the lines R1, R2, and R3 carry a non-synonymous H258Y mutation in subunit B of the succinate dehydrogenase complex that confers resistance to pyflubumide and cyenopyrafen. Each R line (R1 to R3) has a respective paired S control line (S1 to S3) that went through the same introgression crossing scheme, but lacks the target-site mutation. The S lines are susceptible to pyflubumide and cyenopyrafen. All lines were maintained on detached unsprayed bean leaves (*Phaseolus vulgaris* L. cv 'Prelude'). If needed, populations were expanded on 3-week old potted bean plants placed in W47.5 x D47.5 x H47.5 cm cages (Bugdorm-4F4545). All mite populations were kept under laboratory conditions of 25 ± 1 °C, 60% relative humidity and 16:8 h light:dark photoperiod.

3.3.2 Life history traits

3.3.2.1 Total development time, sex ratio and immature stage survivorship

The total development time, sex ratio and immature stage survival (ISS) were calculated based on previously established methods by Bajda et al., (2018) . From the stock population of each of the six near-isogenic lines, four replicates of 100 adult females were randomly selected and transferred to a detached bean leaf. Females were allowed to oviposit for 8 hours. The numbers of eggs were counted and recorded for ISS calculations. Here, ISS is defined as the proportion of mites reaching adulthood. On the 7th day after oviposition, mite development was monitored with 12 hours intervals. Development time is defined as the time taken for eggs to develop into adults. Eclosion was scored

and the sex of adult mites was determined. The sex ratio is defined as the proportion of female adults over all mites that reached adulthood.

3.3.2.2 Oviposition and adult longevity

To generate age-synchronized mites, 200 adult females were placed on a detached bean leaf and allowed to oviposit for 8 hours. The females were then removed and egg development monitored daily. Per line, 25 single-pair crosses were established by placing a teleiochrysalid female with an adult male on a 3 cm² bean leaf disk. Once the females reached adulthood, each pair was transferred daily to a new leaf disk. Males that did not survive for two days after their female partner reached adulthood were replaced. The pre-oviposition, oviposition and post-oviposition periods were monitored for each female as described in Bajda et al., (2018) . Pre-oviposition period is defined as the time span between adult female emergence and first egg laying, as estimated from observations made with 12 hours intervals. After the first egg laying, the number of eggs deposited every day was recorded until no more eggs were laid. This was defined as the oviposition period. Post-oviposition period was determined from the day when no more eggs were deposited by an individual female to the time of her death.

3.3.3. Data analysis

Analysis of all life history data was carried out using R version 4.2.2 (R Core Team, 2021). ISS and sex ratio were analyzed using the glm function with a binomial error distribution (package stats). Development time, longevity and fecundity data were analyzed in a linear model using Im function (package stats). A post hoc Tukey's test was subsequently carried out using the glht function (package multcomp). Correction for multiple comparisons was carried out using Holm-Sidak's method (adjusted p-value < 0.05). When normality was not met, a non-parametric Krustal-Wallis test was carried out with Dunn's multiple comparisons test (adjusted p-value < 0.05). Analysis of life-history traits was conducted using the lifetable R script (Maia et al., 2014). Specifically, mite line, female ID, age, and number of eggs laid per female at each oviposition date, proportion of female offspring and ISS were used as input. The intrinsic rate of increase (rm) was calculated with the equation $\sum_{x=x0}^{\Omega g} e - rmlxmx = rmlxmx$ 1 where Ωg is the oldest age attained, lx is the proportion of females surviving to age x and mx is the mean number of female progeny per adult female at age x. The net reproductive rate (R0) or mean number of daughters produced per female was calculated from $R0 = \sum_{x=x0}^{\Omega g} lxmx$ and the mean generation time from T = $\frac{\ln(R0)}{rm}$. The finite rate of increase and doubling time were inferred from the equations LM = e^{rm} and DT = $\frac{\ln 2}{rm}$, respectively. Variance for the life table parameters (LTP) was estimated with jackknife resampling method (Quenouille, 1956). Mean Jackknife values and their standard errors (SE) were calculated for the five LTP parameters (Meyer et al., 1986). A multiple comparison of the mean jackknife values for the six mite lines was carried out using Tukey's test with correction for multiple comparisons using Holm-Sidak's method (adjusted p-value < 0.05).

3.3.4 Experimental evolution of mixed populations of Y258 and H258 genotypes in an acaricide-free environment

Changes in allele frequency of the H258Y mutation were evaluated for replicated mixed populations of R1 + S1 and R3 + S3. Two hundred adult females were used as the founding population in a 50:50 ratio and maintained on potted 3-week old bean plants placed in cages. The experimental setup was carried out in three replicates per mixed line, making a total of six bi-allelic populations at the H258Y locus. Every two weeks (approximately one generation), 200 adult females were collected from four leaves outside the initial inoculation area. Populations were allowed to propagate for a total of 24 weeks, corresponding to approximately 12 generations. Pooled mite samples were stored at -80 °C. DNA was extracted using a DNeasy blood and tissue kit (Qiagen) following the manufacturer's protocol. DNA quality was verified using a Denovix DS-11 spectrophotometer (DeNovix, Willmington, DE, USA) and by running a 2% agarose gel electrophoresis (30 min at 100 V). The Y258 allele frequency was estimated using both proportional sequencing and droplet digital PCR (ddPCR).

3.3.4.1 Proportional sequencing

A fragment of the *sdhB* gene was amplified by a standard PCR approach using GoTaq G2 DNA polymerase (Promega). Forward and reverse primer were 5'- AGTTGCTTTCCTTGGCTTCA-3' and 5'- ACCAGTTACTTGGGGGCTTT-3', respectively (Fotoukkiaii et al., 2019). Amplification conditions were: 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s with a final extension at 72 °C for 5 min. PCR products were sequenced at LGC Genomics (Germany). Sequencing data was visualized using Bioedit version 7.2. Frequencies of the Y258 allele were calculated from the double peak observed in the trace data as follows: $\left(\frac{Y258 \text{ peak height}}{Y258 \text{ peak height}}\right) * 100$. Based on the trace data of the forward and reverse primer, an average of the frequencies was calculated and used for subsequent analyses. Final plots were made in GraphPad prism version 9.1.2.

3.3.4.2 H258Y TaqMan assay design

A new TaqMan assay was designed based on the *sdhB* gene sequences of *T. urticae*. The assay consisted of two primers (forward primer: 5'- CTGAAAAAAGGYTGGATCAATTG -3', reverse primer: 5'- CGACCAGGATTCAAGTTCTTAGGA -3') and two probes (H258 probe: 5'-HEX- TCTCACTTTATCGATGYCAT -MGB-3', Y258 probe: 5'- FAM- TCTCACTTTATCGATGYTAT -MGB-3'). For validation purposes, the H258Y TaqMan assay was tested with qPCR in individual mites of known genotypes (N=20 susceptible, N=20 resistant and N=14 heterozygous samples) and was able to correctly genotype all samples (Figure

S3-1). The qPCR instrument was a CFX Connect, Real-Time PCR System (Bio-Rad). The qPCR reaction volume was 10 μL and consisted of 5.0 μL 2×TaqMan[™] Universal PCR Master Mix (Applied Biosystems), 1000 nM of primers, 400 nM of probes. Concentrations were adjusted to final reaction volumes with DEPC-treated water. The qPCR thermal protocol was: 95 °C for 10 min, and 50 cycles of 95 °C for 15 s, 60 °C for 1 min. Sample calling was performed using the Allelic Discrimination module of the Bio-Rad CFX Maestro 2.3 software.

3.3.4.3 Droplet digital PCR (ddPCR)

Concentrations of dsDNA were measured with the Qubit[™] dsDNA BR Assay on a Qubit fluorometer 2.0 (Invitrogen, Carlsbad, CA). The ddPCR was performed according to Mavridis et al., (2021), using the primers and probe of the TagMan assay described in 3.3.4.2. Briefly, 20 µL reactions consisting of 1x ddPCR Supermix for probes, 5U restriction enzyme EcoRI-HF® (New England Biolabs), 1 ng of dsDNA, 900 nM of primers and 500 nM of probes were prepared. The final volume to 20 μL was adjusted with DEPC-treated water. Samples were mixed with 70 µL of droplet generator oil for probes (Bio-Rad), inserted in the QX200 droplet generator (Bio-Rad) and transferred to 96-well plates (Bio-Rad), where PCR was performed on a C1000 Touch thermal cycler (Bio-Rad) with the following thermal protocol: 95 °C for 10 min, and 40 cycles of 94 °C for 30 s, 58 °C for 1 min, and a final step of 98 °C for 10 min; the ramp rate was set at ramp rate = 2 °C/sec. A gradient ddPCR (52 °C to 62 °C) was used to find the optimal annealing temperature. Endpoint fluorescence was measured in the FAM and HEX channels in the QX200 droplet reader (Bio-Rad). Raw data was processed with the QuantaSoft Analysis Pro Software (v.1.0.596). The detection limit of the assay (defined as the lowest mutant allele frequency that can reliably be detected and is distinguishable from the wild type background), was calculated using synthetic double stranded DNA sequences (gBlocks[™] gene fragments, IDT) of known H258 and Y258 sequence concentration and copy number in the following frequencies: 100%, 50%, 10%, 1%, 0.5%, 0.2%, 0.1%, and 0%. The experimental DNA samples were assayed in a similar way as the synthetic standards.

3.3.5 In vitro activity assays of the mitochondrial complexes I, II and III

To determine whether the H258Y mutation affects succinate dehydrogenase enzyme activity, biochemical assays were carried out using mitochondria extracted from the six near-isogenic lines following a modification of the protocol described in Schägger and von Jagow., (1991). Mitochondria were prepared as described previously (Chapter 2). Protein concentration was determined using Pierce BCA protein assay (Thermo Fisher, Germany) with BSA as the reference. Aliquots of mitochondrial pellets were stored at -80 °C. For complex II activity assay, myxothiazol (Sigma-Aldrich, Germany) and atpenin A5 (Enzo Life Sciences, USA) were dissolved in DMSO to yield 10 mM stock solutions. *In-vitro*-

activity of succinate: ubiquinone oxidoreductase (SQR) was assessed using a modified protocol of Spinazzi et al., (2012), as described in chapter 2. Electron transfer from succinate to ubiquinone via mitochondrial complex II (SQR activity) was monitored as ubiquinol-dependent reduction of DCPIP. Absorption changes (λ = 600 nm, 25 flashes per second, 60 seconds intervals for 30 minutes) were measured in a M1000 Pro microplate photometer (Tecan Group, Switzerland) and wells without mitochondria or with atpenin A5 (1 µM) served as negative and specificity controls, respectively. Absorption changes between three and 17 minutes were linear ($R^2 > 0.95$) and considered in the final evaluation. Testing of both Y258 (R lines) and H258 (S lines) replicates always took place in parallel on each plate. Michaelis-Menten constant and Vmax were calculated and plotted with GraphPad Prism version 9.1.2. Raw data were expressed as specific activities (nanomoles DCPIP reduced per mg protein and minute) with the molar extinction coefficient of DCPIP ($\varepsilon = 19.1 \text{ mM}^{-1} \text{ cm}^{-1}$). In total, 102 technical replicates per succinate concentration were averaged for the S or R lines. To specify the consequences of the H258Y mutation on the activity of other mitochondrial electron transport protein complexes, and to serve as an extra control for the amount of mitochondrial membranes in preparations, coupled NADH-cytochrome c oxidoreductase (NCCR) activity was assayed. By supplementing NADH as substrate, complex I (NADH dehydrogenase) of the mitochondrial fractions reduces intrinsic ubiquinone to ubiquinol, which is subsequently reoxidized by complex III (cytochrome c oxidoreductase) to finally reduce cytochrome c (Hatefi and Stiggall, 1978; Medja et al., 2009; Spinazzi et al., 2012). In brief, mitochondrial pellets were thawed on ice, suspended in buffer (4 mM CHAPS, 250 mM potassium chloride, 1 mg mL⁻¹ BSA in 50 mM potassium phosphate buffer, pH 7.4) and transferred to clear 384 well microtiter plates (1 µg well⁻¹). 100 µM equine cytochrome c (Sigma-Aldrich, Germany) with 300 μ M sodium cyanide in 50 mM potassium phosphate buffer pH 7.4 was added and stable background absorption was monitored. Immediately after addition of reduced β -NADH (158 nM - 500 μ M in 50 mM potassium phosphate buffer, pH 7.4), absorption increments of cytochrome c (λ = 550 nm, 25 flashes per second, 30 seconds intervals for 20 minutes) were measured in a M1000 Pro microplate photometer (Tecan Group, Switzerland). Cyanide blocked the physiological electron transfer from cytochrome c to complex IV (cytochrome c-oxidase) to ensure that cytochrome c remained in its reduced form. Reactions were linear for 22 minutes ($R^2 > 0.95$) and considered in the final evaluation. Michaelis-Menten constant and V_{max} were calculated and plotted with GraphPad Prism version 9.1.2. Therefore, raw data were expressed as specific activities (nanomoles cytochrome c reduced per mg protein and minute) with the molar extinction coefficient of cytochrome c ($\varepsilon = 18.5$ mM⁻¹ cm⁻¹). In total, 16 technical replicates per succinate concentration were averaged for the S or R lines.

3.4 Results

3.4.1 H258Y does not influence single-generation life history traits and fertility lifetable parameters in *T. urticae*

The impact of the target-site H258Y mutation on nine single-generation life history traits was studied using independently constructed and paired near-isogenic H258 (S1 to S3) and Y258 (R1 to R3) lines. No significant differences were observed for the median development time of females. However, males of R2 exhibited a significantly longer median development time than their susceptible counterparts (p = 0.0042). Males of S2 matured on average 15 hours earlier than those of R2 (Table 3-1). It took approximately 12 days for mites from all lines to reach adulthood (Figure S3-2). ISS estimates of line R3 differed significantly from line S3 (p = 0.0167), with 88% of the eggs reaching adulthood while only 65% of the S3 eggs developed into adult mites. Sex ratio values (the proportion of female adults over all mites that reached adulthood) did not differ significantly between the S and R lines (p = 0.0917). All lines exhibited a similar survival pattern, with no significant differences observed in the lifespan (Table 3-2, Figure 3-1a). The daily oviposition trends for all lines are shown in Figure 3-1b. Values for the daily and total ferences in the duration of pre-oviposition, oviposition and post oviposition period (Table 3-2).



Figure 3-1 Visualization of female adult longevity and daily egg laying per *T. urticae* female. Panel a represents the adult longevity while panel b depict the number of eggs/female/day for the lines carrying H258Y (solid lines) and their susceptible control lines (dashed lines).

Table 3-1: Mean values \pm SE of development time, immature stage survivorship (ISS), offspring sex ratio (FR), daily (FFD) and total (TF) fecundity in *T. urticae* near isogenic lines. Means followed by a different letter within a column indicate significant difference (adjusted p value < 0.05). Where a resistant line and its control differed significantly is indicated in bold. R and S lines followed by the same number (1, 2 or 3) indicate a resistant line (R) and its control susceptible line (S). Development time (d): time required for 50% ≥ males and females to emerge, TF: eggs/female, FFD: eggs/female/day within the oviposition period, ISS: % males and females reaching adulthood, FR: % females in the progeny, N: initial number of eggs.

Line	Ν	Development time ± SE		ISS ± SE	FR ± SE	FFD ± SE	TF ± SE
		Male	Female				
S1	939	$8.45\pm0.06^{\rm ab}$	$8.81\pm0.12^{\text{a}}$	0.78 ± 0.03^{ab}	0.63 ± 0.02^{ab}	6.16 ± 0.67^{a}	49.90 ± 7.94ª
R1	555	$8.50\pm0.00^{\text{ab}}$	8.94 ± 0.06ª	$0.85 \pm 0.05^{\circ}$	$0.54\pm0.03^{\text{ab}}$	7.10 ± 0.75^{a}	69.29 ± 11.85°
S2	744	8.25 ± 0.14^{b}	$9.0 \pm 0.00^{\circ}$	$0.80\pm0.02^{\text{ab}}$	$0.70\pm0.03^{\text{a}}$	$5.04 \pm 0.48^{\circ}$	45.43 ± 8.00ª
R2	770	8.88 ± 0.07^{a}	9.0 ± 0.00^{a}	$0.84\pm0.02^{\text{ab}}$	0.62 ± 0.02^{ab}	6.87 ± 0.66^{a}	$60.32 \pm 11.41^{\circ}$
S 3	834	$8.63\pm0.13^{\text{ab}}$	$9.0 \pm 0.00^{\circ}$	0.65 ± 0.08 ^b	$0.52\pm0.07^{\text{b}}$	5.54 ± 0.61ª	54.96 ± 8.70ª
R3	649	$8.63\pm0.13^{\text{ab}}$	$9.0\pm0.00^{\mathrm{a}}$	0.88 ± 0.03^{a}	$0.60\pm0.01^{\text{ab}}$	5.56 ± 0.68^{a}	79.52 ± 11.78ª

Table 3-2: Mean values \pm SE of female adult longevity, duration of pre-oviposition (Pre-OP), oviposition (OP) and post oviposition periods (Post-OP) obtained for *T. urticae* near isogenic lines. Time is expressed in days. N: number of females. R and S lines followed by the same number (1, 2 or 3) indicate a resistant line (R) and its control susceptible line (S).

Line	Ν	Longevity ± SE	Pre-OP ± SE	OP ± SE	Post-OP ± SE
S1	20	11.25 ± 1.99ª	1.60 ± 0.09 ^a	7.00 ± 1.00 ^a	2.40 ± 0.80^{a}
R1	21	10.16 ± 1.33ª	1.52 ± 0.11ª	8.38 ± 1.31ª	1.57 ± 0.51ª
S2	21	9.80 ± 1.25ª	1.71 ± 0.09ª	7.57 ± 1.23ª	1.29 ± 0.35ª
R2	19	9.32 ± 1.09^{a}	$1.66 \pm 0.09^{\text{a}}$	7.74 ± 1.25 ^a	0.95 ± 0.28^{a}
S3	24	11.44 ± 1.38ª	1.65 ± 0.11^{a}	8.38 ± 1.15ª	2.04 ± 0.80^{a}
R3	25	12.88 ± 2.00 ^a	1.52 ± 0.07ª	9.76 ± 1.48^{a}	1.96 ± 0.78ª

Mean jackknife values of life table parameters for the six lines are summarized in Table 3-3. Since the fecundity data was only moderately skewed ($-1 \le x \ge 1$, Table S3-1, Figure S3-3), jackknife resampling method was used to estimate the life table parameters. Lines R1 and R2 did not differ significantly from their susceptible control lines S1 and S2 in any of the five fertility life table parameters. Line R3 had significantly higher values of intrinsic rate of increase (rm, p = 0.0006) and finite rate of increase (LM, p = 0.0005) and consequently shorter doubling time (DT, p = 0.0018) as compared to its control line S3. This line was also characterized with a higher net reproductive rate (R0, p = 0.0052).

Table 3-3: Jackknife estimates \pm SE of five FLT parameters. Means followed by a different letter within a column indicate significant difference (Tukey's test, adjusted p value < 0.05). Where a resistant line and its control differed significantly is indicated in bold. R and S lines followed by the same number (1, 2 or 3) indicate a resistant line (R) and its control susceptible line (S). Abbreviations: reproductive rate (RO), intrinsic rate of increase (rm), mean generation time (T), finite rate of increase (LM) and doubling time (DT) obtained for near isogenic *T. urticae* lines. N is the number of females.

Group	N	R0 ± SE	T ± SE	DT ± SE	rm ± SE	LM ± SE
S1	20	20.46 ± 3.43 ^b	15.90 ± 0.39°	3.62 ± 0.17^{ab}	0.191 ± 0.009^{ab}	1.210 ± 0.011^{ab}
R1	21	26.86 ± 5.16 ^{ab}	16.78 ± 0.39ª	$3.51\pm0.16^{\text{ab}}$	0.197 ± 0.009 ^{ab}	1.218 ± 0.011^{ab}
S2	21	21.41 ± 4.21 ^b	16.51 ± 0.47ª	$3.70\pm0.18^{\text{ab}}$	0.187 ± 0.009^{b}	1.205 ± 0.011 ^b
R2	19	23.84 ± 5.25 ^{ab}	$16.88 \pm 0.47^{\circ}$	3.65 ± 0.20^{ab}	0.189 ± 0.010^{ab}	1.209 ± 0.012^{ab}
S 3	24	18.02 ± 2.91 ^b	16.94 ± 0.39°	4.03 ± 0.17 ^a	0.172 ± 0.007 ^b	1.187± 0.008 ^b
R3	25	41.85 ± 6.20 ^a	16.98 ± 0.36ª	$3.14\pm0.07^{\text{b}}$	0.221 ± 0.005ª	1.247 ± 0.006ª

3.4.2 Y258 allele frequency steadily decreases in H258/Y258 T. urticae populations

We adopted two independent methods to quantify the Y258 allele frequency in populations from an experimental evolution setup: proportional Sanger sequencing and droplet digital PCR (ddPCR). A TaqMan assay was custom designed, validated against individual controls using qPCR (Figure S3-4) and adapted for a more sensitive ddPCR assay for use in pooled samples. Analysis of the control samples using ddPCR showed that the detection limit was 1%.

Propagation of 50:50 mixed experimental populations of R1+ S1 and R3 + S3 on non-sprayed bean plants showed a steady decrease in frequency of the mutant Y258 allele over a period of 24 weeks, strongly suggesting a fitness cost. With proportional sequencing, the allele frequency of Y258 could no longer be detected after 12 weeks (Figure 3-2D), 16 weeks (Figure 3-2B), 18 weeks (Figure 23-F), 20 weeks (Figure 3-2A and 3-2E) and 22 weeks (Figure 3-2C). In contrast, using ddPCR, allele frequencies were detected throughout the experiment (Figure 3-2). By the end of 24 weeks (approx. 12 generations), the allele frequency of Y258 had decreased from the initial 50% to 4.93%. The divergent 2-D ddPCR plots for population samples from week 0 and 24 are shown in Figure S3-4.



Figure 3-2 Decline in Y258 allele frequency over a period of 24 weeks. Panels A, B and C represent cage 1, 2 and 3 respectively of the mixed population 50R1:50S1 (founding population of 100 adult females of line R1 and 100 adult females of line S1). Panels D, E and F represent cage 1, 2 and 3 respectively of the mixed population 50R3:50S3 (founding population of 100 adult females of line R3 and 100 adult females of line S3). Solid lines depict droplet digital PCR (ddPCR) data while dashed line show the data obtained by proportional sequencing.

3.4.3 Activity of complex II is significantly reduced in Y258 lines of T. urticae

To assess whether H258Y results in lower complex II activity, *in vitro* assays with extracted mitochondria were carried out. Succinate dehydrogenase oxidizes succinate to fumarate with FAD as cofactor to generate reduction equivalents in form of FADH₂. Electrons are then sequentially passed over to three iron-sulfur clusters and finally accepted by ubiquinone or its derivative decyl-ubiquinone with better water solubility used in this assay. However, instead of feeding electrons in the physiological electron acceptor complex III (cytochrome c oxidoreductase), decyl-ubiquinol is forced to deliver the electrons to the dye DCPIP in this assay setup, as complex III is inhibited by myxothiazol. DCPIP reduction did not occur when no mitochondrial sample was added. Furthermore, after addition of 10 mM succinate to wells containing mitochondria, DCPIP reduction was inhibited in both H258 and Y258 samples by more than 93 % when 1 μ M atpenin A5 was present. Hence, this assay specifically assessed succinate:ubiquinone oxidoreductase (SQR) activity.

Mitochondrial preparations from the control H258 (S) lines reached a maximum SQR velocity of 462 nmol min⁻¹ mg⁻¹. In comparison, the Y258 (R) lines showed significantly lower specific SQR activity, amounting to 221 nmol min⁻¹ mg⁻¹ (p < 0.0001, paired two-tailed t-test, n = 714) (Figure 3-3a). The K_m for the SQR reaction was however not altered (S = 195 pmol min⁻¹ µg⁻¹, R = 190 pmol min⁻¹ µg⁻¹, p > 0.05, paired two-tailed t-test, n = 714). In contrast, maximum NCCR activity was 18 % higher when mitochondria from the R lines were compared to the control S lines (p = 0.0021, paired two-tailed t-test two-taile

test, n = 112). 82 nmol cytochrome c were reduced per minute and μ g mitochondrial protein when they carried Y258, whereas the maximum rate in H258 mitochondria amounted to 67 nmol min⁻¹ μ g⁻¹ total protein (Figure 3-3b).



Figure 3-3 In panel a, comparison of catalytic SQR activity in *T. urticae* mitochondria extracts from Y258 (R) line and the control H258 (S) lines. K_M (S = 195 pmol min⁻¹ μ g⁻¹, R = 190 pmol min⁻¹ μ g⁻¹) remains unchanged, whereas V_{max} in mitochondria from the R lines is 48 % lower than in S lines (S = 462 nmol min⁻¹ mg⁻¹, R = 221 nmol min⁻¹ mg⁻¹, p < 0.0001, paired two-tailed t-test, n = 714). In panel b, comparison of NCCR activity in R and S lines. Maximum reaction velocity of the S lines accounted 67 nmol min⁻¹ μ g⁻¹, which was significantly lower than 82 nmol min⁻¹ μ g⁻¹ that were calculated for the R lines (p < 0.0021, paired two-tailed t-test, n = 112). Michaelis-Menten constants did not differ significantly (S = 1.17 pmol min⁻¹ μ g⁻¹, R = 1.1 pmol min⁻¹ μ g⁻¹, p > 0.05). Error bars represent the standard deviation. n = 16.

3.5 Discussion

Only a few studies have reported the involvement of target site mutations in resistance to acaricidal SDH inhibitors (Maeoka and Osakabe, 2021; Chapter 2; Sugimoto et al., 2020). The target-site mutations H258Y, I260V/T in subunit B and S56L in subunit C of the succinate dehydrogenase (SDH) complex have been shown to cause high levels of resistance to SDH inhibitors cyenopyrafen, pyflubumide and/or cyflumetofen (>5000mg ⁻¹L⁻¹) (Maeoka and Osakabe, 2021; Chapter 2). In some instances, laboratory selection with SDH inhibitors has led to high cross-resistance and fixation of the resistance mutation (Maeoka and Osakabe, 2021; Chapter 2), but whether there are fitness costs associated with these mutations is yet to be investigated. Target site resistance mutations among fungal SDH inhibitors and especially mutations involving the highly conserved histidine residue in subunit B of SDH appear to be common and have no associated fitness cost (Fraaije et al., 2012; Scalliet et al., 2012; Sierotzki and Scalliet, 2013). Here, we quantified the potential pleiotropic fitness effects of H258Y, a mutation associated with high-cross resistance to cyenopyrafen and pyflubumide in *T. urticae*. As the mutation is located in a highly conserved ubiquinone binding (Q) site, we hypothesized that it could impair the functioning of SDH.

Analysis of individual life history traits and fertility life table parameters did not reveal a consistent significant difference across all Y258 (R) lines. However, line R3 differed significantly from its control

line S3 in reproductive rate, intrinsic rate of increase, finite rate of increase and doubling time. A possible explanation to the different characteristics of the three R lines could be segregating genetic variation still present in the reference London strain used in generating these near isogenic lines. In chapter 2, it was noted that the susceptible London strain was not inbred, and therefore allelic variants still segregate in the population which could result in complex (epistatic) interactions not fully understood. Although we acknowledge that the study of fitness costs using fertility life table parameters provides detailed information about the specific biological components underlying a fitness cost, the method suffers from the fact that only a selection of biological parameters is assessed under constant environment, and is dependent on population/line sampling to control for variation in fertility and mating behaviour (Prout, 1971). Therefore, it remains possible that some pleiotropic effects only appear under specific (stressful) conditions such as food shortage, high rate of migration and at certain population densities (ffrench-Constant and Bass, 2017; McKenzie, 1996).

Indeed, to get a good estimate of fitness costs associated with a resistance mutation, a good experimental design should also include the population-cage approach where resistant and susceptible genotypes are kept in direct competition for several generations without a selection pressure (McKenzie, 1996). This experimental evolution setup better resembles a natural population setting and therefore provides a better estimate of the complete fitness costs (Bourguet et al., 2004). Stability or fluctuations of allele frequencies between generations is used as a measure of presence or absence of fitness costs. Here, a fitness cost of the H258Y mutation was observed when the Y258 (R) and H258 (S) lines were kept in competition for a period of six months without acaricide treatment. From the initial 50SS:50RR genotypes, frequency of the Y258 allele steadily decreased and remained below ≤15% as observed using both proportional Sanger sequencing and droplet digital PCR (ddPCR) methods. Allele frequency estimates determined in pooled DNA sample vary due to differences in DNA yields of individuals in the pooled samples (Rode et al., 2018). This variation can be limited leading to a higher achieved precision by increasing the number of replicates per bulk sample. Unfortunately, this also increases the cost of sample processing and sequencing (Sudo and Osakabe, 2022). The lack of a perfect correlation between allele frequency values obtained using ddPCR and proportional sanger sequencing can be attributed to a number of reasons. The sanger sequencing method has a relatively low sensitivity and suffers from the fact that different sequencing chromatographs, and thus relative abundance of peak residues, are obtained when using the forward and reverse primers (Sudo and Osakabe, 2022). By adopting the highly sensitive ddPCR technique developed by Mavridis et al., (2021), with overall detection limit of 1%, much lower allele frequencies can be detected in the pooled DNA samples. This can be very useful in the field to detect resistance mutations in the early phase of resistance development when resistant allele frequencies are usually very low.

In contrast to life table analysis, but consistent with the competition experiments, the in vitro assays with extracted mitochondria revealed a fitness cost in respect to succinate ubiquinone oxidoreductase (SQR) activity, showing a 48% lower specific activity of SQR in the Y258 (R) lines as compared to the control H258 (S) lines. Such impairment of complex II activity as a result of resistance mutations has previously been reported in the fungal species Mycosphaerella graminicola (Scalliet et al., 2012). Mutation of H267 (corresponding to H258 in T. urticae) to either L,N,Q or Y resulted in reduction of SDH activity to 13%, 20%, 13% and 9% respectively (Scalliet et al., 2012). However, the presence of a fitness penalty in vivo was not demonstrated in spite of the reduced complex II activity (Scalliet et al., 2012). As similar Q site substitutions influence oxidative growth of the fungi Saccharomyces cerevisiae through an increased production of reactive oxygen species (ROS) by the mutated SDH enzyme (Guo and Lemire, 2003; Ishii et al., 1998; Szeto et al., 2007), the lack of a significant growth defect in M. graminicola suggests that the likely increased ROS production is countered by a strong antioxidant defense system in this species. Indeed, *M. graminicola* is a hemibiotrophic fungus, therefore has to survive plant induced oxidative stress in the colonized plant tissue (Scalliet et al., 2012). M. graminicola is equipped with an efficient ROS detoxification system that enables this fungus to survive this stage of its lifecycle (Keon et al., 2007). Additionally, most fungi unlike animals possess a branched respiratory chain consisting of alternative NADH dehydrogenases that enable cytoplasmic NADH to be used directly and alternative oxidases that enable respiration to continue in the presence of complex III and IV inhibitors (Joseph-Horne et al., 2001; Wood and Hollomon, 2003). This metabolic flexibility in fungi could also be the reason why SDH mutations are frequently encountered in the field (see Sierotzki and Scalliet., (2013) for a review on SDH mutations in field populations of fungi) as respiration is expected to continue even with a dysfunctional complex II.

As normalization based on protein content might be insufficient for the mitochondrial SQR assays, which could lead to overall lower respiratory activity in preparations between strains, we also performed a combined complex I + III assay. Interestingly, mitochondria from the resistant strain displayed 18 % higher NCCR activity (coupling complex I and complex III activity *in vitro*), and thus ruling out that the lower complex II activity is a preparation or normalization artefact. In addition, it is biologically conceivable that, to compensate the fitness cost by impaired SQR activity, abundance of the essential electron transferring ubiquinone pool is increased in the mutant strain via complex I and complex III. This is supported by the fact that there was no ubiquinone derivative added in the NCCR assay, but rather the presence of intrinsic ubiquinone was rate limiting, and NCCR activity was found to be significantly higher in Y258 lines. Both SQR and NCCR activity rely on bridging electron transfer mediated by the ubiquinone pool. As the added amount of the water-soluble ubiquinone derivative decyl-ubiquinone was equal in all mitochondrial samples in SQR assays, the resistance conferring

mutation probably decreases ubiquinone affinity of the active site as well. This is supported by cocrystallization data, as the respective acaricide inhibitors and other SDH inhibitors share a common binding motif in the ubiquinone binding site of SDH (Huang et al., 2021). The study in chapter 2 showed that cyenopyrafen, pyflubumide and the ubiquinone analogue atpenin A5 have a decreased affinity for the Q-site as a result of the mutation. The succinate binding site resides in subunit A and electrons are passed through three distinct iron sulfur clusters in subunit B before they reach the heme binding, membrane-bound subunits C and D assisting ubiquinone reduction at their interface. Hence, it is unlikely that succinate oxidation in sdhB is impaired in the mutant strain, but rather the final electron transfer to ubiquinone is impeded because of its lower binding probability. To compensate for this severe fitness cost, increased expression levels of the other respiratory chain complexes NADHdehydrogenase and cytochrome c oxidoreductase might play a role besides raised ubiquinone abundance, but this awaits further investigation.

The mitochondrial respiratory chain (MRC) plays a fundamental role in energy metabolism, maintenance of redox balance and cellular energy levels (Szeto et al., 2010). SDH catalyzes the oxidation of succinate to fumarate, coupled with the reduction of ubiquinone to ubiquinol. As such, SDH is the only mitochondrial complex linking the tricarboxylic acid cycle with oxidative phosphorylation (Sun et al., 2005). Metabolic profiling of yeast revealed significant alterations in the yeast metabolism as a result of mutations in SDH (Szeto et al., 2010). In mammals, studies using mouse models and mammalian cell lines indicate that the loss of SDH enzyme activity and expression due to inactivating mutations in the SDH genes reprograms energy metabolism, altering amino-acid metabolism and hyper-activating glycolysis, TCA cycle and β -oxidation pathways (Lussey-Lepoutre et al., 2015; Mu et al., 2018). It is highly likely that the low SDH activity resulting from H258Y also results in global alterations to metabolism in spider mites. Under optimal conditions, the resources needed to make these metabolic alterations are available, hence the mites are able to maintain the balance between energy storage and conversion. This could also be the reason why no fitness costs were revealed in life table analysis as conditions were optimal. In a population setting however, resources are limited as spider mites are living in competition for food and space, making it difficult to maintain the balance in energy storage and conversion with a deficient SDH. Probably why the fitness costs were apparent in the experimental evolution setup.

H258Y has not yet been reported in recent resistance screens of field populations (inak et al., 2022b; Sugimoto et al., 2020), which might be associated with the high fitness cost. It is likely that the strong impairment of complex II activity by H258Y (and potentially other mutations in complex II) would result in a rapid decrease of allele frequency in the absence of selection pressure, as we have observed in our experimental evolution setup. In support of this hypothesis, a similar intrinsic correlation between
resistant allele frequencies and altered enzyme activity was reported for acetylcholine esterase (AchE) mutations in *Drosophilla melanogaster* (Shi et al., 2004). AchE catalyzes hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, a reaction that is inhibited by organophosphates. *AchE* mutations have been associated with resistance to organophosphates in insects and mites. Four mutations 1161V, G368A, F330Y and G265A are widespread in insect populations (Menozzi et al., 2004). The first three cause a drastic decrease in the activity of AchE, while G265A increases the activity of AchE in *Drosophila melanogaster* (Shi et al., 2004). Consequently, the mutation G265A had the highest allele frequency (20%) in 29 *D. melanogaster* field populations while 1161V, G368A and F330Y had 5.4%, 5.4% and 3.6% respectively (Shi et al., 2004).

The high fitness cost associated with H258Y is very favorable for resistance management strategies for SDH inhibitors in *T. urticae*. In addition, we present here a ddPCR method that can be used to detect resistance alleles at very low frequency. Once detected, resistance management strategies in the sprayed areas can exploit the selective advantage of susceptible individuals by extending periods between acaricides treatments and by alternating SDH inhibitors with a different mode of action group (Bajda et al., 2018; Leeper et al., 1986). Although a reversal to susceptibility in *T. urticae* can occur relatively rapid under laboratory conditions as our results suggest, under field conditions, the migration of susceptible or resistant mites from other host plants may influence the time taken to reestablish susceptibility (Dunley and Croft, 1992; Grafton-Cardwell et al., 1991; Miller et al., 1985; Osakabe et al., 2009).

3.6 Conclusion

In this study, the impact of H258Y on spider mite fitness was quantified by analyzing various biological components of fitness, *in vitro* activity of mitochondrial complexes and allele competition in an experimental evolution setup. The mutation did not induce a reproducible significant change in the single generation life history traits and fertility life table parameters. However, a steady decrease in frequency of the resistant allele was observed when artificial populations of susceptible and resistant genotypes were kept in competition without a selection pressure, suggesting a fitness cost. The presence of a fitness cost was corroborated by the strong impairment of the activity of complex II, and compensatory mechanisms via other mitochondrial complexes might be at play. Our findings can be used for rational management of resistance, which will delay the development of resistance to acaricidal SDH inhibitors.

Funding information

This work was supported by the Research Council (ERC) under the European Union's Horizon 2020 research and innovation program, grant 772026-POLYADAPT to T.V.L. and 773902–SUPERPEST to T.V.L. N.W. was supported by BOF fellowship (Ghent University, 01P03420) throughout this project.

Supplementary information

All supplementary data can be found at https://doi.org/10.1002/ps.7640

Table S3-1. Measure of skewness of the data distribution (total fecundity/ female within a line) for the *Tetranychus urticae* lines used in this study.



Supplementary Figure S3-1 TaqMan assay for the detection of the H258Y mutation in individual mite samples. Graph and sample calling were generated made using the Allelic Discrimination module of the Bio-Rad CFX Maestro 2.3 software.



Supplementary Figure S3-2 Visualization of total development time of *T. urticae* females (left) and males (right) of Y258 (R) lines and H258 (S) lines. Error bars represent standard error of the mean. N = 4



Supplementary Figure S3-3 Visualization of pseudo-values of the five fertility life table parameters reproductive rate (R0), intrinsic rate of increase (rm), finite rate of increase (LM), doubling time (DT) and generation time (T).



Supplementary Figure S3-4 2-D ddPCR amplitude plots (Channel 1-FAM vs Channel 2-HEX) for representative samples at Week 0 (H258Y allelic frequency = 49.05%) (Part A) and Week 24 (H258Y allelic frequency = 4.93%) (Part B).

Authors' contributions

TVL and NW conceived the study. CN, NW and TVL designed the experiments. CN, CS and KM performed the experiments. CN, CS and KM analysed data. CN wrote the manuscript with significant input from NW, SG, JV and TVL. All authors read and approved the final manuscript.

Chapter 4: The host plant strongly modulates acaricide resistance levels to mitochondrial complex II inhibitors in a multi-resistant field population of *Tetranychus urticae*

This chapter has been partially redrafted from:

Christine Njiru*, Marilou Vandenhole*, Wim Jonckheere, Nicky Wybouw and Thomas Van Leeuwen. (2023) The host plant strongly modulates acaricide resistance levels to mitochondrial complex II inhibitors in a multi-resistant field population of *Tetranychus urticae*. Pesticide Biochemistry and Physiology, 196:105591.

*Contributed equally to this study

4.1 Abstract

The two spotted spider mite *Tetranychus urticae* is a polyphagous pest with an extraordinary ability to develop acaricide resistance. Here, we characterize the resistance mechanisms in a T. urticae population (VR-BE) collected from a Belgian tomato greenhouse, where the grower was unsuccessful in chemically controlling the mite population resulting in crop loss. Upon arrival in the laboratory, the VR-BE population was established both on bean and tomato plants as hosts. Toxicity bioassays on both populations confirmed that the population was highly multi-resistant, recording resistance to 12 out of 13 compounds tested from various mode of action groups. DNA sequencing revealed the presence of multiple target-site resistance mutations, but these could not explain resistance to all compounds. In addition, striking differences in toxicity for six acaricides were observed between the populations on bean and tomato. The highest difference was recorded for the complex II inhibitors cyenopyrafen and cyflumetofen, which were 4.4 and 3.3-fold less toxic for VR-BE mites on tomato versus bean. PBO synergism bioassays suggested increased P450 based detoxification contribute to the host-dependent toxicity. Given the involvement of increased detoxification, we subsequently determined genomewide gene expression levels of VR-BE on both hosts, in comparison to a reference susceptible population, revealing overexpression of a large set of detoxification genes in VR-BE on both hosts compared to the reference. In addition, a number of mainly detoxification genes with higher expression in VR-BE on tomato compared to bean was identified, including several cytochrome P450s. Together, our work suggests that multi-resistant field populations can accumulate a striking number of target-site resistance mutations. We also show that the host plant can have a profound effect on the P450-associated resistance levels to cyenopyrafen and cyflumetofen.

4.2 Introduction

The spider mite *Tetranychus urticae* is one of the most polyphagous arthropod herbivores, with the ability to feed on a wide range of plant species including several economically important agricultural crops (Jeppson et al., 1975; Migeon et al., 2010). The major control method for *T. urticae* is based on the use of acaricides that are classified based on the mode of action (MoA), including but not limited to, sodium channel modulators such as bifenthrin; glutamate-gated chloride channel modulators such as abamectin; mite growth inhibitors affecting chitin synthase I such as etoxazole; mitochondrial electron transport complex I inhibitors such as pyridaben, fenpyroximate and tebufenpyrad; complex II inhibitors such as cyenopyrafen and cyflumetofen; complex III inhibitors such as acequinocyl and bifenazate; ATP synthase inhibitors such as fenbutatin oxide; and acetylCoA carboxylase inhibitors such as spiromesifen (Sparks and Nauen, 2015). However, *T. urticae* is able to quickly develop resistance regardless of the chemical class, with the first case of resistance often reported a few years after introduction of a new acaricide (De Rouck et al., 2023).

Utilizing a broad host range comes with a significant challenge as polyphagous herbivores are exposed to divergent mixtures of plant produced defense compounds. These compounds can be extremely diverse and/or highly toxic. The ability to metabolize and detoxify plant chemicals is considered one of the major responses that arthropod herbivores have evolved (Després et al., 2007; Futuyma and Agrawal, 2009; Simon et al., 2015). For spider mites, this is reflected in an exceptionally strong toolkit to detoxify xenobiotic compounds, including laterally acquired genes from microorganisms with novel metabolic abilities, expansion of detoxification gene families and a fine-tuned transcriptional plasticity in response to host plant transfer (Dermauw et al., 2013; Grbić et al., 2011; Snoeck et al., 2018; Wybouw et al., 2016, 2015). Even the mite's salivary composition may be tailored to its current host (Jonckheere et al., 2017, 2016), potentially to optimize interactions with the plant's defense response (Blaazer et al., 2018; Villarroel et al., 2016). Transcriptional plasticity and genetic variation determining gene-expression regulation of these adaptation genes might be a key factor in allowing polyphagous herbivores to colonize diverse host plant species (Brattsten, 1988; Castle et al., 2009; Kurlovs et al., 2022; Liang et al., 2007; Yu, 1986). It has been suggested that the evolutionary history of polyphagy might have led to the ability to better cope with anthropogenic pesticides (Alyokhin and Chen, 2017; Dermauw et al., 2018). Indeed, several studies have shown that adaptation to different host plants or even short term exposure to different plant chemicals alters the herbivore's sensitivity to pesticides (Castle et al., 2009; Gould et al., 1982; Liang et al., 2007; Pym et al., 2019; Yang et al., 2001).

Resistance mechanisms to natural and synthetic toxins can be broadly classified into (i) toxicodynamic changes that involve a reduction in the sensitivity or availability of the target-site due to point mutations, gene knockout or amplification; and (ii) toxicokinetic changes that reduce the amount of toxic chemicals that reach the target-site through changes in metabolism, penetration, transportation, exposure and excretion (Feyereisen et al., 2015). Metabolic resistance to natural and synthetic xenobiotics is known to commonly rely on increased expression of genes that belong to large multi-gene families such as cytochrome P450 monooxygenases (CYPs), carboxyl/cholinesterases (CCEs), glutathione-S-transferases (GSTs), UDP-glycosyl transferases (UGTs) and xenobiotic transporters such as ABC-transporters (De Rouck et al., 2023; Van Leeuwen and Dermauw, 2016). Novel gene families have also been implicated in xenobiotic metabolism and transport, including: intradiol ring cleavage dioxygenases (DOGs), lipocalins, short chain dehydrogenases (SDRs) and the major facilitator superfamily (MFS) (Dermauw et al., 2013; Wybouw et al., 2015; Zhurov et al., 2014). Recent work has provided formal evidence that some of these detoxification enzymes can metabolize plant allelochemicals (Chapter 5).

In *T. urticae* and other agricultural pests, high levels of pesticide resistance has often been attributed to a combination of target site mutations and detoxification enzymes, which suggests that resistance

traits can involve multiple genetic factors (for a review see De Rouck et al., 2023). Here, we used various approaches to characterize a population of *T. urticae* collected from a tomato greenhouse near Antwerp (Belgium) that could no longer be controlled by the registered available acaricides, resulting in crop failure. Upon arrival in the laboratory, we created two sub-populations: one on tomato as the original host and one on bean as the standard laboratory host of *T. urticae*. First, the efficacy of 13 commercially important acaricides from different MOA groups was investigated in toxicity assays performed on both hosts. Next, molecular assays were used to uncover known target-site mutations. To explain the observed patterns, synergism experiments were performed together with transcriptome sequencing of the resistant population on both hosts and a susceptible reference. The results were discussed in the light of a pest management strategy.

4.3 Materials and methods

4.3.1 T. urticae populations

The German susceptible strain (GSS) is a reference strain that has been reared without pesticide exposure for more than five decades (Stumpf et al., 2001). A red morph *T. urticae* which we named <u>very r</u>esistant <u>Be</u>lgian (VR-BE) population, was collected from a tomato greenhouse near Antwerp (Belgium) in 2021 where the grower was unsuccessful in controlling it using commercial formulations of abamectin, hexythiazox, bifenazate, spirodiclofen and cyflumetofen. Upon arrival in the laboratory, VR-BE was transferred to unsprayed potted tomato plants cv. 'Moneymaker' and bean plants cv. 'Prelude' and maintained separately on these two hosts throughout the experiments. Both populations will be referred to as VR-BE tomato and VR-BE bean, respectively. All mite populations were reared in climatically controlled chambers maintained at 25 ± 1°C and 60% relative humidity (RH) with a 16:8 light:dark photoperiod.

4.3.2 Chemicals

Thirteen formulated acaricides/insecticides were used for toxicity bioassays: abamectin (Vertimec 1.8% EC), acequinocyl (Kanemite 164 g L⁻¹ SC), bifenazate (Floramite 240 g L⁻¹ SC), bifenthrin (Talstar 80 g L⁻¹ SC), cyenopyrafen (Kunoichi 30% SC), cyflumetofen (Scelta 20% SC), etoxazole (Borneo 110 g L⁻¹ SC), fenbutation oxide (Acrimite 550 g L⁻¹ SC), fenpyroximate (Kiron 51.2 g L⁻¹ SC), azadirachtin A (NeemAzal-T/S 10 g L⁻¹ EC), pyridaben (Sanmite 150 g L⁻¹ SC), spiromesifen (Oberon 240 g L⁻¹ SC) and tebufenpyrad (Masai 20 WP).

4.3.3 Toxicity and synergism assays

Dose-response assays on adults or larvae were conducted with a slight modification of the standard method described by Van Leeuwen et al., (2004). Briefly, 20-25 adult females of VR-BE bean and GSS

were placed on the upper side of a 9 cm² kidney bean leaf disc prepared on wet cotton wool. Using a custom-built spray tower (Van Laecke and Degheele, 1993), plates were sprayed with 870 µl of at least five serial dilutions of each acaricide and a control (distilled water) at 1 bar pressure to obtain a homogenous spray film (2 mg aqueous deposit/cm²). At least four replicates were used for each acaricide dose. Female adults of VR-BE tomato were assayed in a similar way but using 9 cm² tomato leaf discs. For the larval bioassays with spiromesifen and etoxazole, 20-30 adult females were placed on 9 cm² tomato or bean leaf discs and allowed to lay eggs for 6 h in a climatically controlled chamber (25 ± 1 °C and 60% RH with a 16:8 light: dark photoperiod). After hatching (3-4 days), larvae were counted and sprayed with 870 μ L of at least five serial dilutions of each acaricide and a water control as previously described. Mortality was assessed after one day for abamectin, cyflumetofen, cyenopyrafen and tebufenpyrad, after two days for azadirachtin A, acequinocyl, bifenthrin, fenpyroximate and pyridaben, after three days for fenbutatin oxide and bifenazate, and after four days for etoxazole and spiromesifen. Mortality on the control replicates never exceeded 10%. For adulticidal assays, mites were considered dead when not being able to walk their own body length within 10 seconds after prodding with a fine brush. For larval bioassays, mites were considered unaffected if they displayed the same development stage as a water treated control at the time of scoring. When 5000 mg L⁻¹ acaricide did not cause 50% mortality, higher concentrations were not tested. Lethal concentration killing 50% of the population (LC_{50}) values, slopes, resistance ratios (RR) and 95% confidence intervals (CI) were calculated by probit analysis using Polo Plus 2.0 software. The RR was considered significant if the 95% CI did not include the value 1 (Robertson et al., 2017).

Synergism assays were conducted as described in Van Pottelberge et al., (2009). Briefly, the synergist piperonyl butoxide (PBO), a P450 mono-oxygenase inhibitor, was dissolved in a 200 μ L mixture of N,N-dimethylformamide and emulsifier W (alkarylpolyglycoether) in a 3:1 w/w ratio and subsequently diluted 100-fold in demineralized water to 1000 mg L⁻¹. Adult female mites of VR-BE _{bean} and VR-BE tomato were transferred to 9 cm² leaf discs and sprayed with the synergist mixture. After 24 h, the surviving mites (about 90% of PBO treated mites) were transferred to fresh leaf discs and used in cyflumetofen toxicity bioassays as described above. The synergism ratios (SR, calculated as LC₅₀ obtained after cyflumetofen treatment alone divided by the LC₅₀ obtained after cyflumetofen and synergist pretreatment) and 95% CI were calculated by probit analysis using Polo Plus 2.0 software (LeOra Software, USA). The SR was considered significant if the 95% CI did not include the value 1.

4.3.4 Detection of target-site mutations

Upon arrival and establishment in the laboratory, approximately 200 adult female mites were collected from the VR-BE tomato population. Genomic DNA was extracted using the DNA blood and tissue kit (Qiagen, Belgium), according to the manufacturer's instructions, and was used as a template for PCR

amplification. PCR amplification was used to screen for known or novel target-site mutations, with a set of well validated primers (De Beer et al., 2022b; İnak et al., 2022a; Khalighi et al., 2015; Simma et al., 2020). Primers used for the amplification and sequencing of different target-site regions are provided in Table S4-1.

For all PCR setups except for *cytb*, the reactions were performed using the Promega GoTaq® G2 DNA polymerase kit in 50 μ l reactions containing 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.3 μ M forward and reverse primer, 1.25 u GoTaq DNA polymerase and 1-2 μ L gDNA template. Cycling conditions were 2 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 52-55°C and 30-120 s at 72 °C; and final extension of 7 min at 72°C. For *cytb*, the Expand Long Range dNTPack was used in 50 μ L reactions containing 1.25 mM MgCl₂, 0.5 mM of each dNTP, 0.3 μ M forward and reverse primer, 3.5 u Expand Long Range Enzyme mix and 2 μ l gDNA template. Cycling conditions were 2 min at 92°C; 40 cycles of 30 s at 92°C, 15 s at 54°C and 2 min at 58°C (with extension time increasing 10 s/cycle after the 10th cycle); and final extension of 7 min at 58°C.

Amplicon purification and sequencing was performed at LGC Genomics GmbH (Germany). The sequencing data were analyzed using BioEdit v.7.0.5 software (Hall, 1999), while visual inspection of chromatograms for segregating SNPs was performed using Unipro UGENE v.37.0 (Okonechnikov et al., 2012). To check the persistence of these target site mutations over time, 200 adult females of VR-BE population were collected after one year on bean. DNA extraction and mutation screening was carried out as described above.

4.3.5 RNA isolation

RNA was extracted from a pool of 150-200 adult females using the RNeasy plus mini kit (Qiagen, Belgium), according to the manufacturer's instructions. Five independent extractions were performed for each population (GSS, VR-BE _{bean} and VR-BE _{tomato}). VR-BE _{bean} was sampled a second time after six months using five replicates. The concentration and integrity of RNA samples were assessed by a DeNovix DS-11 spectrophotometer (DeNovix, USA) and by running a 2 µL aliquot on 1% and 2% agarose gel.

4.3.6 RNA sequencing, mapping and principal component analysis (PCA)

From all RNA samples, Illumina libraries were constructed using the NEBNext Ultra II RNA Library Prep Kit for Illumina. Libraries were sequenced using Illumina NovaSeq6000 generating an output of paired reads of 2 × 150 bp (library construction and sequencing was performed at Genewiz (Germany)). The quality of the RNA reads was verified using FASTQC v.0.11.9 (Andrews, 2010) and reads that passed the quality control were aligned to the annotated *T. urticae* three-chromosome genome assembly

using the two-pass alignment mode of STAR v.2.7.9a with a maximum intron size set to 20 kb (Dobin et al., 2013; Wybouw et al., 2019). Resulting BAM files were sorted by chromosomal coordinate and indexed using SAMtools v.1.11 (Li et al., 2009). HTSeq v.0.11.2 performed read-counting on a per-gene basis with the default settings (Anders et al., 2015). The total read-counts per gene were used as an input for the R-package (R v.4.2.0) DESeq2 v.1.36.0 to perform a PCA analysis; VR-BE bean month 1, VR-BE bean month 6 (referring to samples collected six months apart), VR-BE tomato and GSS. Read counts were normalized via the regularized-logarithm (rlog) transformation function of the DESeq2 package. Using these values, a PCA was performed and plotted for the 5000 most variable genes across all RNA samples using the DESeq2 function PlotPCA (Love et al., 2014).

4.3.7 Identification of viral contaminants

As a drastically lower number of reads of all VR-BE bean samples mapped against the *T. urticae* genome, unmapped reads across all ten VR-BE bean samples were pooled and used as an input for Trinity (v.2.13.2) to construct a *de novo* assembly under default conditions. An NCBI BLASTn search against the non-redundant nucleotide collection database was performed using a random subset of 100 unmapped reads in order to identify the most abundant contaminants present in the RNA samples. Based on relative abundance of the best blast hits with > 95% query cover and > 95% identities with the read, we could identify three main virus species present, and their respective genomes were used for further analysis; *Tetranychus urticae*-associated picorna-like virus 1 isolate Lisbon, complete genome (MK533147.1); *Aphis glycines* virus 1 isolate Lisbon, partial genome (MK533147.1); *Aphis glycines* virus 1 isolate Lisbon, partial genome of the full-length genomes. All reads that could not be mapped against the *T. urticae* genome were mapped against the three viral genomes using the same methods as described above but without setting the maximum intron size.

4.3.8 Differential expression analysis and Gene ontology (GO) enrichment analysis

Differential expression (DE) analysis was performed with DESeq2 v.1.36.0 using the total per-gene read counts generated by HTSeq as input (Love et al., 2014). In first instance, gene expression changes associated with different hosts compared to GSS as a reference was assessed by identifying significantly differentially expressed genes (DEGs, Log_2 Fold Change $|Log_2FC| > 1$, Benjamini-Hochberg adjusted *p* value < 0.05) in the VR-BE bean vs GSS and VR-BE tomato vs GSS comparisons (Benjamini and Hochberg, 1995). From these lists of DEGs, subsets of genes belonging to important detoxification families were made (Kurlovs et al., 2022). A plot showing commonly overexpressed genes in both VR-BE tomato and VR-BE bean was produced with the ggplot2 package v.3.3.6 (Wickham, 2009). To assess the

intrinsic expression change of the VR-BE population due to the host plant change from tomato to bean, the same method was used to identify DEGs in the VR-BE _{bean} vs VR-BE _{tomato} comparison of which a volcano plot, color coded by detoxifying gene family was made using ggplot2 package.

A gene ontology (GO) enrichment analysis was performed on the DEGs in the pairwise comparisons between VR-BE _{bean} and GSS and VRBE _{tomato} and GSS using the R function "enricher" from the package clusterProfiler (v.4.2.2). The GO terms for Biological Processes (BP) and Molecular Functions (MF) were collected based on the *T. urticae* annotation (v 20190125) from the Orcae database (Sterck et al., 2012). Benjamini-Hochberg correction for multiple testing was done by assigning the argument "pAdjustMethod = 'BH'".

4.4 Results

4.4.1 VR-BE has a multi-resistant profile on bean and tomato hosts

Toxicity bioassays revealed that in comparison to GSS, VR-BE tomato and VR-BE bean exhibited resistance to all compounds, except for azadirachtin (Table 4-1). Resistance ratios ranged between 28-8300 fold on tomato and 8.6-3900 fold on bean. Resistance to the mite growth inhibitor etoxazole was extremely high in populations on both hosts, with a RR of > 13000. Comparing the LC₅₀ values of VR-BE tomato and VR-BE bean (RR host), clear differences could be seen with the mitochondria complex II inhibitors cyflumetofen and cyenopyrafen (> 3-fold RR host), complex III inhibitors acequinocyl and bifenazate (~2 fold RR host) and the acetyl-coA carboxylase inhibitor spiromesifen (2-fold RR host), suggesting a (strong) effect of the host plant on acaricide toxicity.

Table 4-1. Probit analysis of mortality data of 13 acaricides on GSS and VR-BE populations.

		GSS susceptible strair	ſ		VR-BE _{bean}			-	VR-BE tomato			
Acaricide	Slope ± SE	LC _{50^a} (95%CI)	χ^2 (df) ^b	Slope ± SE	LC ₅₀ ª (95%CI)	χ^2 (df) ^b	RR°(95%CI)	Slope ± SE	LC ₅₀ ª (95%CI)	χ^2 (df) ^b	RR° (95%CI)	RR ^d host
Abamectin	5.1 ± 0.73	0.76 (0.67 - 0.86)	30 (25)	2.3 ± 0.17	66 (55 - 79)	30 (21)	87 (73 - 100)	4.6 ± 0.76	91 (70 - 110)	32 (22)	120 (99 - 150)	1.4 (1.1-1.7)
Acequinocyl	3.4 ± 0.25	9.3 (8.3 - 10)	22 (18)	3.7 ± 0.28	240 (210 - 270)	24 (18)	26 (23 - 30)	1.8 ± 0.16	440 (360 - 560)	26 (18)	47 (39 - 58)	1.8 (1.5-2.2)
Azadirachtin	2.6±0.21	120 (100 -140)	18 (18)	4.1 ± 0.64	120 (90 - 150)	28 (18)	1.0 (0.80 - 1.2)	4.0 ± 0.34	120 (110 -140)	19 (16)	1.0 (0.84 - 1.2)	1.0 (0.8-1.2)
Bifenazate	2.7 ± 0.41	2.2 (1.3 - 2.8)	27 (18)	0.83 ± 0.05	180 (130 - 250)	45 (33)	84 (59 - 120)	1.2 ± 0.11	440 (330 - 570)	36 (30)	200 (140 - 280)	2.4 (1.7-3.4)
Bifenthrin	1.4 ± 0.11	3.3 (2.7 - 4.1)	34 (26)	0.95 ± 0.08	360 (250 - 490)	26 (26)	110 (73 - 150)	0.83±0.07	310 (210 - 440)	36 (30)	92 (63 - 130)	0.9 (0.5-1.4)
Cyenopyrafen	9.7 ± 1.2	0.64 (0.58 - 0.70)	20 (18)	1.8 ± 0.10	39 (34 - 45)	26 (25)	52 (44 - 62)	2.3 ± 0.23	170 (130 - 210)	32 (21)	230 (190 - 280)	4.4 (3.5-5.5)
Cyflumetofen	2.1±0.17	6.2 (5.4 - 7.3)	16 (22)	3.5 ± 0.23	54 (48 - 59)	27 (22)	8.6 (7.2 – 10)	1.6 ± 0.16	180 (140 - 220)	14 (25)	28 (22 - 37)	3.3 (2.6-4.1)
Etoxazole	1.1 ± 0.18	0.38 (0.27 - 0.55)	5.3 (16)	1.1 ± 0.21	>5000		>13000	0.06 ± 0.05	>5000		>13000	
Fenbutatin	2.7 ±0.23	80 (68 - 93)	19 (18)	1.1 ± 0.21	>5000		> 63	1.1 ± 0.19	>5000		> 63	
oxide												
Fenpyroximate	1.4 ± 0.15	61 (49 - 79)	13 (18)	1.5 ± 0.59	>5000		>82	0.65 ± 0.21	>5000		>82	
Pyridaben	3.2 ± 0.24	50 (46 - 56)	14 (18)	0.63±0.38	>5000		>100	2.6 ± 0.41	>5000		>100	
Spiromesifen	3.4±0.38	0.28 (0.22 - 0.33)	28 (18)	0.89 ± 0.13	1100 (460 -	33 (24)	3900 (2300-	1.6 ± 0.23	2300 (1700 -	16 (18)	8300 (6300 -	2.1 (1.2-3.7)
					1800)		6700)		2800)		11000)	
Tebufenpyrad	2.8±0.23	30 (27 - 33)	19 (30)	1.2 ± 0.17	>5000		>167	2.3 ± 0.66	>5000		>167	
^a LC ₅₀ is expresse	d in mg active	ingredient L ⁻¹										
$^{ m b}\chi^2$ is the Chi squ.	are goodness	of fit value and (df) is	the degrees	of freedom								
^c Resistance ratio	= LC ₅₀ VR-BE/	LC ₅₀ GSS										
^d Resistance ratio	host = LC ₅₀ V	R-BE tomato / LC ₅₀ VR-B	E _{bean}									

71

4.4.2 Target-site resistance mutations of VR-BE

PCR screening of VR-BE tomato upon establishment in the laboratory revealed the presence of several known target site resistance mutations (Table 4-2, see Table S4-1 for all target site mutations screened). Specifically, the I1017F substitution in chitin synthase 1, which is associated with high resistance to mite growth inhibitors (Demaeght et al., 2014; Van Leeuwen et al., 2012), was fixed. Similarly, the acetylcholinesterase mutation F331W associated with resistance to organophosphates and carbamates (Anazawa et al., 2003; Khajehali et al., 2010; Kwon et al., 2010b); the PSST homologue mutation H92R, associated with resistance to fenbutatin oxide (De Beer et al., 2022); the ATP synthase mutation V89A, associated with resistance to fenbutatin oxide (De Beer et al., 2022b) were also found to be fixed in the 200 mites sampled from VR-BE tomato. The recently identified abamectin resistance mutation I321T (Xue et al., 2020) found in subunit 3 of the glutamate chloride channel was segregating in the population. The screening did not reveal any known or novel candidate non-synonymous resistance mutations in the voltage gated sodium channel, mitochondrial succinate dehydrogenase subunits (complex II) and acetyl-CoA carboxylase. After one year on bean, estimated allele frequencies of the fixed mutations I1017F, F331W, H92R and V89A remained at 100%, while allele frequency of the segregating I321T mutation decreased from 50% to 20% (Table 4-2).

		Freq	uency (%)			
Target gene	Substitution	Initial	After one	Status	Compounds	
			year			
tetur03g08510 (CHS1)ª	I1017F	100	100	Fixed	Etoxazole, clofentezine,	
					hexythiazox	
tetur10g03090 (GluCl3)ª	I321T	50	20	Segregating	Abamectin	
tetur06g03780 (ATP	V89A	100	100	Fixed	Fenbutatin oxide	
synthase)ª						
tetur07g05240 (PSST) ^b	H92R	100	100	Fixed	Fenpyroximate, pyridaben,	
					tebufenpyrad	
tetur19g00850 (AChE)°	F331W	100	100	Fixed	Organophosphates	
^a Numbering of the substitution according to the reference species <i>Tetranychus urticae</i>						
^b Numbering of the substitut	ion according to th	e reference	species Yarrow	via lipolytica		
^c Numbering of the substitut	ion according to th	e reference	species Torped	o californica		

Table 4-2. Target site mutations identified in VR-BE.

4.4.3 PBO synergizes cyflumetofen toxicity on VR-BE tomato

To assess whether metabolic detoxification, in particular cytochrome P450 metabolism, could be at least partially responsible for the observed decrease in sensitivity to some acaricides in VR-BE tomato

relative to VR-BE _{bean}, synergism assays with PBO were carried out. Pre-treatment with PBO enhanced the toxicity of cyflumetofen by 3-fold in VR-BE _{tomato} but not in VR-BE _{bean} (Table 4-3), suggesting the increased metabolic detoxification by P450s of cyflumetofen in the population on tomato.

Population	Treatment	Slope ± SE	LC ₅₀ ^a (95% CI)	χ ^{2 b} (df)	SR º (95% CI)		
VR-BE _{bean}	Cyflumetofen	3.5 ± 0.23	54 (48-59)	27 (22)			
	PBO + cyflumetofen	3.8 ± 0.33	45 (39 - 50)	25 (18)	1.2 (1.0 - 1.3)		
VR-BE _{tomato}	Cyflumetofen	1.6 ± 0.16	180 (140 - 220)	14 (25)			
	PBO + cyflumetofen	6.2 ± 0.75	52 (46 - 58)	28 (18)	3.4 (2.7 - 4.2)		
^a LC ₅₀ is expressed i	n mg active ingredient L-1						
${}^{b}\chi^2$ is the Chi square goodness of fit value and (df) is the degrees of freedom							
^c Synergism ratio =	LC ₅₀ without PBO treatmen	t/ LC ₅₀ after PBO	treatment				

Table 4-3. Probit mortality of cyflumetofen in the VR-BE populations after pretreatment with synergist PBO

4.4.4 RNA sequencing reveals presence of viruses in VR-BE bean and GSS populations

Illumina sequencing resulted on average in approximately 30 million paired reads per sample (raw reads were deposited on NCBI SRA database under BioProject PRJNA1006202). Alignment of RNAseq reads against the *T. urticae* annotation resulted in an overall mapping rate of uniquely mapped reads of 73.59% for VR-BE tomato, 64.66% for GSS and 46.27% for VR-BE bean (Table S4-2). Noteworthy, a large fraction of the reads for VR-BE bean (~ 40%) and GSS (~ 18%) did not map against the T. urticae genome which hints towards a potential contamination. An NCBI BLASTn search of a random subset of the unmapped reads was performed in order to identify the most abundant contaminants present in the RNA samples which identified three virus species; Tetranychus urticae-associated picorna-like virus 1 isolate Lisbon, complete genome (MK533157.1), Tetranychus urticae-associated dicistrovirus 1 isolate Lisbon, partial genome (MK533147.1) and Aphis glycines virus 1 isolate Lisbon, partial genome (MK533146.1) as the main reason for the lower mapping rates against the *T. urticae* genome. Next, a de novo transcriptome assembly of the unmapped reads from VR-BE bean was built under default conditions and a BLASTn search of the viral genomes against the *de novo* assembly identified multiple contigs of > 4 kb that give hits with > 95% identity for each of the three viral genomes studied. For the Tetranychus urticae-associated dicistrovirus 1 isolate Lisbon, partial genome there is even a contig spanning the genome (8290 bp) with 96% identities and with an additional 564 bp in the assembly (Supplementary file S4-1). All reads that did not map against the *T. urticae* genome in both VR-BE bean and GSS samples were then mapped against the three viral genomes to estimate the relative abundance of each virus species (Figure 4-1, Table S4-2). Interestingly, the largest fraction of reads mapped against Tetranychus urticae-associated picorna-like virus 1 for VR-BE bean, whereas for GSS the

largest fraction mapped against *Tetranychus urticae*-associated dicistrovirus 1. *Aphis glycines* virus 1 was only present in VR-BE _{bean} and none of the identified viruses were present in VR-BE _{tomato}. Resampling and sequencing of the VR-BE _{bean} population six months after arrival in the laboratory and transfer to bean yielded a similar result, with a 44.64% uniquely mapped reads matching to the three viruses identified in the first sequencing (Table S4-2).



Figure 4-1. Percentage frequency of uniquely mapped reads. All genes in VR-BE _{bean} that could not be mapped to the *T. urticae* transcriptome were mapped to three viral genomes: picornavirus which was the highest contaminant, followed by the dicistrovirus and aphis glycines virus. GSS was also contaminated with picornavirus and dicistrovirus while VR-BE _{tomate} was not contaminated with the viruses. Error bars represent standard error of the mean. N=5 for GSS and N= 10 for VR-BE _{bean}.

4.4.5 Effect of the host plant on gene expression

Principal component analysis revealed that 72% of the total variation could be explained by principal component 1 (PC1) while 15% could be explained by PC2 (Figure 4-2). Replicates clustered by population and the groups were clearly separated from each other. The two batches of VR-BE _{bean} samples collected six months apart clustered together on PC1. VR-BE _{tomato} was positioned far away from VR-BE _{bean} on PC1, which clearly indicates the dramatic effect the host plant has on gene expression.



Figure 4-2. Principal component analysis (PCA) of gene expression among GSS, VR-BE bean and VR-BE tomato populations of *T. urticae*.

Gene expression patterns in the resistant VR-BE populations were compared to the susceptible GSS. Differential expression analysis identified 1423 upregulated genes in VR-BE bean population compared to GSS, and 80% of these genes (1145 genes) overlapped with VR-BE tomate vs GSS comparison (Figure 4-3a, Table S4-3). A total of 1693 genes were downregulated in VR-BE bean vs GSS, with 82% of these genes (1392 genes) overlapping with the VR-BE tomato vs GSS comparison. 301 genes were overexpressed specifically in the VR-BE tomato vs GSS comparison only, while 650 genes were downregulated in this comparison only (Figure 4-3a, Table S4-3). The overexpression plot shown in Figure 4-3a clearly indicates that multiple gene families that have been implicated in detoxification (CYPs, CCEs, GSTs, UGTs and DOGs) or transport of xenobiotics (MFS and ABCs) were amongst the overexpressed genes in both comparisons, with CYPs showing the highest Log₂ fold changes. Moreover, a gene ontology (GO) enrichment analysis revealed a statistically significant (p adj < 0.05) enrichment of GO terms associated with detoxification and metabolic processes. Results in Table S4-4 show several GO terms associated with cytochrome P450s amongst the enriched GO terms with highest significance in both comparisons (e.g. GO:0055114 "oxidation-reduction process"; GO:0016705 oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen"; GO:0020037 "heme binding"; GO:0005506 "iron ion binding"). To identify the genes differentially expressed in the population on bean versus tomato, gene expression patterns in VR-BE tomato were directly compared to VR-BE bean. Similar to the comparison with GSS, multiple detoxification genes and transporters were differentially expressed in VR-BE tomato vs VR-BE bean (Figure 4-3b). Overexpressed detoxification genes and transporters with a Log₂FC of ≥2 in VR-BE tomato vs VR-BE bean are shown in (Table 4-4).



Figure 4-3. Overview of differentially expressed genes ($|Log_2 FC| \ge 1.0$, *p* adj < 0.05). A) Overexpression plot of differentially expressed genes in VR-BE _{bean} and VR-BE _{tomato} compared to the susceptible GSS; Venn diagram depicting overlap among differentially expressed genes from VR-BE _{bean} vs GSS and VR-BE _{tomato} vs GSS comparisons. B) A volcano plot of the differentially expressed genes in a pairwise comparison of the VR-BE _{tomato} vs VR-BE _{bean}. Genes known to be implicated in detoxification and xenobiotic transport are shown in colours in the two plots: red, cytochrome P450 monoxygenases (CPPs); yellow, carboxyl choline esterases (CCEs); dark blue, intradiol ring cleavage dioxygenases (DOGs); green, glutathione-s-transferases (GSTs); blue, major facilitator superfamily transporters (MFS); pink, short chain dehydrogenases/reductases (SDRs); maroon, UDP-glycosyl transferases (UGTs); and purple, ATP-binding casette transporters (ABC transporters).

Table 4-4. List of highly overexpressed (Log₂FC \geq 2) detoxification genes and transporters in VR-BE tomato compared to VR-BE bean

tetur ID	Description	Log ₂ FC
tetur11g05520	CYP385C4	4.2
tetur20g00290	СҮР392В3	4.3
tetur11g05540	CYP385C3	3.9
tetur03g02810	CCEincTu04	3.3
tetur11g05760	TuCCE34	2.2
tetur05g00060	UGT20	4.5
tetur22g00270	UGT59	3.9
tetur13g04550	TuDOG11	3.3
tetur29g00220	TuGSTd14	2.0
tetur28g01720	SDR	2.5
tetur46g00180	MFS	4.7
tetur40g00030	MFS	3.7
tetur03g09800	TuABCC-10	3.1
tetur03g09880	TuABCC-11	2.4

4.5 Discussion

In this study, we characterized VR-BE, a *T. urticae* field population collected from a tomato greenhouse in Belgium. The grower had reported loss of efficacy after treatment with commercial formulations of abamectin, hexythiazox, bifenazate, spirodiclofen and cyflumetofen, and the crop was lost to spider mites. Occasionally, low efficacy of acaricides results from operational factors such as incorrect spray technique, the use of tank mixes or inappropriate application time (Khajehali et al., 2011). We therefore first confirmed resistance in the laboratory with toxicity bioassays with acaricides of different mode of action groups, revealing that VR-BE was indeed resistant to almost all acaricides tested.

Resistance to a mite growth inhibitor etoxazole and the METI-I acaricides pyridaben, tebufenpyrad and fenpyroximate may be fully explained by the target site mutations I1017F and H92R respectively, which were fixed in VR-BE. However, other mechanisms might also contribute to the high cross-resistance observed with METI-Is. High resolution QTL mapping in *T. urticae* has revealed that cross resistance to METI-Is is not only associated with the target-site resistance mutation, but also possibly cytochrome P450 metabolism (Snoeck et al., 2019a). Validation of the mutation in *T. urticae* by marker assisted back-crossing indeed revealed that the mutation alone only contributes up to 22-fold resistance to fenpyroximate and 30-60 fold resistance to tebufenpyrad and pyridaben (Bajda et al., 2017). A study with resistant field populations has also revealed that additive or synergistic effects of multiple mechanisms most likely determine the phenotypic strength (Xue et al., 2022). As CYPs were among the most highly overexpressed detoxification genes in both VR-BE populations, they might be contributing to the extremely high resistance levels to METI-Is in addition to the H92R mutation.

High resistance to the mitochondrial ATP synthase inhibitor (fenbutation oxide) was recorded in both VR-BE populations, and can be attributed to the fixed mutation V89A but also to metabolic detoxification by CYPs. Using QTL mapping, De Beer et al., (2022b) recently characterized resistance to fenbutatin oxide, revealing that high resistance is likely achieved by a combination of V89A and metabolic detoxification by the P450s *CYP392E4* and *CYP392E6*, which were overexpressed in both VR-BE populations. Similarly, resistance to abamectin may be attributed to I321T mutation in the GluCl3 subunit, which we identified in VR-BE. Since the mutation was not fixed in VR-BE, additional mechanisms might contribute to the observed abamectin resistance. Previous studies have indicated that detoxification enzymes, especially CYPs and UGTs are also involved in abamectin resistance (Çağatay et al., 2018; Riga et al., 2014; Xue et al., 2020). Specifically, functionally expressed CYP392A16 has been shown to hydroxylate abamectin *in vitro* (Riga et al., 2014), but was only moderately overexpressed in both VR-BE populations (~1.6-fold in VR-BE bean and ~1.4-fold in VR-BE tomato) and therefore other P450s might be involved. Similarly, recombinant UGT10 (*tetur02g09830*) and UGT29 (*tetur05g05060*) enzymes have been shown to glycosylate abamectin *in vitro* (Xue et al., 2020). The

genes encoding these enzymes were also overexpressed in both VR-BE populations (2-fold in VR-BE tomato for both UGTs, 2-fold for UGT10 and 3.8-fold for UGT29 in the VR-BE bean), and might be contributing to resistance.

The high levels of resistance to bifenthrin and spiromesifen can likely be exclusively attributed to metabolic detoxification as no target-site mutations were identified in the target sites of these acaricides. De Beer et al., (2022a) recently showed that recombinant CCEinc18 could metabolize bifenthrin, and UGT10 could glycosylate bifenthrin-alcohol. The genes encoding these two enzymes were overexpressed in both VR-BE populations (2-fold in VR-BE tomato for both genes, 1.5-fold for CCEinc18 and 2-fold for UGT10 in VR-BE populations (2-fold in VR-BE tomato for both genes, 1.5-fold for CCEinc18 and 2-fold for UGT10 in VR-BE bean), and potentially contribute to the observed resistance. Previous studies have shown that the P450 enzyme CYP392E10 can metabolize spirodiclofen and spiromesifen (Demaeght et al., 2013), but *CYP392E10* was not among the differentially expressed P450s in our study, suggesting that other mechanisms might be responsible for the observed resistance. Synergism studies have indicated that, in addition to CYPs, CCEs also play an important role in resistance to tetronic/tetramic acid derivatives (lnak et al., 2022; Van Pottelberge et al., 2009b; Wei et al., 2020). Several CCEs were overexpressed in both VR-BE populations (*TuCCE48, TuCCE42, TuCCE04, TuCCE71, TuCCE05, TuCCE49, TuCCE33, TuCCE50* and *TuCCE27*) and might play a role in spiromesifen resistance.

We did not identify any mutation in *cytb* in spite of the moderate resistance recorded with acequinocyl and bifenazate. Although most often associated with maternal inheritance and point mutations in *cytb*, some genetic studies in combination with genome-wide gene expression analysis have revealed that acequinocyl and bifenazate resistance can also have a polygenic inheritance pattern, involving both mutations in the mitochondrial *cytb* gene and overexpression of detoxification genes, especially CYPs (Lu et al., 2023). The involvement of P450-based increased detoxification is further supported by strong synergism with the P450 inhibitor piperonyl butoxide (Sugimoto and Osakabe, 2019). Functionally expressed CYP392A11 has been shown to metabolize bifenazate (Lu et al., 2023), but this P450 was downregulated in both VR-BE populations, likely suggesting alternative mechanisms.

VR-BE also showed moderate resistance to cyflumetofen and cross-resistance to cyenopyrafen, which is not yet registered in Europe. None of the previously reported resistance mutations were detected in VR-BE (mutations reviewed in De Rouck et al., 2023). In contrast, synergism assays with PBO indicated the involvement of P450 detoxification in cyenopyrafen resistance, which is in line with previous studies (Khalighi et al., 2015, 2014; Riga et al., 2015). Functional expression studies have shown that at least CYP392A11 can hydroxylate cyenopyrafen (Riga et al., 2015). However, in VR-BE *CYP392A11* was downregulated versus GSS, and no significant expression differences between hosts

were detected. Other P450s from the 392A subfamily including *CYP392A14, CYP392A9, CYP392A13, CYP392A15* and *CYP392A10*, next to *CYP392D8* and *CYP392D7*, were highly expressed in both VR-BE populations and should be functionally characterized to understand their role in resistance. In addition, TuGST05, a GST enzyme shown to metabolize cyflumetofen (Pavlidi et al., 2017) was not overexpressed in VR-BE, but other GSTs were highly overexpressed in VR-BE on both hosts. These include: *TuGSTd08* (5.5-fold on both hosts), *TuGSTd12* (4.2-fold on bean and 3.2-fold on tomato), *TuGSTd10* (3.5-fold on bean and 2.5-fold on tomato), and *TuGSTd14* (1.3-fold on bean and 3.3-fold on tomato). The functional role of these GSTs should be further investigated.

Although both VR-BE tomato and VR-BE bean were highly resistant to acaricides of different MOA groups, we still observed a strikingly decreased toxicity for six acaricides in the tomato population compared to bean. A caveat of the population comparisons within this study is the lack of replication, since drift and other factors might result in different responses to acaricides. Since specifically the bean population (and not tomato) was contaminated with viruses, we considered the possibility that the presence of viruses might have an influence on acaricide toxicity. Previous studies have identified the presence of viruses in arthropods (Berman et al., 2023; Niu et al., 2019; Wu et al., 2020). Even so, the presence of such large amounts of viral RNA reads has not been reported in previous RNA sequencing datasets of T. urticae (De Beer et al., 2022a, 2022b; Fotoukkiaii et al., 2021; Kurlovs et al., 2022; Lu et al., 2023), although viral infections have been documented in other Tetranychidae such as the Panonychus species (Bajda et al., 2015; Putman, 1970; Reed and Desjardins, 1982; Smith et al., 1959). As such, whether infection occurred by chance or is related to the host plant shift, remains unclear and little is known on how these viruses interact with their hosts. However, all viruses require the hosts machinery to be able to synthesize viral proteins. Indeed, some RNA viruses such as dicistroviridae have evolved elegant strategies to hijack the hosts ribosome (Warsaba et al., 2019). By redirecting the hosts translation machinery, the entire cellular response to stress is compromised, which can include the response to xenobiotic stress. However, synergism assays showed that piperonyl butoxide (PBO), a P450 inhibitor, synergized cyflumetofen toxicity in the tomato population and had no significant effect on the bean population, suggesting that the increased resistance in VR-BE tomato is more likely due to increased detoxification, even though the VR-BE populations were not replicated on both hosts. Indeed, detoxification gene response with host change between bean and tomato has previously been associated with altered acaricide toxicity in T. urticae (Dermauw et al., 2013). Moreover, the fact that cyenopyrafen and cyflumetofen are vulnerable to metabolic attack was indeed already documented in a previous study, where some multi-resistant field populations of T. urticae showed cross-resistance to cyenopyrafen and cyflumetofen, without prior to exposure to these compounds in the field. Cyenopyrafen cross-resistance was specifically linked to the overexpression of P450s (Khalighi et al.,

2015, 2014). Interestingly, of all P450s differentially expressed in VR-BE in comparison to GSS, only *CYP392B3, CYP385C3 and CYP385C4* were specially upregulated in the VR-BE tomato vs VR-BE bean comparison. These P450s were indeed also previously shown to be induced upon mite transfer from bean to tomato (Wybouw et al., 2015). Despite the fact that there is no functional validation at present, these P450s might further elevate resistance levels to complex II inhibitors conferred by other P450s.

Because of the effect of the host on detoxification enzyme activity and acaricide toxicity, we also included a plant derived acaricide containing azadirachtin in toxicity bioassays. Surprisingly, this was the only compound effective on both populations, suggesting that secondary plant metabolites are not necessarily more vulnerable to metabolic attack by higher detoxification associated with different host plants in *T. urticae*.

It has been proposed that, due to their ability to cope with diverse plant defense chemicals encountered during feeding, generalist herbivores such as T. urticae are pre-adapted to evolve pesticide resistance (Alyokhin and Chen, 2017; Dermauw et al., 2013). But, resistance is also known to mainly result from a strong selection imposed by intensive pesticide use, and the relative importance of the evolutionary history associated with polyphagy on resistance development is still a matter of debate (Dermauw et al., 2018). Dermauw et al., (2013) observed highly coordinated changes in gene expression for many genes in tomato-adapted mites and in pesticide-resistant strains, suggesting that adaptation to tomato would also increase tolerance to pesticides. In the current study, a multiresistant field population of T. urticae showed remarkable differences in gene expression when maintained on tomato or bean, and toxicity of some acaricides was reduced in the population on tomato. Similar to Dermauw et al., (2013), most of the differentially expressed genes belonged to gene families that have been commonly implicated in detoxification (CCEs, P450s, GSTs and UGTs) or xenobiotic transportation (ABC transporters). This shows that the host plant influences gene expression in T. urticae, and these host-specific changes in transcript levels of detoxification enzymes influence acaricide toxicity and resistance levels. This observation is further supported by synergism assays, where we show that inhibiting P450s in the tomato population increases toxicity of cyflumetofen, reaching the same level of toxicity as the bean population. Host plant responses have also been shown to affect the toxicity of insecticides to insects. In relation to this, a study with the polyphagous whitefly Trialeurodes vaporariorum revealed considerable differences in transcriptional responses to various host plants, and these changes in gene expression were associated with significant shifts in tolerance of the host-adapted T. vaporariorum lines to pesticides (Pym et al., 2019). Additionally, the role of detoxification enzymes in pesticide resistance and tolerance to plant allelochemicals is well established in insects and mites (Dermauw and Van Leeuwen, 2014; Després et al., 2007; Feyereisen et al., 2015; Heidel-Fischer and Vogel, 2015), especially the functionally diverse

P450s which are expressed in response to phytochemicals (Vandenhole et al., 2021), and whose role in detoxification of xenobiotics has been widely studied (Feyereisen, 2012; Nauen et al., 2021).

Similar to Dermauw et al., (2013), we observed strong differential expression of genes not previously implicated in detoxification. These included lipocalins, small extracellular proteins with the ability to bind hydrophobic molecules (Ahnström et al., 2007; Flower et al., 2000). Therefore, they may bind acaricides or plant toxins, resulting in sequestration of these normally hydrophobic molecules (Dermauw et al., 2013). Genes belonging to the major facilitator superfamily (MFS) were especially highly upregulated in the VR-BE tomate compared to the VR-BE bean. Upregulation of these single polypeptide carriers might result in a higher efflux of acaricides or toxic plant metabolites out of spider mite cells as previously suggested by Dermauw et al., (2013). Additionally, two intradiol ring cleavage dioxygenases (DOGs): TuDOG1 (tetur01q00490) and TuDOG11 (tetur13q04550) were upregulated in VR-BE tomato relative to VR-BE bean (1.6-fold and 7-fold respectively). TuDOG11 has recently been shown to detoxify the tomato metabolites caffeic acid and chlorogenic acid (Chapter 5), and is therefore important in adaptation to tomato. We also observed upregulation of two transcription factors tetur07q01800 and tetur36q00260. The latter belongs to the nuclear receptors family, that is known to be involved in response to stress and xenobiotics in vertebrates and insects (Misra et al., 2011; Pascussi et al., 2008). The two transcription factors were also upregulated in resistant strains and upon adaptation to tomato in Dermauw et al., (2013), and could be playing a role in regulation of gene expression in response to plant allelochemicals or acaricides. Indeed, a recent study quantifying the extent of cis-versus trans-regulation on a genome-wide basis in a collection of multi-resistant T. urticae strains revealed that trans-effects are most abundant, especially for P450s and DOGs (Kurlovs et al., 2022).

4.6 Conclusion

In this study, we confirmed that field failure of a tomato crop to spider mites was due to high levels of resistance to all tested registered acaricides. The presence of target-site mutations could explain resistance to some acaricides, but not all. In addition, resistance levels differed between the population kept on bean or on tomato. This was likely not associated with the presence of large amount of virus in the bean population, but with the induction of detoxification genes on tomato. Further, RNA sequencing revealed large transcriptional differences between the population grown on bean or on tomato, and P450s were shown to contribute to increased resistance levels on tomato.

Funding information

This work was supported by the Research Council (ERC) under the European Union's Horizon 2020 research and innovation program, grant 772026-POLYADAPT to T.V.L. and 773902–SUPERPEST to T.V.L. N.W. was supported a BOF fellowship (Ghent University, 01P03420) throughout this project.

Supplementary information

All supplementary data can be found at <u>https://doi.org/10.1016/j.pestbp.2023.105591</u>

Supplementary file S4-1. Partial genome sequence of Tetranychus urticae associated dicistrovirus 1 isolate Belgium.

Supplementary Table S4-2. Percentage number of uniquely mapped reads and unmapped reads after RNA sequencing.

Supplemenray Table S4-3. Normalized read-counts of all samples and Log_2 fold changes of all differentially expressed genes in all studied comparisons.

Supplementary Table S4-4. Significantly enriched GO terms in VR-BE bean and tomato populations in comparison to GSS.

Gene	Amplicon size (nt)	Mutation(s) screened for	Primer name	Primer sequence (5'-3')	Primer reference
ACCase Tetur21g02170	1550 1296 1510	F1656L ^{Tet} , (A2060V ^{Tet})	ACC_A_FW ACC_A_RV ACC_A_int1 ⁵⁸ ACC_B_int1 ⁵⁸ ACC_B_BFW ACC_B_RV ACC_B_INT1 ⁵⁸ ACC_B_INT1 ⁵⁸	ATATITIGGTCCCCGFGAAGA CGTCATTTCTGTGCATAGCTT AAAGGGGCTGGCTTTATTTT GCCTTTACCGATAATGGAACC AGCCAGAAGGTTCAGTCGAA AGCCAGGAATGATACCTCTAA CTTCGCTATTCCGCCTTTCT TTGATAAAACGCCTTGAGCA	(inak et al., 2022a; Lueke et al., 2020)
AChE Tetur19g08500	965	G1195, A2015, G328A, F331W/C/Y ^{Tor}	TuAChE_one_exon_1kb_F TuAChE_one_exon_1kb_R	TTCAGGTGCATGTTACCAAGTC TCAGTTGCTTCACGATTCTCA	(Simma et al., 2020)
ATP synthase Tetur06g03770 Tetur06g03780	361 399	V89A ^{Tet}	Tetur06g03770_Fw Tetur06g03770_Rv Tetur06g03780_Fw Tetur06g03780_Fw	AACAATTCATCAGTCCTTTTCCA AAGCTAAGCCAATTTGTTGAA CAATTCATCAGTCCTTTCCAA CAATTCATCAGTCCTTTCCAA CAATTCAACCTTTCATTCTTCA	(De Beer et al., 2022b)
CHS1 Tetur03g08510 Cytb (mitochondrial)	543 1577	l1017F ^{ret} G1265 + A1337/136T/5141F/1258F, G132A, I260V (+ N326S), P262T ^{ret}	TuCHS1_dia_F TuCHS1_dia_R Cytbdia2R Cytbdia2R cytbWTF ^{Seq} PEWYF1 ^{Seq} PEWYF1 ^{Seq}	TGTCCGCTTGTTATGCACTACTG GCCACCAAGTGGGGTCAAGAT TTAAGAACTCCTAAAACTTTTCGTTC GAAACAAAATTATTATTTCCCCCAAC GGAATAATTTTACAAATAACTCATGC CGGAATAATTTACAAATAACTCATGC TGGTACAGATCGTAGAATTAGG AAAGGCTCATCTAACCAAATAGG AAAGGGCTCATCTAACCAAATAGG AAAGGGTCATTCTGTAAAAAGGGTATTC	(Van Leeuwen et al., 2012) (Nieuwenhuyse et al., 2009)
GluCl1 Tetur02g04080 GluCl3 Tetur10g03090	262 250	G314D ^{Tet} I321T, G326E, V327G+L329F ^{Tet}	Tu_GluCI1_diag_F Tu_GluCI1_diag_R Tu_GluCI3_diag_F Tu_GluCI3_diag_R Tu_GluCI3_diag_R	TTGGATTGACCCTAACTCAGCA TTGCACCAACAATTCCTTGA CCGGGTCAGTCTTGGTGTTA CACCAAGAACCTGTTGA	(Dermauw et al., 2012)
PSST Tetur07g05240	543	H92R ^{Yar} (=H110R ^{Ter})	PSST_Exon_New_F PSST_Exon_New_R	ACAGGTCAGCCAATCGAATC ATACCAAGCCTGAGCAGTGG	(Bajda et al., 2017)
SdhB Tetur01g15710	895	H258Y/L, I260V/T ^{Tet}	SdhB_F SdhB_R	AGTTGCTTTCCTTGGCTTCA ACCAGTTACTTGGGGGGGCTTT	(Khalighi et al., 2015)

Table S4-1. An overview of target site mutations that the VR-BE population was screened for.

83

	(Khajehali et al., 2011) (Simma et al., 2020)	
AAATCATGTTATTTCCACGTTTGA GCAATTGGTTACGGGTAGTTTAGTAT	CAACATTCAAAGGTTGGACAAT TCTTCCGTCATCAACATCTCC TGATTGTTTTCCGTGTCCTG CGCGGAAGCTGCTTAAGTCC CACGGGACAACGAAACAATC TGCAAACATTGAAG	
sdhC_F SdhC_R	kdrF4 kdrR1 kdrF5 kdrF5 kdrR2 34g00970_super_kdr_exon_F 34g00970_super_kdr_exon_F	
S56L ^{Tet}	L925M, L1024F, F1538I, M918L + F1534S/F1538I, F1534S + F1538I ^{Mus}	
580	226 255 420	
SdhC Tetur30g00210	VGSC Tetur34g00970	

Authors' contributions

TVL and NW conceived the study. CN, NW and TVL designed the experiments. CN, MV and WJ performed the experiments. CN, MV and WJ analysed data. CN

wrote the manuscript with significant input from NW, MV and TVL. All authors read and approved the final manuscript.

Chapter 5: Intradiol ring cleavage dioxygenases from herbivorous spider mites as a new detoxification enzyme family in animals

This chapter has been partially redrafted from:

Christine Njiru, Wenxin Xue, Sander De Rouck, Juan Alba, Merijn Kant, Maksymillian Chrusz, Bartel Vanholme, Wannes Dermauw, Nicky Wybouw and Thomas Van Leeuwen. (2022) Intradiol ring cleavage dioxygenases from herbivorous spider mites as a new detoxification enzyme family in animals. BMC Biology, 20:131.

5.1 Abstract

Generalist herbivores such as the two spotted spider mite Tetranychus urticae, thrive on a wide variety of plants and can rapidly adapt to novel hosts. What traits enable polyphagous herbivores to cope with the diversity of secondary metabolites in their variable plant diet is unclear. Genome sequencing of T. urticae revealed the presence of 17 genes that code for secreted proteins with strong homology to "intradiol ring cleavage dioxygenases (DOG)" from bacteria and fungi, and phylogenetic analyses show that they have been acquired by horizontal gene transfer from fungi. In bacteria and fungi, DOGs have been well characterized and cleave aromatic rings in catecholic compounds between adjacent hydroxyl groups. Such compounds are found in high amounts in solanaceous plants like tomato, where they protect against herbivory. To better understand the role of this gene family in T. urticae, we studied the phylogeny and the transcriptional patterns across tissues and in mites from different plant hosts. We subsequently functionally characterized the most notable DOG genes by recombinant expression and metabolomics. DOGs expression differed between mites from different plant hosts and was induced in response to jasmonic acid defense signaling. In consonance with a presumed role in detoxification, expression was localized in the mite's gut region. Silencing selected DOGs expression by dsRNA injection reduced the mites' survival rate on tomato, further supporting a role in mitigating the plant defense response. Recombinant purified DOGs displayed a broad substrate promiscuity, cleaving a surprisingly wide array of aromatic plant metabolites, greatly exceeding the metabolic capacity of previously characterized microbial DOGs. Our findings suggest that the laterally acquired spider mite DOGs function as detoxification enzymes in the gut, disarming plant metabolites before they reach toxic levels. We provide experimental evidence to support the hypothesis that this proliferated gene family in T. urticae is causally linked to its ability to feed on an extremely wide range of host plants.

5.2 Introduction

Plants produce a diverse set of aromatic compounds, ranging from aromatic amino acids to secondary metabolites that often function as defense compounds against herbivores and pathogens (e.g., flavonoids, terpenes and phenolics) or as building blocks for structural polymers (e.g., lignin and suberin) (Dao et al., 2011; Guzik and Hupert-kocurek, 2013; Mazid et al., 2011; Neish, 1960). In addition, human activities have led to persistent and toxic man-made aromatic pollutants such as benzene, nitrophenols, organophosphates, chlorinated phenols and hydrocarbons (Bugg and Winfield, 1998; Wells and Ragauskas, 2012). Some species of bacteria and fungi are able to degrade these often complex organic and synthetic aromatic compounds, thus are important players in the global carbon cycle (Bugg and Winfield, 1998).

Microbial degradation of aromatic compounds involves a series of endogenous funneling reactions that convert a wide variety of organic compounds to either 3,4-dihydroxybenzoate (protocatechuate) or 1,2-dihydroxybenzene (catechol), simple monocyclic aromatic compounds (Figure S5-1). Both compounds are catabolized by the β -ketoadipate pathway (β -KAP), which first step is a ring cleavage reaction catalyzed by a class of non-heme iron-containing enzymes referred to as ring cleavage dioxygenases (MacLean et al., 2006; Wells and Ragauskas, 2012). Based on their cleavage mechanism, these enzymes are classified as either extradiol or intradiol ring cleavage dioxygenases.

The extradiol ring cleavage dioxygenases cleave protocatechuate and catechol rings adjacent to either of the hydroxyl groups (meta cleavage) to form semialdehydes (Cha, 2006; Tsai and Li, 2007). This class of enzymes is widespread across the tree of life. In addition, they typically exhibit large substrate promiscuity and catalyze multiple reactions in addition to the oxygen-mediated ring splitting, including epimerization, isomerization, and nucleophilic substitution (Bugg and Winfield, 1998; Fetzner, 2012; Guzik and Hupert-kocurek, 2013; Harayamas and Rekik, 1989; Sherr, 1994).

Intradiol ring cleavage dioxygenases (hereafter abbreviated as DOGs) cleave protocatechuate and catechol rings between the two neighboring hydroxylated carbons (ortho-cleavage) to form dicarboxylic acid. Based on their substrate specificity, they are further classified as catechol 1,2-dioxygenases, protocatechuate 3,4-dioxygenases or hydroxyquinol 1,2-dioxygenases (Guzik and Hupert-kocurek, 2013). In contrast to extradiol ring-cleavage dioxygenases, DOGs are believed to only catalyze oxygen-mediated ring cleavage reactions and are not as widespread across the tree of life. In the past they were considered to be restricted to the genomes of bacteria and fungi. However, in 2011, Grbić et al. reported the presence of a *DOG*-like family in the spider mite *Tetranychus urticae*, a polyphagous arthropod herbivore. Further studies showed that these *DOG* genes had been acquired by a single horizontal gene transfer (HGT) event from fungi and subsequently proliferated (Dermauw et al., 2013b; Grbić et al., 2011; Wybouw et al., 2018). However, the timing and functional importance of the transfer event remains elusive. Schlachter et al., (2019) uncovered that one paralog (*tetur07g02040*) codes for a DOG enzyme capable of cleaving the model substrates catechol and 4-methyl catechol *in vitro*. Additionally, this enzyme is active in a monomeric state, unlike bacterial and fungal DOGs (Bianchetti et al., 2013; Ferraroni et al., 2006; Matera et al., 2010).

The presence of a proliferated *DOG* gene family (with 17 paralogs in the London reference genome) in *T. urticae* suggests that these horizontally acquired genes functionally diversified and could contribute to the ability of *T. urticae* to metabolize the myriad of different aromatic compounds it encounters in its plant diet (Bennet and Wallsgrove, 1994; Erb and Kliebenstein, 2020). Previous work supports this hypothesis by uncovering strong transcriptional responses of *DOG* genes upon short- and long-term

transfers from kidney bean to other host plants that are defended by a diverse blend of aromatic compounds (Dermauw et al., 2013; Snoeck et al., 2018; Wybouw et al., 2015).

Studies on fungi and bacteria have reported that certain secreted DOGs exhibit the rare ability to cleave non-model complex substrates like procyanidins and catecholic intermediates of the lignin biosynthetic pathway such as caffeic acid and caffeoyl-CoA (Bianchetti et al., 2013; Roopesh et al., 2010). In the current study, we functionally characterized the spider mite DOGs and investigated the hypothesis that they can cleave plant-derived aromatic compounds. We first examined the evolutionary histories of DOGs within the Tetranychoidea superfamily, including spider mites and false spider mites. We then assessed DOG transcript accumulation in different feeding and non-feeding mite developmental stages as well as in adults in response to various plant diets. We also established in which mite tissues DOGs are expressed via in situ hybridization. Furthermore we assessed the effect of DOG knockdown via RNAi on spider mite performance on different host plants. We subsequently produced recombinant protein for 7 DOGs and tested to what extent they can cleave 33 aromatic plant compounds, many of which are known to be toxic to herbivores. Understanding the functional role of this unique enzyme family might not only help to understand why certain spider mite species like T. urticae thrive on so many host plants with markedly different chemical profiles, but could also be a stimulus for the development of commercial applications that can efficiently degrade environmental pollutants.

5.3 Materials and methods

5.3 1 Mite strains and tomato cultivars

The London strain is an outbred reference laboratory *T. urticae* strain (Grbić et al., 2011) and was maintained on potted bean plants (*Phaseolus vulgaris* L. cv "Prelude"). The AT-London strain is derived from London and is adapted to tomato (Wybouw et al., 2015). AT-London was maintained on potted tomato plants (*Solanum lycopersicum* L. cv "Moneymaker"). The METI-resistant strain MR-VP was maintained on kidney bean leaves treated with foliar applications of a commercial formulation of tebufenpyrad (Van Pottelberge et al., 2009). Houten-1 was originally collected in a tomato greenhouse in Houten (The Netherlands) (Kant et al., 2004). Santpoort-2 was collected from a spindle tree located in a natural park near Santpoort (The Netherlands) (Kant et al., 2008), whereas DeLier-1 was collected from *Ricinus communis* in a rural area close to De Lier (The Netherlands) (Alba et al., 2015). Houten-1 mites were maintained on detached tomato leaves (cv "Castlemart" hereafter referred to as CM), whereas Santpoort-2 and Delier-1 were maintained on detached bean leaves (*P. vulgaris* L. cv "Speedy"). The Lahijan strain of *Panonychus citri* was originally collected in citrus orchards in Guilan (Iran) (Alavijeh et al., 2020) and was maintained on detached sour orange leaves (*Citrus aurantium L*.).

Mite strains were reared under laboratory conditions of 25 ± 1 °C, 60% relative humidity and a 16:8 h light:dark period. Here, we used tomato plants (CM) and the jasmonic acid mutant within a CM genetic background (hereafter referred to as *def-1*) in our experiments. Tomato plants were reared under greenhouse conditions (25:18 °C day:night, 50-60% relative humidity and 16:8 h light:dark photoperiod). Experiments involving potted tomato plants were carried out in a climate room (25 °C, 60% relative humidity and 16:8 h light:dark photoperiod), to which the potted plants were transferred two weeks prior to mite infestation.

5.3.2 Spider mite DOG nomenclature

In the current study, we have given specific gene identifiers to the *DOG* genes of *T. urticae* and *P. citri*. The nomenclature of our *T. urticae DOGs* corresponds to the following gene identities in the ORCAE database (Sterck et al., 2012); (*TuDOG1-tetur01g00490, TuDOG2-tetur04g00150, TuDOG3-tetur04g08620, TuDOG4-tetur06g00450, TuDOG5-tetur06g00460, TuDOG6-tetur07g02040, <i>TuDOG7-tetur07g05930, TuDOG8-tetur07g05940, TuDOG9-tetur07g06560, TuDOG10-tetur12g04671, TuDOG11-tetur13g04550, TuDOG12-tetur19g03360, TuDOG13-tetur19g02300, TuDOG14-tetur20g01160, <i>TuDOG15-tetur20g01790, TuDOG16-tetur28g01250* and *TuDOG17-tetur44g00140*). The nomenclature of the *P. citri DOGs* in this study corresponds to the following identities in the *Panonychus* transcriptomic data (Bajda et al., 2015); (*PcDOG5-Pc_IDRCD16, PcDOG6-Pc_IDRCD9*).

5.3.3 Confirmation of horizontal gene transfer and phylogenetic analysis of tetranychoid DOGs

Coverage plots for 17 *T. urticae DOG* genes were generated using the methodology described in Wybouw et al., (2018) to confirm that they are real HGT genes and not genome contaminants. Three DOG genes (*TuDOG7*, *TuDOG10* and *TuDOG11*) were also selected for PCR verification. A single PCR amplicon for each of these genes and their neighboring intron containing eukaryotic genes was generated using GoTaq G2 DNA polymerase (Promega). Primers sequences are listed in Table S5-1. The PCR conditions were initial denaturation at 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s and a final extension at 72 °C for 5 min. PCR products were purified using E.Z.N.A[®] Cycle pure kit (Omega-Biotek) and sanger sequenced (LGC genomics, Germany). The sequenced data was analyzed in BioEdit version 7.2.

T. urticae DOG protein sequences were used as query in a tBLASTn search (E-value threshold of E-5) against transcriptomes of 69 of the 72 Tetranychoid species (excluding *T. urticae, Panonychus ulmi* and *P. citri*) previously described in Matsuda et al., (2018). The *getorf* command from the EMBOSS package (Rice et al., 2000) was used to extract open reading frames (ORFs, translations between START and STOP, minimum length of 375 nucleotides) from the unique tBLASTn hits. Subsequently, ORFs were

translated to protein sequences and T. urticae DOGs were used in a BLASTp search (E-value threshold of E-5) against these translated ORFs to identify tetranychoid DOGs. DOGs (> 125 AA) of P. citri and P. ulmi were previously identified (Bajda et al., 2015) while those of Brevipalpus yothersi were identified by a BLASTp search (E-value threshold of E-5) against the manually curated proteome of B. yothersi (Navia et al., 2019) using T. urticae DOG proteins as queries. DOG proteins of each of the 73 tetranychoid species were filtered for identical sequences using the cd-hit program (Li and Godzik, 2006) with the "-c 0.98" option and DOGs of a given tetranychoid species as input. Filtered DOGs of each species were merged (496 sequences in total (see File S5-1), aligned using the online version of MAFFT version 7 with G-INS-I settings (Katoh and Standley, 2013) and revealed that two DOGs (S_lesp_12131_2 and P_late_308_1) disturbed the alignment. These two DOGs were removed from the set of DOG sequences and a final alignment was generated in an identical way as the preliminary alignment. According to the online version of ModelFinder (Stamatakis, 2014; Trifinopoulos et al., 2016) and using the Akaike Information Criterion, the WAG+G+F model was optimal for phylogenetic reconstruction. A maximum likelihood analysis was performed using RAxML v8 HPC2-XSEDE (Stamatakis, 2014) on the CIPRES Science Gateway (Miller et al., 2010) with 1,000 rapid bootstrapping replicates ("-p 12345 -m PROTGAMMAWAGF -f a -N 1000 -x 12345"). The tree was midpoint rooted and visualized using Iroki (Moore et al., 2020).

5.3.4 *T. urticae DOG* transcript analysis in different life stages, resistant strains and strains adapted/acclimatized to different host plants

Protein sequences of the 17 *T. urticae* DOGs (Grbić et al., 2011) were aligned using the online version of MAFFT v7 (Katoh and Standley, 2013) with default settings. The resulting alignment was used in a maximum likelihood analysis using RAxML v8 HPC2-XSEDE (Stamatakis, 2014) on the CIPRES Science Gateway (Miller et al., 2010), with 1,000 rapid bootstrapping replicates and protein model set to "auto" ("-p 12345 -m PROTGAMMAAUTO -f a -N 1000 -x 12345" option). The tree was midpoint rooted, visualized using MEGA 6.0 (Tamura et al., 2013) and used to order *DOG* genes in the DOG expression heatmap. Gene expression microarray expression data of *T. urticae* populations resistant to acaricides and *T. urticae* lines adapted/acclimatized to different host plants were derived from previous studies (Demaeght et al., 2013; Dermauw et al., 2013; Jonckheere et al., 2016; Khalighi et al., 2015; Pavlidi et al., 2017; Snoeck et al., 2018; Wybouw, 2015; Wybouw et al., 2014) and gene expression analysis was performed as in Snoeck et al., (2019b). In addition, we re-analyzed the microarray gene expression data of Bryon et al., (2013) based on the methodology of Snoeck et al., (2019b) but using non-diapausing LS-VL as the reference. Fold changes for *T. urticae* DOG genes were extracted and a heatmap was constructed using the ComplexHeatmap version 2.4.3 package (Gu et al., 2016) in R. RNAseq reads of *T. urticae* stages (Illumina trimmed reads with a length of 60 bp) and *T. urticae*

males/females (Illumina paired-end, strand specific 100 bp reads) were aligned as described in Wybouw et al., (2018) and Ngoc et al., (2016), respectively. RNAseq read counts per gene, based on the annotation of August 11, 2016 were obtained using the default settings of HTSeq 0.6.0 (Anders et al., 2015) with the 'FEATURE' flag set to 'exon' and the 'ORDER' flag set to 'pos'. For stage-specific RNAseq read count data, technical replicates data were collapsed using the DESEQ2 v. 1.28.1 package (Love et al., 2014) in R and genes with no read counts for any of the four stages were excluded from further analysis, while genes with no read counts for any of the four biological replicates were excluded from the male/female RNAseq read count data. Next, read counts were normalized by the method of trimmed mean of M-values (TMM) and log2 (counts per million (CPM)) were calculated using the edgeR version 3.30.2 package (Robinson et al., 2010). For the male/female RNAseq data, log2(CPM) values were averaged across biological replicates (four biological replicates/sex). Finally, log2(CPM) values were extracted for *T. urticae DOG* genes and a heatmap was constructed using the ComplexHeatmap version 2.4.3 package (Gu et al., 2016) in R.

5.3.5 T. urticae DOG transcript analysis in response to tomato defense

To establish the transcriptional changes that JA-mediated defenses induce in mites, *T. urticae* adult females were transferred from common bean to 21 days old tomato plants, CM and *def-1*. To obtain age-synchronized females, adult females were transferred from the stock cultures to detached bean leaves and removed after 48 hours. Offspring was allowed to reach adulthood. About fifteen two-day old females were transferred to a tomato leaflet, and three leaflets per plant were infested (45 mites in total). Mites from 5 plants were pooled (max of 225 mites per pool) and constitute a biological replicate. Experiments were repeated 4 times in 4 consecutive weeks. Mites were frozen in liquid nitrogen and kept at -80 °C until RNA extraction.

Total RNA from frozen mite samples was isolated using the RNeasy Mini Kit from Qiagen (Venlo, The Netherlands) following the manufacturer's guidelines. 1 μ g RNA was DNAse-treated, and 500 ng was used for cDNA synthesis. cDNA was diluted 10 times and 1 μ L of this dilution was used as template for a quantitative polymerase chain reaction (qPCR) using the Platinum SYBR Green qPCR-SuperMix-UDG kit (Invitrogen, Thermo Fisher Scientific, USA) and the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Primers used in qPCR experiments are provided in Table S5-1. The normalized expression (NE) data were calculated by the Δ Ct method (Livak and Schmittgen, 2001). Using 18S (tetur01g03850) as the reference gene (Yang et al., 2015), NE of each target gene was compared using a nested ANOVA with 'Mite Strain' and 'Plant Genotype' were included as factors and 'technical replicate' (i.e. two for each reaction) nested into the corresponding biological replicate (cDNA sample). Means of each group were compared by Fisher's LSD post hoc test using PASW Statistics 17.0 (Chicago: SPSS Inc). To plot the relative expression, NE-values and SEM were scaled to

the treatment with the lowest average NE. To test statistical significance, a t-test was performed for each mite line, comparing *DOG* expression in defenceless tomato line (*def-1*) vs wild type (CM). Pvalues were corrected for false positives using Holm-Sidak method.

5.3.6 In situ hybridization

In situ hybridization (ISH) was carried out to identify the in vivo localization of the transcription of TuDOG1, TuDOG11 and TuDOG16 in T. urticae. We based our protocols on previous work (Jonckheere et al., 2016). RNA was extracted from a pool of 100-120 adult females of the London strain using the RNeasy plus mini kit (Qiagen)). Approximately 2 µg of RNA was used to synthesize cDNA with a Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Primers were designed using Primer3 (Untergasser et al., 2012) and amplified a fragment of approximately 300 bp (Table S5-1). After purification with an E.Z.N.A. Cycle Pure Kit (Omega Biotek, GA, USA), PCR products were cloned into pGEM-T plasmids (Promega) and transformed into *E. coli* DH5α cells using heat-shock method (Froger and Hall, 2007). Based on colony PCR assays, eight positive colonies for TuDOG1, TuDOG11 and TuDOG16 were grown in 5 mL LB broth (Duchefa Biochemie) at 37 °C with shaking at 250 rpm for 16 h. Plasmids from these liquid cultures were purified with an E.Z.N.A. Cycle Pure Kit (Omega Biotek, GA, USA). Insert orientations and nucleotide sequences were determined by Sanger sequencing (LGC Genomics, Germany). PCR assays were performed on the purified plasmids using pUC/M13 primers (Table S5-1) to generate amplicons that contain our approximately 300 bp insert flanked by the T7 and SP6 promoter sites. The cycling conditions were 95 °C for 2 min, 35 cycles of 30 s at 95 °C, 45 s at 57 °C, 1 min at 72 °C and 5 min at 72 °C. After verification by agarose gel electrophoresis, PCR products were purified using an E.Z.N.A. Cycle Pure Kit (Omega Biotek, GA, USA). An in vitro labeling reaction was performed using T7 or SP6 RNA polymerase (Roche), digoxigenin-uridine triphosphate (DIG-UTP, Roche) and the purified PCR product, to generate sense or anti-sense DIG-labeled probes. Probes were purified using Sigma Spin [™] Sequencing Reaction Clean-Up Columns (Sigma) supplemented with hybridization buffer (50% formamide (Sigma), 2x SSC (Sigma), 1x Denhardt's solution (Sigma), 200 µg mL⁻¹ tRNA (wheat germ type V, Sigma), 200 µg mL⁻¹ ssDNA (boiled salmon sperm DNA, Sigma), 50 µg mL⁻¹ heparin (sodium salt, Sigma), 10% dextran sulfate (sodium salt, Sigma) and stored at -20 °C until use.

Adult female mites of the AT-London strain were collected in an Eppendorf tube with 30% sucrose in 1x PBS (0.85% NaCl, 1.4 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.1) and kept at 4 °C for 1-2 h. Specimens were mounted in an Optimal Cutting Temperature (OCT) compound (Tissue-Tek; Sakura), sectioned into 7 μ m thickness using a *Leica* cryostat CM1860 and then mounted on silanized slides. After air drying for 15 min, the slides were fixed in 4% formaldehyde at 4 °C for at least 30 min, then followed with washing in PBS (1 min), 0.6% HCl (10 min) and PBS with 1% TritonX-100 (2 min). After two times

30 s washes in PBS, slides were pre-hybridized in 100-150 μL of hybridization buffer at 52 °C for 30-60 min. Slides were covered with coverslips and hybridized overnight (20-24 h) at 52 °C in a closed container that contained 2x SSC. Coverslips were subsequently removed and the slides were washed in 0.2× SSC for 2× 30min. Slides were subsequently rinsed shortly in TBS (100 mM Tris, pH 7.5, 150 mM NaCl) after which 1 mL of 0.1% BSA in TBS with 0.03% Triton X-100 was added on each slide followed by incubation at room temperature for 30 min. After the solution was discarded, the slides were incubated with the antibody mixture (anti-DIG-AP: BSA buffer = 1:500) at 37 °C for 1 hour. Slides were subsequently washed three times in TBS, 0.05% Tween-20 for 5 min and washed in DAP-buffer (100 mM Tris, 100 mM NaCl, 10 mM MgCl₂, pH 8.0), and in TBS, 0.05% Tween for 5 min. Fast Red/HNNP mix (Roche) was added to the slides, followed by incubation at room temperature for 30 min in the dark. After careful removal of the coverslip, slides were washed three times for 5 min in TBS, 0.05% Tween-20. The slides were mounted with antifade mounting medium (Vectashield) and covered with coverslips for further microscopic investigation (Nikon A1R fluorescence confocal microscope; emission at 500-530 nm and acquisition at 488 nm for spider mite auto-fluorescence and emission at 570-620 nm and acquisition at 561.7 nm for FastRed signal). Z-stacks were created using 4 slices with 2-3 µm distance between slices. All images were processed with Fiji and CorelDRAW Home & Student ×7.

5.3.7 DOG silencing by dsRNA injection

To further investigate the functional importance of DOGs to host plant use, we selected two key DOG genes (TuDOG11 and TuDOG16) for transcriptional silencing by dsRNA injection. Total RNA of 200 adult females of the MR-VP strain was extracted using RNeasy Plus Mini kit (Qiagen, Belgium), reverse transcribed using the Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific), and amplified using gene-specific primers (Table S5-1) that contain 23 bases of the T7 promoter. The PCR conditions were as follows; initial denaturation at 95 °C for 2 min, followed by 35 cycles of amplification (95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min) and a final extension at 72 °C for 5 min. PCR products were purified using E.Z.N.A. Cycle Pure kit (Omega Bio-Tek, USA), sub-cloned in pJET/Blunt 2.0 Vector, and transformed by heat shock into E. coli DH5a component cells. Selection was performed on LB agar plates with 50 µg mL⁻¹ carbenicillin. Positive colonies were confirmed by colony PCR and Sanger sequencing (LGC Genomics, Germany). To obtain pure and highly concentrated PCR template for dsRNA synthesis, a second PCR amplification step was performed on the plasmids. PCR products were purified using E.Z.N.A. Cycle Pure kit (Omega Bio-Tek, USA). The quantity and quality of the PCR products were assessed by DeNovix DS-11 spectrophotometer (DeNovix, USA) and by running an aliquot on a 2% agarose gel. Next, dsRNA was synthesized using Transcript Aid T7 High Yield Transcription kit (Thermo Fisher Scientific). After synthesis, dsRNA was purified by isopropanol

precipitation (Fisher, 2019). The quantity and quality of dsRNA were assessed by a DeNovix DS-11 spectrophotometer (DeNovix, USA) and by running an aliguot on a 2% agarose gel. Injections of dsRNA were carried out as described by Dermauw et al. (2020a). Briefly, 200 two-day old adult females of the MR-VP strain were immobilized on Alura red stained 2% agarose gel platforms for each treatment. Females were injected with 3 nL of a 1 μ g μ L⁻¹ dsRNA solution targeting either *GFP*, *TuDOG11*, or TuDOG16. The dsRNA solution was injected into the ovary (near the third pair of legs) under a Leica S8 APO stereomicroscope using a Nanoject III injector (Drummond Scientific, USA). Needles used for injections were made from 3.5" glass capillaries (Drummond Scientific, USA) using a PC-10 Dual-Stage Glass Micropipette Puller (Narishige, Japan) with 2-steps and following settings for step 1 and 2 respectively: 101.1 and 96.4. Eight batches of 200 females were injected per dsRNA treatment. Each mite batch was transferred to a detached kidney bean leaf and allowed to recover. After 72 hours, approximately 40-80 mites were collected per treatment and RNA was extracted using the RNeasy plus kit (Qiagen). RNA integrity was verified on 1% agarose gel after which cDNA was prepared from 1 μ g RNA using the maxima cDNA synthesis kit (Thermo Fisher Scientific). gPCR assays were performed using GoTaq®qPCR master mix (Promega corporation). The thermal profile for qPCR was: 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 60 °C for 30 s. Silencing efficiency was determined using qbase+ with ubiquitin (tetur03q06910) and glyceraldehyde-3-phosphate (tetur25q00250) as the reference genes (An M value of 0.6 was obtained, reflecting stability of the housekeeping genes). Primers for dsRNA synthesis and qPCR are outlined in Table S5-1. The remaining mites (about 60-100 mites per batch) were transferred to tomato leaves cv "Moneymaker". Mortality and fecundity were scored at different time points (24, 48 and 72 hours) on tomato. Mortality was defined as the percentage of dead mites at each time point relative to the total number of founding mites, whereas fecundity was defined as the total number of eggs divided by the surviving mites at each time point. To test statistical significance, a paired t-test was performed to compare each DOG treatment with GFP treatment using rstatix package in R (R development core team 2017) and GraphPad prism v.6.01 softwares.

5.3.8 Functional expression of recombinant DOGs

The enzymatic abilities of spider mite DOGs were investigated by recombinant expression of some key DOGs in *E. coli*. To see whether the aromatic substrate ranges of *DOG* genes differed across species of the Tetranychidae family, we selected two DOGs (*PcDOG5* and *PcDOG6*) from the citrus red mite, *P. citri* that were specific to the *Panonychus* clade (Bajda et al., 2015). All expression constructs were designed using SnapGene [®] viewer v. 5.1.6, and added an N-terminal 6x His-tag for purification and detection. Signal peptides were predicted using SignalP v.5.0 (Almagro Armenteros et al., 2019) and removed from the final expression constructs to allow for cytoplasmic expression. The coding sequences for *TuDOG1*, *TuDOG11* and *TuDOG16* were retrieved from ORCAE (Sterck et al., 2012),

codon optimized for expression in E. coli and produced by ATUM (Newark, CA) in the expression vector pJ Express 411. Additional DOGs constructs were designed from cDNA amplified from spider mite strains. The London strain was used to generate TuDOG7 and TuDOG15 constructs while the Lahijan strain was used to obtain PcDOG5 and PcDOG6 constructs. For cDNA preparation, total RNA was extracted from approximately 200 mites using RNeasy plus mini kit (Qiagen) and reverse transcribed using Maxima[®] first strand cDNA synthesis kit (Thermo Fisher Scientific). Full-length cDNAs (from start to stop codon) were amplified by a Phusion high fidelity DNA polymerase (Thermo Fisher Scientific). Primers sequences are listed in Table S5-1. The PCR conditions were 98 °C for 30 s, 30 cycles of 98 °C for 10 s, 60 °C for 30 s,72 °C for 30 s and a final extension at 72 °C for 5 min. PCR products were purified using E.Z.N.A® Cycle pure kit (Omega-Biotek), cloned into PJET 1.2/blunt vector, transformed into E.coli DH5 α cells using the heat shock method and grown on LB agar plates substituted with 50 μ g mL⁻¹ carbenicillin. Five colonies per DOG gene were selected, grown in 5 mL LB broth with 50 µg mL⁻¹ carbenicillin, plasmid extracted using the E.Z.N.A® plasmid mini kit II (Omega-Biotek) and Sanger sequenced (LGC genomics, Germany). Based on the obtained sequences (File S5-2a), codon optimized constructs of TuDOG7, TuDOG15, PcDOG5 and PcDOG6 without the signal peptide were generated by Genscript (The Netherlands), cut from the storage vector puC57 using Ndel and BamHI restriction enzymes, and cloned into the final expression vector PJ Express 411 (for codon optimized sequences see File S5-2b).

Protein expression conditions were optimized in 5 mL liquid cultures to maximize protein yields. For large scale expression, E. coli BL-21 (DE3) cells were transformed by heat shock, grown in 1 liter LB broth with 50 µg mL⁻¹ kanamycin at 37 °C and shaken at 250 rpm in a 311DS Environmental shaking incubator (Labnet international, USA) until OD_{600nm}= 0.8 was reached. After cooling the cultures to 16 °C, protein expression was induced with 4 mL of 100 mM isopropyl β-D-1 thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. To promote the incorporation of an iron cofactor into the recombinant DOG proteins, 3 mg of ferrous sulphate heptahydrate was added. The induced cultures were grown for an additional 24 h at 16 °C with shaking at 180 rpm in Innova 40/40R-benchtop orbital shaker (Eppendorf, Germany). Cells were harvested by centrifuging at 8000 g for 10 min. The pellet was stored at -80 °C overnight, thawed and suspended in 100 mL lysis buffer (0.1 M PBS pH 7.5, 500 mM NaCl, 10 mM imidazole, 2% glycerol and 0.5 mM PMSF) and lysed by sonication on ice for 30 min. The lysate was centrifuged at 8000 g for 30 min and the supernatant passed through NiNTA column equilibrated with wash buffer (0.1 M PBS pH 7.5, 500 mM NaCl and 20 mM imidazole). Unbound proteins were washed off the column twice; first with the wash buffer containing 20 mM imidazole then with the wash buffer containing 30 mM imidazole. The protein was eluted with elution buffer (0.1 M PBS pH 7.5, 500 mM NaCl) containing increasing concentrations of imidazole between 50 mM and
200 mM. Following an SDS-PAGE, imidazole fractions containing the recombinant protein were pooled together, concentrated and desalted using Amicon[®]Ultra-15 centrifugal filter units (Sigma Aldrich). Protein concentration was determined using Pierce [™] Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific). The concentrated protein was stored at -20 °C with addition of glycerol to a final concentration of 25%.

5.3.9 Ferrozine assay

The ability of our recombinant DOGs to incorporate iron was verified using a modification of the ferrozine assay described by Ring et al. (2018). Briefly, 10 μ M protein in 150 μ L distilled water was hydrolyzed in an equal volume of acid digestion solution (a mixture of 1:1 ratios of 1.2 M HCl and 1.2 M ascorbic acid) at 60 °C for 3 hours. The hydrolyzed protein was cooled to room temperature prior to adding 150 μ L of iron chelating reagent (freshly prepared mixture of 5 M ammonium acetate, 2 M ascorbic acid, 6.5 mM ferrozine and 13.1 mM Neocuproine). 210 μ L of the reaction mixture was transferred into a 96 well plate, covered in foil and incubated at room temperature for 30 min. The absorbance at 562 nm was recorded and iron content calculated from iron standards of 0-20 μ M prepared from Mohr's salt subjected to the same hydrolysis and detection procedure as the samples.

5.3.10 Spectrophotometry

As a first step to characterize the substrate range of our recombinant DOG enzymes, we defined their kinetic parameters using the model substrates catechol, 4-methyl catechol, and 4-chlorocatechol. All substrates were purchased from Sigma-Aldrich (Belgium). The reactions were carried out in triplicate in a 96 well UV-Star[®] Microplate (Greiner Bio-one), with a total reaction volume of 200 µL per well consisting of 0.1 M PBS pH 7.5, 1 μg or 5 μg enzyme and varying substrate concentrations of 5-500 μM. The reaction was initiated by adding the aromatic substrate to a mixture of buffer and enzyme. The formation of muconic acids at 260 nm (catechol and 4-chlorocatechol) and 255 nm (4-methyl catechol) was followed for 5 min. For negative controls, the recombinant enzyme was omitted. The initial velocity (vo) with each substrate concentration was calculated using molar extinction coefficients of 16800 M⁻¹ cm⁻¹ for catechol, 14300 M⁻¹ cm⁻¹ for 4-methyl catechol (Schlachter et al., 2019) and 12400 M⁻¹ cm⁻¹ with 4-chlorocatechol (Kaulmann et al., 2001). Curves of initial velocity vs substrate concentration were plotted using Sigma Plot v.13 (Systat Software Inc., USA) and kinetic parameters were determined for each enzyme with the three model substrates. The total protein used in k_{cat} calculations was corrected for iron content using an iron factor calculated for each protein in the ferrozine assay (see above). As such, only the total amount of iron bound enzyme was used in the k_{cat} calculations.

5.3.11 Oxygen consumption

We further investigated the substrate range of our recombinant DOGs using other more complex organic compounds in addition to the model substrates. All substrates were purchased from Sigma-Aldrich (Belgium) except trans-clovamide which was purchased from Cayman Chemical (The Netherlands). Enzymatic activity was tested against a total of 33 plant secondary metabolites, five model substrates, and two pesticides. All substrates are listed in Table S5-2. Using the Oxytherm+System (Hansatech, UK), ortho-cleavage (i.e. cleavage of the aromatic ring between two neighboring hydroxyl groups) was evaluated by recording oxygen consumption rates following incubation with the recombinant T. urticae DOGs. The electrode was prepared and calibrated to 25 °C before use. All reactions were carried out in three to four replicates in a total reaction volume of 1 mL, with the stirrer speed set to approximately 700 rpm (75% steps) for optimal oxygen circulation. Briefly, 984 µL of 0.1 M PBS pH 7.5 was added to the reaction chamber, stirred and the signal allowed to equilibrate for 90 s after which 5 μ L of 20 mM substrate stock prepared in methanol was added and the chamber sealed from external oxygen. After signal equilibration for 90 s, 2 µL of boiled enzyme was injected into the chamber. Oxygen signal was recorded for 2 min at intervals of 10 s prior to injection of 9 μ L active enzyme (3 or 5 μ g). Oxygen consumption with the active enzyme was measured for 5 min. The oxygen consumption rate (OCR) was determined from the slope of the linear part of the graph after addition of active enzyme and corrected for background oxygen consumption with the OCR observed for the boiled enzyme control. Rates above 1 nmol mL⁻¹ min⁻¹ were scored as activity while lower rates were scored as background noise. A detailed graphical representation of the assay is provided in Figure S5-2. The OCRs for various substrates were compared and visualized using R package ggplot2 (R Development core Team 2017).

5.3.12 Identification of reaction products by UPLC-MS

To confirm ortho-cleavage of the aromatic rings by *T. urticae* DOGs, a selection of eleven plant secondary metabolites that showed oxygen consumption using the Oxytherm+System (see above), were subjected to further metabolomic analysis by UPLC-MS to identify the reaction products (Figure S5-3). Analysis was carried out at the VIB Metabolomics Core Facility (Ghent, Belgium). Prior to metabolite analysis, all substrates were standardized to ensure their detectability using a similar protocol as described by Vanholme et al. (2019). Briefly, 20 μ L of a 1 mM stock methanol solution was mixed with an equal volume of distilled water, then 10 μ L of the mixture was injected into the UPLC-MS system (Waters Corporation) and the detectability checked in both positive and negative modes. The metabolomics reactions were carried out in five replicates with either boiled or active DOG enzyme in a 1 mL reaction volume. Specifically, to a 2 mL sterile Eppendorf tube, 986 μ L of 0.1M PBS pH 7.5 was added, 9 μ L active or boiled enzyme (a total of 3 μ g) and 5 μ L of 20 mM substrate stock. The tubes

were loosely capped to allow oxygen diffusion and incubated at 25 °C with shaking at 300 rpm for 1 h. Reactions were stopped by adding 500 μ L urea to a final concentration of 7 M. The samples were stored at -20 °C until analysis. Reaction mixtures were analyzed on a UPLC-MS system as described in Vanholme et al. (2019). Briefly, 2 µL of the mixture was injected on a Waters Acquity UPLC® equipped with a UPLC BEH C18 column (130 Å, 1.7 μm, 2.1 mm x 50 mm) and coupled to a SynaptXS Q-Tof (Waters Corporation). A gradient of two buffers was used: buffer A (99/1/0.1 H₂O/acetonitrile/formic acid, pH 3), buffer B (99/1/0.1 acetonitrile/H₂O/formic acid, pH 3); 99% A for 1 min decreased to 50% A in 10 min (500 µL/min, column temperature 40 °C). The flow was initially diverted to waste for 0.5 min, and then to the MS equipped with an electrospray ionization source and lock spray interface for accurate mass measurements operating in negative ionization mode. The MS source parameters were capillary voltage, 2.5 kV; sampling cone, 40 V; source temperature, 120 °C; desolvation temperature, 550 °C; cone gas flow, 50 L h⁻¹; and desolvation gas flow, 800 L h⁻¹. The collision energy for the trap and transfer cells was 4 V and 2 V, respectively. For data acquisition, the dynamic range enhancement mode was activated. Full scan data were recorded in negative centroid sensitivity mode; the mass range was set between m/z 50 and 1,200, with a scan speed of 0.1 s scan⁻¹, with Masslynx software v4.1 (Waters). Leucin-enkephalin (250 pg μ L⁻¹ solubilized in water/acetonitrile 1:1 (vol:vol), with 0.1% formic acid) was used for the lock mass calibration with a scan time of 0.5 s. Data processing was done with Progenesis QI v2.4 (NonLinear Dynamics).

5.4 Results

5.4.1 The evolutionary history of spider mite DOG genes

First, the *T. urticae* genome assembly was studied in detail in order to verify that *DOG* genes indeed reside within the mite genome. Coverage plots for all the 17 *DOG* genes (File S5-3) showed equal coverage of DNA reads aligned to the assembled reference genome. In addition, single PCR amplicons of *TuDOG7*, *TuDOG10* and *TuDOG11* with canonical intron containing eukaryotic genes were also generated (Figure S5-4 for the PCR amplified bands and Files S5-4,S5-5 and S5-6 for the DNA alignments of the three DOGs respectively). Together, these analysis provide sound evidence for the genomic integration of *DOG* genes in the *T. urticae* genome.

Next, to obtain genome-wide insight in the evolutionary history of spider mite *DOG* genes, a phylogenetic analysis was carried out using transcriptomic and genomic data. *DOG* homologs were identified in all 72 spider mite species of the Tetranychidae family and in one false spider mite species of the Tenuipalpidae family (Figure 5-1, File S5-7). Together with previous work that screened a wider range of metazoan genomes, these findings are in line with the previously proposed scenario that *DOG* genes restricted to bacteria and fungi were acquired by an ancestral mite species by horizontal gene

transfer from fungi (Dermauw et al., 2013b; Grbić et al., 2011; Schlachter et al., 2019). Current findings suggest that this transfer event occurred before the formation of the Tetranychidae and Tenuipalpidae families. Although caution is warranted for the comparison of gene numbers and orthologs detected in transcriptomic data (Bajda et al., 2015; Matsuda et al., 2018) and genomic data (Grbić et al., 2011; Navia et al., 2019), as recent gene duplications and genes with very low expression levels might be missed in transcriptomic data, some general patterns can be deducted from our analyses (Figure 5-1, File S5-7). First, the number of identified DOGs does not appear to be correlated with the number of host plants the mite species is reported to feed on. For instance, the polyphagous T. urticae and Oligonychus biharensis have a similar number of DOGs as Amphitetranychus quercivorus and Schizotetranychus shii, which specialize on oak and Castanopsis sp., respectively (Table S5-3, spidermite web) (Migeon et al., 2010). Second, tetranychoid DOGs can either be lineage- (a3, d1, D) (Matsuda et al., 2018), genus- (e.g. Panonychus sp.) or species-specific (A. quercivorus and S. shii). Last, based on the phylogenetic analysis we classified T. urticae DOGs into 13 orthologous clusters and for eleven clusters an orthologue could be detected in a specialist spider mite species (Figure 5-1, File S5-1, Table S5-4). On the other hand, three of these eleven clusters contained duplicated T. urticae DOGs (TuDOG12/TuDOG13; TuDOG7/TuDOG8/TuDOG9; TuDOG4/TuDOG5) but whether these are duplicated in specialist spider mite species could not be clearly determined based on transcriptomic data alone.



Figure 5-1: Phylogenetic reconstruction of horizontally acquired tetranychoid DOGs. Maximum-likelihood phylogenetic analysis of 494 tetranchoid DOGs. The 14 tetranychoid genera and *T. urticae* are colour coded. Arches indicate lineage, genus, or species-specific clades. We named these lineage-specific clades (D, d1 or a3) in accordance with Matsuda et al. (2018). An asterisk indicates that the *T. urticae DOG* was functionally expressed in this study. Only bootstrap values above 65 and at phylogenetically relevant nodes are shown. The scale bar represents 0.43 substitutions per site. A dashed branch indicates that this branch was shortened to improve figure clarity. See File S5-1 for DOG sequences used for phylogenetic analysis and File S5-7 for an expanded version of the phylogenetic tree.

5.4.2 Transcriptional responses of T. urticae DOGs

Previously published life stage- and sex-specific RNAseq data were analyzed to investigate *DOG* expression in *T. urticae* (Figure 5-2a). For thirteen of the 17 *T. urticae DOG* genes we observed lower

transcript levels in embryos than in any of the three feeding stages (larvae, nymphs or adults), while for three T. urticae DOGs (TuDOG6, TuDOG14 and TuDOG11) levels were higher in the embryo than in any of the feeding stages. Noteworthy, the transcript levels of TuDOG15, TuDOG3 and TuDOG16 were on average the highest of all the *DOG* genes during feeding stages (average \log_2 CPM > 6, Table S5-5). T. urticae DOG genes showed similar transcript accumulation patterns in males and females, with TuDOG15, TuDOG3 and TuDOG16 transcripts being the most abundant (log₂CPM > 6) of all DOG genes in both males or females. All T. urticae DOG genes, except TuDOG6, TuDOG14 and TuDOG11, were significantly downregulated in adult diapausing females compared to non-diapausing females (Table S5-6) (Bryon et al., 2013). Multiple T. urticae DOG genes responded to the mites being transferred from common bean to another host plant (i.e. tomato, maize cotton, soy bean or lima bean), with six DOG genes being upregulated ($log_2FC > 1.5$, adjusted p-value < 0.05) in mites after transfer to lima bean compared to 14 after transfer to tomato. Of note, TuDOG16 was the only DOG gene that was always upregulated in spider mites upon transfer from common bean to another host plant, while TuDOG11 displayed the highest level of upregulation (fold change) in mites transferred from common bean to tomato. TuDOG11 was also the strongest upregulated gene in three pesticide resistant strains (MAR-AB, MR-VP and JP-R) (Figure 5-2a, Table S5-6). Based on the phylogenetic tree (Figure 5-1) and expression data (Figure 5-2a), we selected some highly expressed lineage specific DOG genes for recombinant expression. The selected genes are marked with an asterisk in Figure 5-1 and indicated in bold font in Figure 5-2a. Three of the highly expressed lineage specific DOGs (TuDOG1, TuDOG8 and TuDOG16) were further investigated for their transcriptional response to tomato jasmonate defenses using three mite strains (Houten-1, Santpoort-2, and DeLier-1) that interact differently with jasmonic acid mediated tomato defenses (Alba et al., 2015; Kant et al., 2008). Houten-1 is a mite strain that resists the induced jasmonate defenses; Santpoort-2 is susceptible to these defenses; and DeLier-1 is susceptible to these yet is able to suppresses them before taking effect (Alba et al., 2015; Kant et al., 2008). We found that in the resistant strain Houten-1, only TuDOG16 expression was significantly different in wildtype CM tomato plants vs def-1 mutants (P value = 0.009), suggesting that this DOG could have an important role in overcoming tomato jasmonate defenses. As previously noted, TuDOG16 was also the only DOG differentially expressed in all mites transferred from bean to another host (Figure 5-2a). Interestingly, all three DOGs were significantly upregulated in the JA-defense susceptible Santpoort-2 strain on CM tomato plants compared to def-1 plants (P values = 0.002, 0.0001 and 0.0001 with TuDOG1, TuDOG8 and TuDOG16 respectively). On the other hand, in the defense suppressor DeLier-1 strain none of the DOGs responded to the absence or presence of jasmonate defenses (P values > 0.05) (Figure 5-2b). Together, these data indicate that the expression of TuDOG1, TuDOG8 and TuDOG16 is upregulated in spider mites that induced jasmonate defenses, especially in strains that are susceptible to these defenses.



Figure 5-2: Host plant dependent DOG transcript accumulation in T. urticae. A) Heatmap of T. urticae DOG transcript accumulation determined by RNAseq (left panel), or by microarray experiments (right-panel). For microarray experiments, expression data from either non-diapausing LS-VL adult females on common bean (diapause), London adult females on common bean (host plant data, MAR-AB, MR-VP, JP-R, Akita and Tu008R) or LS-VL deutonymph females on bean (SR-VP, SR-TK) were taken as reference to calculate log₂FC values. Gray boxes indicate that for a specific DOG gene no probes were included in the respective T. urticae microarray design, and hence expression could not be estimated. T. urticae DOGs in bold font were functionally characterized in this study. B) Transcriptional response of DOGs to tomato defence. The expression of three DOG genes (TuDOG1, TuDOG8 and TuDOG16) was quantified by gPCR in three mite strains that differentially interact with jasmonic acid (JA) mediated tomato defence. Houten-1 induces the JA defences of tomato and is resistant to it, Santpoort-2 induces JA-defences and is susceptible to it while DeLier-1 suppresses tomato defence. Error bars represent the standard error of the mean (n = 4). An asterisk indicates a significant difference (alpha = 0.05). Expression of the three DOG genes was significantly upregulated in the JA-defense susceptible strain Santpooort-2 feeding on the wild type plants compared to the def-1 mutants (P values = 0.002, 0.0001 and 0.0001 with TuDOG1, TuDOG8 and TuDOG16 respectively). The expression of TuDOG16 was also significantly upregulated in the resistant strain Houten-1 feeding on the wild type plants compared to the def-1 mutants (P value = 0.009). The three DOGs were not differentially expressed in the suppressor strain Delier-1 feeding on either wild type plants or *def-1* mutants (P values > 0.05).

5.4.3 Spatial expression pattern of DOGs in T. urticae

The localization of transcription of three *T. urticae DOG* genes (*TuDOG1*, *TuDOG11*, and *TuDOG16*) was evaluated using whole-mount *in situ* hybridization (ISH). In this ISH approach, the target transcripts are

visible as a red signal, while the spider mite body shows green auto-fluorescence. Red auto-fluorescence of the cuticle was also seen in both antisense and sense (control) treatment (Figure 5-3, Figure S5-5). Using mites that were adapted to tomato and overexpress *DOGs* (Dermauw et al., 2013; Wybouw et al., 2015), ISH revealed that *TuDOG11* and *TuDOG16* were mainly transcribed in the digestive system, more precisely the posterior midgut epithelium, ventricular epithelium and dorsal epithelium. Some staining was also seen in the developing eggs (Figure 5-3). No clear staining was observed for *TuDOG1*, which could be as a result of poor probe design.



Figure 5-3: In situ localization of the transcription of T. urticae DOG genes. A) a schematic representation of a mid-sagittal section of an adult T. urticae female redrafted from Alberti and Crooker (1985) with the permission from Elsevier. The digestive and reproductive system is highlighted in dashed red and green lines, respectively. B) A virtual mid-sagittal section obtained by a sub-micron CT scan of an adult T. urticae female. The internal morphology is as described by Jonckheere et al. (2016). C) A DIG-labelled antisense probe for TuDOG16 was used for hybridization and the signal was developed using anti-DIG-AP and FastRed as substrate. The reaction product is visible as a red signal (indicating regions where DOGs are expressed) while the spider mite body shows green auto fluorescence. The cuticle stained red in both antisense and sense treatment so we considered this as a false signal. D) The control signal developed from a DIG-labelled sense probe for TuDOG16. Abbreviations: C, cuticle; PME, posterior midgut epithelium; OV, ovary; PM, posterior midgut; V, ventriculus; VE, ventricular epithelium; DE, dorsal epithelium; Scale bars: 100 µm.

5.4.4 Effect of DOG silencing on mite performance on a challenging host plant

To gain insights in DOG function and overall contribution to mite fitness, we used a reverse genetic RNAi approach to silence two tomato-induced *DOG* genes (see Figure 5-2a), *TuDOG11* and *TuDOG16*, for which expression was shown in the digestive track of mites feeding on tomato. Micro-injection of 3 nL dsRNA that targets *TuDOG11* and *TuDOG16* resulted in a silencing efficiency of 89% (SE \pm 9%) and 96% (SE \pm 3%), respectively (Figure 5-4a). However, we observed that each *TuDOG* dsRNA was not specific and silenced both *DOG* genes, limiting our ability to disentangle the effect of a single *DOG*

gene. The relative expression of *TuDOG11* and *TuDOG16* after silencing was significantly different from mites treated with GFP dsRNA (P value = 0.005 and 0.006 with *TuDOG11* and *TuDOG16* respectively). Upon transfer to tomato, a significantly higher mortality was observed with *TuDOG* dsRNA-injected mites compared to GFP dsRNA-injected control mites (Mortality of *TuDOG11 vs GFP*, P value = 0.037 and 0.004 with *TuDOG11* and *TuDOG16* respectively). (Figure 5-4b, Table S5-7). *DOG* silencing did not significantly affect daily fecundity (P values > 0.05), although after 48 hours on tomato *TuDOG* dsRNA-injected females laid a slightly lower number of eggs on average compared to GFP dsRNA-injected females (Figure 5-4c). Of note, we observed high variations in the numbers of eggs deposited (SE-values ranged from 0.163 to 0.514) and hypothesize that this could have resulted from different degrees of wounding by needle injection.



Figure 5-4: Silencing of *DOG* **genes by RNA interference. A)** The relative expression of *TuDOG11* and *TuDOG16* was quantified by qPCR in adult female mites injected with dsRNA of either *DOG* or *GFP***. B)** Cumulative mortality. **C)** Daily fecundity defined as the number of eggs laid by surviving mites after 24, 48 and 72 hrs on detached tomato leaves. Error bars represent the standard error of the mean (n = 8). Asterisks indicate a significant difference (alpha = 0.05). The relative expression of *TuDOG11* and *TuDOG16* after silencing was significantly different from mites treated with GFP dsRNA (P values = 0.005 and 0.006 with *TuDOG11* and *TuDOG16* respectively). Mortality was also significantly higher in *TuDOG16* respectively). Daily fecundity was not affected by the dsRNA treatments (P values = 0.037 and 0.004 with *TuDOG11* and *TuDOG16* respectively). Daily fecundity was not affected by the dsRNA treatments (P values > 0.05).

5.4.5 Functional characterization of spider mite DOG proteins

The coding sequences of five *T. urticae* and two *P. citri DOGs* were cloned into the expression plasmid pJExpress 411. As expression of the two *P. citri DOGs* was unsuccessful with the pJExpress 411 plasmid, the *P. citri DOGs* were recloned into pCold-SUMO expression plasmids (Takara Bio, France). Although this strategy improved the solubility, it failed to improve protein yield (≤ 1 mg). IPTG induction of the *T. urticae* constructs resulted in protein yields ranging between 2 and 12 mg from 1 L cultures (Table S5-8). All proteins were purified close to homogeneity as evidenced by a main band between 25 and 37 kDa on both SDS-PAGE and Western-blot with anti-His-tag primary antibody (Figure S5-6). Following chromatography purification, all proteins exhibited a dark red coloration on the NiNTA column. The

red coloration remained visible in the final purified proteins that had a high yield (TuDOG11, TuDOG15 and TuDOG16) but was not apparent in the proteins of low yield batches (TuDOG1, TuDOG7, PcDOG5 and PcDOG6). Ferrozine assays confirmed that the purified DOG proteins contained iron, ranging from 0.3-1.0 nmol iron/nmol protein (Table S5-8), needed for catalytic activity.

The kinetic parameters of recombinant T. urticae DOGs were determined with spectrophotometric assays using model substrates catechol, 4-methyl catechol and 4-chlorocatechol. Due to the low protein yield and low activity observed with model substrates, the kinetic parameters of P. citri proteins were not characterized. TuDOG1 and TuDOG11 cleaved catechol with a higher efficiency as compared to 4-methyl catechol and 4-chlorocatechol (k_{cat}/K_M 1.2-2.5 fold higher with catechol as the substrate for the two enzymes). TuDOG7, TuDOG15 and TuDOG16 showed preference for the substituted catecholates (Table 5-1). Under similar buffer and temperature conditions, four of the recombinant T. urticae DOGs had a much lower affinity to catechol (K_M 3-6 times higher) and 4-methyl catechol (K_M 2-5 times higher) compared to mTuIDRCD (TuDOG6) that was previously characterized by Schlachter et al. (2019). TuDOG1 had a relatively similar affinity to catechol as mTuIDRCD, and cleaved this substrate with a 4 times higher efficiency as compared to that of mTuIDRCD under similar buffer and temperature conditions. TuDOG15 was the most efficient in cleaving 4-methyl catechol $(k_{cat}/K_M = 0.134 \ \mu M^{-1} \ min^{-1})$ and 4 chlorocatechol $(k_{cat}/K_M = 0.076 \ \mu M^{-1} \ min^{-1})$ compared to the other four recombinant T. urticae DOGs. Compared to bacterial homologs of Rhodococcus opacus and fungal homologs of Candida albicans and Aspergillus niger, the recombinant T. urticae DOGs were less efficient in cleaving these three model substrates (k_{cat}/K_M values approximately 2 to 4 orders of magnitude lower) (Ferraroni et al., 2006; Matera et al., 2010; Semana and Powlowski, 2019; Tsai and Li, 2007). In comparison to two hydroxyquinol dioxygenases (named NRRL3_05330 and NRRL3_02644) from Aspergillus niger, four of the recombinant T. urticae DOG enzymes were about 3-7 fold less efficient in cleaving 4-methyl catechol while TuDOG15 was about 1.4 fold more efficient than NRRL3 05330 in cleaving this substrate (Semana and Powlowski, 2019). Additionally, TuDOG15 and NRRL3_05330 have relatively the same affinity towards 4-methyl catechol, 117 and 119 μ M respectively.

200	Culture	K (B.0)) (R	h. (I. (V. (
DOG	Substrate	κ _м (μινι)	v _{max} (µivi min ⁻ -)	K _{cat} (min ⁻	K _{cat} /K _M (μινι ⁻ min ⁻)
	catechol	15.2 ± 4.5	0.14 ± 0.01	2.269 ± 0.162	0.149 ± 0.036
TuDOG1	4-methyl catechol	15.1 ± 2.7	0.12 ± 0.01	1.945 ± 0.162	0.129 ± 0.060
	4-chlorocatechol	21.2 ± 7.4	0.08 ± 0.01	1.297 ± 0.162	0.061 ± 0.022
	catechol	74.9 ± 30.9	0.42 ± 0.06	1.340 ± 0.191	0.018 ± 0.006
TuDOG7	4-methyl catechol	62.2± 42.3	0.53 ± 0.23	1.691 ± 0.734	0.027 ± 0.017
	4-chlorocatechol	115.2 ± 35.7	0.96 ± 0.23	3.062 ± 0.734	0.027 ± 0.021
	catechol	64.9 ± 24.3	0.56± 0.04	3.202 ± 0.229	0.049± 0.009
TuDOG11	4-methyl catechol	153.9 ± 24.6	0.58 ± 0.04	3.316 ± 0.229	0.022 ± 0.009
	4-chlorocatechol	25.2 ± 4.6	0.09 ± 0.01	0.515 ± 0.057	0.020 ± 0.012
	catechol	91.0 ± 21.2	0.09± 0.01	0.797 ± 0.089	0.009 ± 0.004
TuDOG15	4-methyl catechol	117.4 ± 46.5	1.77 ± 0.37	15.678± 3.277	0.134 ± 0.070
	4-chlorocatechol	72.0 ± 21.3	0.62 ± 0.09	5.492 ± 0.797	0.076 ± 0.037
	catechol	51.0 ± 23.4	0.06 ± 0.01	0.483 ± 0.081	0.009 ± 0.003
TuDOG16	4-methyl catechol	52.4 ± 19.6	0.14 ± 0.02	1.127 ± 0.161	0.022 ± 0.008
	4-chlorocatechol	154.3 ± 20.9	0.31 ± 0.01	2.496 ± 0.081	0.016 ± 0.004

Table 5-1 Steady state kinetic parameters (25 °C) of recombinant T. urticae DOG enzymes with catechol, 4-methyl catechol, and 4-chlorocatechol

5.4.6 Substrate diversity of T. urticae DOGs

The ability of *T. urticae* and *P. citri* DOGs to cleave more complex substrates in addition to the classical model substrates was evaluated by measuring oxygen consumption using a Clark-type electrode. As DOG enzymes consume oxygen to cleave aromatic rings, oxygen depletion can be linked to the conversion of substrates to their dicarboxylic acid derivatives. First, we checked oxygen depletion with the three classical model substrates assayed earlier in spectrophotometry (catechol, 4-methyl catechol and 4-chlorocatechol) and two additional model substrates of microbial dioxygenases (hydroxyquinol and protocatechuic acid). In this setup, the three classical model substrates showed high OCR with the *T. urticae* proteins while the *P. citri* protein *pcDOG5* had a low OCR of 1.113 nmol mL⁻¹min⁻¹ with 4-methyl catechol, confirming the finding from spectrophotometric assays. In addition, TuDOG11 and TuDOG15 showed activity towards hydroxyquinol while TuDOG16 was active on protocatechuic acid (Figure 5-5).

Next, fourteen monocyclic and nineteen polycyclic compounds (Table S5-2) of plant origin containing adjacent hydroxyl groups were screened in oxygen consumption assays. Here, sixteen compounds resulted in oxygen consumption rates (OCRs) higher than 1 nmol mL⁻¹ min⁻¹ (Figure 5-5), indicative of enzymatic activity towards a high number of newly uncovered DOG substrates. Interestingly, only six of the sixteen compounds cleaved by recombinant DOGs were monocyclic while the rest were

polycyclic with varying degree of polymerization, ranging from dicyclic (e.g. transclovamide, chlorogenic acid, fraxetin and rosmarinic acid) to more complex structures (e.g. epicatechin, catechin, eriodictoyl, ellagic acid and procyanidins A1 and B2). It is noteworthy that some of these polycyclic compounds namely eriodictoyl, epicatechin and catechin had similar or even higher OCRs compared to the model substrates, an indication that these complex plant metabolites could be better substrates for *T. urticae* DOGs. Our data revealed a low level of specificity of the *T. urticae* DOGs towards their substrates, as only 24% of all the metabolized substrates were cleaved by one particular DOG enzyme (Figure 5-5). These include the model substrate protocatechuic acid cleaved by TuDOG16, ellagic acid (TuDOG1), caffeoyl malic acid (TuDOG16) and procyanidins A1 and B2 (TuDOG15). Procyanidin A1 was also cleaved by the *P. citri* protein pcDOG6, with an OCR of 1.691 nmol mL⁻¹ min⁻¹.



Figure 5-5: Substrate screening by oxygen consumption assay. Color map indicating oxygen consumption rates observed in a screening assay to identify substrates cleaved by spider mite DOGs. 5 model substrates (left) and 16 of 33 plant secondary metabolites (right) screened were cleaved by our recombinant *T. urticae* DOGs. See Table S5-2 for a detailed list of the substrates screened. White boxes mean the substrate was not cleaved by that DOG while various shades of blue indicate ortho-cleavage of the substrate. The higher the color intensity, the more the OCR and therefore the higher the activity of that DOG on the said substrate. Abbreviations: OCR; oxygen consumption rate.

Last, to confirm that recombinant DOGs only cleave between adjacent hydroxyl groups, we also included two pesticides, all of which contain aromatic rings but lack adjacent hydroxyl groups. (i) 2,4-

dichlorophenoxyacetic acid (2,4-D) is a phenoxy herbicide reported to be metabolized by dioxygensases (Kumar et al., 2016; Pimviriyakul et al., 2020) and (ii) Carbaryl is an insecticide also metabolized by dioxygenases (Swetha and Phale, 2005). None of them were cleaved by recombinant DOGs.

5.4.7 Metabolite identification

As the quantification by OCR is an indirect method, the actual ortho-cleavage of the plant secondary metabolites was confirmed by UPLC-MS analysis. Eleven of the plant metabolites/DOG mixtures that showed oxygen consumption with the OCR measurements were subjected to UPLC-MS analysis. The substrate structures and predicted cleavage positions are shown in Figure 5-6a. Since the substrate is continuously cleaved to a muconic acid derivative during the reaction with active enzyme, the percentage metabolized substrate was estimated from a comparison of the residual substrate in heat inactivated (control) and active enzyme treatments, revealing that eight of the eleven substrates were fully cleaved after 20 minutes of incubation with the active enzyme (Figure 5-6b). To confirm cleavage, metabolites in the form of muconic acid derivatives were detected using mass spectrometry. Muconic acids are important intermediates towards the formation of succinyl-CoA and acetyl-CoA, the final products of the β-KAP that converts toxic aromatic compounds into safe metabolites of the tricarboxylic acid cycle (Wells and Ragauskas, 2012). Additionally, their structures can be predicted based on the cleavage position in the catecholic ring of the aromatic compound, allowing for their detection in mass spectrometry. All ortho-cleavage reactions occurred at the predicted positions, with the predicted muconic acid derivatives being formed as confirmed by structure elucidation with the UPLC-MS. Interestingly, recombinant DOGs were able to cleave transclovamide and procyanidins at the two possible positions containing adjacent OH groups, thus yielding multiple metabolites. TuDOG16 assays yielded two metabolites (detected at 390.08 and 422.07 m/z) with transclovamide as substrate and TuDOG15 assays also yielded two metabolites (detected at 609.12 and 641.11 m/z) for procyanidins B2. Although at low OCR, pcDOG6 cleaved procyanidin A1 at the two possible positions simultaneously, yielding three different metabolites (two isomers detected at 607.11 m/z and a third metabolite detected at 639.10 m/z). All UPLC-MS peaks and chemical structures of the detected products are provided as a supplemental figure (Figure S5-3).

Monocyclic compounds

A



Figure 5-6: Confirmation of ortho-cleavage activity of *T. urticae* DOGs. A) Chemical structures of the monocyclic and polycyclic catecholic compounds subjected to UPLC-MS. The ortho-cleavage positions are indicated with a red thunderbolt sign. B) Percentage metabolized substrate as estimated from residual substrate in active and inactive enzyme treatments after 20 min incubation at 25 °C. The enzyme used with the various substrates is indicated under the curve. Error bars represent the standard error of the mean (n=5).

5.5 Discussion

The complexity of plant-herbivore interactions is considered to be shaped by coevolutionary armsraces between plants and herbivores (Ehrlich and Raven, 1964; Howe and Jander, 2008). Plants have evolved strategies to defend themselves against pests, including signaling pathways that can activate defenses specifically upon attack, and establish a plethora of physiological responses that decrease their palatability. These include the accumulation of defense metabolites such as inhibitors of herbivore digestion and a wide range of toxins and antifeedants (Halkier and Gershenzon, 2006; Poulton, 1990). Herbivore feeding damage also induces structural changes that serve to repair wounds or to reinforce tissues and often linked to lignin biosynthesis which takes place downstream of the phenylpropanoid pathway. Many early lignin precursors (e.g. caffeic, ferulic, and chlorogenic acids) can also function as defense compounds or have a role in defense signaling (Bianchetti et al., 2013). The ability of the feeding herbivore to cope with these compounds is therefore paramount for its survival and reproductive success. Overall, arthropods have developed a myriad of defense strategies, ranging from insensitivity of target-proteins and receptor sites (pharmacodynamic mechanisms), to detoxification, sequestration and secretion (pharmacokinetic mechanisms) (Feyereisen et al., 2015; Li et al., 2007; Van Leeuwen and Dermauw, 2016). A number of enzyme families have been well-studied for their role in detoxification in arthropods, including those in spider mites. Previous studies have suggested that the evolution of a herbivorous generalist lifestyle might be linked to the expansion of several detoxification enzyme families, including cytochrome P450 mono-oxygenases, carboxylcholinesterases, glutathione-S-transferases, UDP-glycosyltransferases, and xenobiotic transporters (Bensoussan et al., 2018; Dermauw et al., 2013b; Grbić et al., 2011; Wybouw et al., 2015). Surprisingly, a number of horizontally transferred genes were also uncovered in the T. urticae genome, and are suggested to play a crucial role in the spider mite's adaptation potential (Dermauw et al., 2013b; Grbić et al., 2011; Wybouw et al., 2018). This includes DOG genes that encode enzymes which were suggested to directly ortho-cleave aromatic defense compounds of the host plant (Dermauw et al., 2013; Schlachter et al., 2019), a metabolic activity unique in animals.

In this study, we used multiple complementary approaches to gather evidence for the involvement of DOGs in the xenobiotic metabolism of spider mites. First, a phylogenetic analysis provided evidence that the horizontal gene transfer event occurred before the split of Tetranychidae and Tenuipalpidae families (Figure 5-1). We identified two clades containing DOGs from both mite families that could be more ancestral and represent a good candidate set to study the initial enzymatic abilities of the transferred *DOG* gene. Lineage specific expansion was also observed with *DOG* genes, most notably in the genus *Tetranychus*, where these patterns are also seen in other detoxification gene families (Ahn et al., 2014; Van Leeuwen and Dermauw, 2016). It is remarkable that an ortholog of *TuDOG3* could be

found in nearly all (66/70) Tetranychinae species, suggesting this gene is highly conserved and could play an essential role in the (eco)physiology of this genus. However, the identification of this gene in the transcriptomes of so many species could also be related to a relatively high transcript abundance, which is at least the case for T. urticae (see Figure 5-2a). The gene expression data in Figure 5-2a further revealed that only three DOG genes (TuDOG6, TuDOG11 and TuDOG14) are highly expressed exclusively in the embryos, suggesting a conserved role unrelated to feeding / diet for these T. urticae DOG genes especially for TuDOG6 and TuDOG14 which were not lineage specific. Previous studies have suggested the involvement of extradiol ring cleavage dioxygenases in the development process by cleaving aromatic amino acids (Sterkel and Oliveira, 2017). As such, the observed increase in expression level of the three dioxygenases could be an indication of a role in embryonic development, although this would be extraordinary for an intradiol enzyme. Furthermore, in situ hybridization also localized TuDOG11 and TuDOG16 to the egg before cell division, potentially indicating maternal import of DOG mRNA in the developing oocyte. This could either be consistent with a role in early development, but also could serve as maternally provided protection strategy. This was recently also suggested for cytochrome P450 mono-oxygenases (CYPs), well known detoxification enzymes, where maternally inherited CYP activity in the eggs was correlated with acaricide resistance in the egg (Itoh et al., 2021). The three dioxygenases were also highly expressed in diapausing mites that are characterized by an increased carotenoid biosynthesis (Bryon et al., 2017, 2013). As previous studies have reported the involvement of extradiol dioxygenases in the metabolism of carotenoids in plants, fungi and bacteria (Ahrazem et al., 2016; Meng et al., 2020; Von Lintig and Wyss, 2001), the observed increase in DOG expression could point to a role in carotenoid metabolism in the diapausing mites. The remaining DOG genes were more highly expressed in the feeding stages (larvae, nymph and adult) of mites feeding on different host plants than in the non-feeding stages, which makes a role in digestion and/or detoxification processes more likely. In this study, transcriptional responses of different spider mite strains feeding on wild-type tomato and a tomato mutant impaired in JA-induced defenses indicate that, upregulation of some DOGs depends on JA-responsive compounds in the host plant, and suggests that spider mites may do so to directly detoxify compounds after ingestion. In support of this idea, the in situ hybridization localized DOGs mainly in the gut (Figure 5-3), also suggesting a primary function in detoxification.

We observed that all five recombinant DOGs exhibited activity against at least three of the five tested classic model substrates (catechol, 4-methyl catechol, 4-chlorocatechol, hydroxyquinol and protocatechuate). Schlachter et al. (2019) observed that *TuDOG6* cleaves catechol and 4-methyl catechol with much less efficiency than the bacterial and fungal homologs. A similar low efficiency was observed with the five *T. urticae* DOGs in our enzymatic assays, suggesting that catechol and 4-methyl

catechol might not be the primary substrates of T. urticae DOGs. We therefore performed a high throughput substrate screening with an oxygen consumption assay to identify other substrates of T. urticae DOGs. We screened a panel of 33 plant metabolites as potential substrates, selected based on their presence in *T. urticae* infested plants, their reported toxicity in arthropods (Kortbeek et al., 2019; Misra et al., 2010; Mohammed AlJabr et al., 2017; Movva and Pathipati, 2017; Onkokesung et al., 2014) and/or since they are plant metabolites that possess two adjacent hydroxylated carbons (ortho) in the chemical structure. Of the 33 tested metabolites, 16 gave rise to detectable and reproducible oxygen consumption rates (OCR), ranging between 1 to 40 nmol mL⁻¹ min⁻¹ (Figure 5-5). Ortho-cleavage in 11 of the 16 detectable substrates was confirmed with UPLC-MS, providing first evidence that this family of DOGs can metabolize a plethora of natural substrates besides the few model substrates. Although formal evidence of a shift in toxicity between compounds and their ring-cleaved metabolites should be provided, this was technically not feasible. Nevertheless, it is clear that splitting an aromatic ring has a huge effect on the stability and the potential interactions of these molecules with their molecular targets. The wide array of substrates observed with the five recombinant T. urticae DOGs is surprising and these results are in sharp contrast to the restricted enzymatic capabilities of DOGs of microbial organisms. We therefore hypothesize that T. urticae DOGs, after the transfer event from a fungal donor species have evolved to metabolize a wide range of structurally unrelated plant metabolites. Indeed, we observed activity on compounds ranging from simple monocyclic organic compounds such as hydroxyquinol, caffeic and gallic acids, to complex polycyclic organic compounds such as phenolics (chlorogenic acid and rosmarinic acid) and flavonoids (epicatechin, eriodictoyl, catechin and procyanidins), with an even higher efficiency than the classic model substrates catechol and substituted catechols. These results are consistent with the TuDOG6 (mTUIDRCD) structure as previously characterized by Schlachter et al. (2019). Their study revealed a relatively large opening at the active site that might accommodate complex substrates as compared to the bacterial DOGs that usually have a smaller active site. Enzyme activity was also observed with dopamine, a plant growth regulator that responds to biological stressors (Liu et al., 2020). TuDOG16 showed high enzymatic activity for cleaving caffeic acid and chlorogenic acid (Figure 5-5 and 5-6), both of which are well-known tomato defense compounds against a wide range of insect herbivores, and whose accumulation in tomato leaves is induced by spider mite feeding (Kielkiewicz, 2002; Kundu and Vadassery, 2019). Even though most substrates were cleaved by more than one of the recombinant T. urticae DOGs, substrates like procyanidins (A1 and B2) and caffeoyl malic acid were only cleaved by TuDOG15 and TuDOG16 respectively. Previously, only fungal DOGs of plant pathogenic/associated fungi were reported to have the ability to degrade plant procyanidins (Roopesh et al., 2010; Wadke et al., 2016). Caffeoyl malic acid on the other hand is degraded by Streptomyces sp., a bacterial species capable of degrading a variety of phenylpropanoids (Bianchetti et al., 2013). The observation that horizontally acquired T. urticae

DOGs of likely fungal origin degrade natural plant compounds strengthens the hypothesis that horizontal gene transfer is a driving force in the evolution of arthropod herbivory (Wybouw et al., 2016). Within the context of benefiting from the enzymatic abilities of microbial organisms, it is interesting to note that a fungal DOG was found to play a central role in the association between the bark beetle Ips typographus and the fungus Endoconidiophora polonica. The beetle is a vector of the fungus and both attack Norway spruce. When feeding, the beetle induces an accumulation of defensive organic compounds that are subsequently cleaved by intradiol catechol dioxygenases of the fungus that utilizes them as a carbon source, thus enabling the bark beetle to escape the defense compounds of Norway spruce (Wadke et al., 2016). Transcriptional silencing of T. urticae DOGs further supports a role in host plant utilization. RNAi-mediated silencing of TuDOG16 via dsRNA injection significantly affected the survival rate of *T. urticae* when transferred to tomato (Figure 5-4). Together, these findings suggest that TuDOG16 is very important for the mite's ability to cope with the tomato defensive chemistry, possibly detoxifying caffeic and chlorogenic acids produced by the plant in response to spider mite attack. On the other hand, the omnipresence of DOGs in tetranychid species might also point towards a function for these enzymes across diverse plant hosts. All tetranychid mite species damage plant cells by piercing the cell wall with its stylets (Bensoussan et al., 2016) and plant cells are known to initiate lignin production upon cell wall damage (Denness et al., 2011). An increase in lignin production might in turn result in an increase in phenylpropanoid pathway intermediates, including 5-hydroxy-ferulic-acid and caffeic acid (Moura et al., 2010; Xie et al., 2018). Such catecholic compounds were often shown to exhibit defensive properties (Duffey and Stout, 1996). They are produced throughout the phenylpropanoid pathway and are believed to be converted into guinones by plant polyphenol oxidases (PPOs) in the herbivore gut. This is a two-component defense system where the plant accumulates herbivore-induced PPOs in vacuoles and ortho-dihydroxyphenolics in the cytosol, keeping them compartmentalized until they are ingested by a feeding herbivore and mixed in the gut. There is evidence that quinones generated by the PPOs after mixing them with these compounds damage the herbivore gut (Constabel and Barbehenn, 2008). Interestingly, PPOs are among the best documented mite- and jasmonate-inducible enzymes in plants such as tomato (Martel et al., 2015). Their expression is much lower in *def-1* (Howe and Ryan, 1999) and they act on catecholic compounds (Constabel and Barbehenn, 2008) as do the DOGs. Therefore we hypothesize that some DOGs may compete with plant PPOs in the mite gut to divert the production of dangerous quinones by PPOs towards the relatively harmless aliphatic acids. The accumulation of ortho-dihydroxyphenolics such as 5-hydroxy-ferulic-acid and caffeic acid has been previously observed in mite infested leaves (Kielkiewicz, 2002) and tetranychid DOGs might then be key in neutralizing these compounds directly, or indirectly by substrate competition with PPOs in the herbivore gut (Richter et al., 2012).

Although some *DOGs* were highly expressed in pesticide resistant strains (Figure 5-2a), the two pesticides tested in this study (2,4-D and carbaryl) were not metabolized, reinforcing the notion that DOGs act on aromatic rings with two adjacent hydroxyl groups. Given that in polyphagous herbivores and spider mites such as *T. urticae* in particular, large coordinated responses in gene expression were observed in response to pesticide selection and host plant use (Dermauw et al., 2013), we hypothesize that the observed increased expression of *DOGs* is probably a result of co-regulation in a more general stress response. Described coordinated responses have included genes involved in xeno-sensing, detoxification and transport (Dermauw et al., 2013; Wybouw et al., 2015), and is likely that DOGs further metabolize pesticides after the introduction of additional hydroxyl groups by CYPs. The DOG-mediated catabolism of catecholic intermediates formed downstream of the activation steps is typical for bacterial β -ketoadipate pathway, for example the degradation of the herbicide 2,4-D that is first converted to 3,5-dichlorocatechol (Kumar et al., 2016) or hydroxyquinol (Pimviriyakul et al., 2020). The insecticide carbaryl is first converted to 1,2-dihydroxynapthalene (Swetha and Phale, 2005) prior to ortho-cleavage by bacterial DOGs.

To see whether substrates of DOG proteins differ between species of the Tetranychidae, we also expressed two DOGs (PcDOG5 and PcDOG6) from the citrus red mite (*Panonychus citri*), a citrus specialist, that were specific to the *Panonychus* clade (Figure 5-1) (Bajda et al., 2015). Unfortunately, we experienced difficulties in obtaining high yield pure recombinant protein for these genes. Nevertheless, oxygen consumption assays showed activity on procyanidin A1 with PcDOG6. UPLC-MS analyses with PcDOG6 and procyanidin A1 also identified three metabolites formed from orthocleavage of the two hydroxyl positions present in procyanidin A1. Procyanidins function in many plants, including citrus, in defense pathways against biotic stress (Dixon et al., 2004; Rao et al., 2019). Given that they are synthesized from polymerization of catechin or epicatechin units, we also expected activity on these compounds as the *T. urticae* DOGs were active on these subunits as well as on procyanidins but this was not the case, possibly due to low activity of the two *P. citri* proteins. Nevertheless, as both *TuDOG15* and *PcDOG6* can metabolize procyanidin A1, we can conclude that DOGs from different tetranychid species can act on the same substrates, while establishing their degree of specificity should be a subject of future study.

Variations in the observed enzymatic properties (iron content and low activity with some proteins) could be attributed to the bacterial expression system we used for their production. Spider mites and fungi are eukaryotic and hence recombinant protein production in a prokaryote, albeit codon optimized, may suffer from misfolding or other post translational differences. Producing mite DOGs in a eukaryotic expression system, for example using insect cell lines, may result in enzymes that exhibit a less variable activity and that provide a more apparent substrate-specific contrast. Additionally, in

bacteria and fungi, DOGs often consist of heteropolymers (Ferraroni et al., 2006, 2005; Orville et al., 1997; Semana and Powlowski, 2019; Vetting and Ohlendorf, 2000). We did not investigate this property for the recombinant DOG enzymes, as the first structure elucidation of TuDOG6 (named mTUIDRCD) by Schlachter et al. (2019) revealed that this particular *T. urticae* DOG is active as a monomer. Co-crystallization of spider mite DOG enzymes with their substrates would be a step forward in understanding the substrate-enzyme interaction dynamics of these DOGs.

5.6 Conclusion

In this study, we examined the evolutionary history of *DOGs* and deduced that *DOG* genes were horizontally acquired before the formation of the (phytophagous) Tetranychidae family. Based on phylogeny and expression profile, a selection of tetranychid *DOGs* was expressed in *E. coli*, purified and functionally characterized with oxygen consumption assays while ring cleavage was confirmed by UPLC-MS. We show here that spider mite DOGs metabolize a wide variety of complex organic phytochemicals, a unique property for this class of enzymes. Substrates include many plant metabolites associated with inducible defenses, suggesting that DOGs function as detoxification enzymes. This hypothesis was re-enforced by the gut specific expression of some DOGs, their JA-dependent expression and their effect on spider mite survival on tomato when silenced by RNAi.

Funding information

This work was supported by the Research Council (ERC) under the European Union's Horizon 2020 research and innovation program, grant 772026-POLYADAPT to T.V.L. and 773902–SUPERPEST to T.V.L. N.W. was supported by a Marie Skłodowska-Curie Action Individual Fellowship (658795-DOGMITE) and a BOF fellowship (Ghent University, 01P03420) throughout this project. M.C. was funded by USDA's National Institute of Food and Agriculture, award # 2020-67014-31179 through the NSF/NIFA Plant Biotic Interactions Program.

Supplementary information

All supplementary data can be found at https://doi.org/10.1186/s12915-022-01323-1



Figure S5-1. Chemical structures of the three products formed from funneling reactions utilized by microorganisms in the breakdown of organic compounds. Catechol and protocatechuic acid degradation proceeds via the β -ketoadipate pathway

while hydroxyquinol is formed from protocatechuate and degraded in an alternative pathway found in some bacteria and fungi.



Figure S5-2. Schematic of oxygen consumption assay with Clark-type electrode. A) The Clark type electrode reaction chamber. The rubber seal replaces the microsyringe after injection of the various components into the system. It serves to seal the chamber from external oxygen. B) A zoom into the electrode unit. The platinum cathode is surrounded by a well that serves as a reservoir for electrolyte solution (50% potassium chloride). During the reaction, the electrolyte is ionized and initiates a current flow from anode to cathode. The current is equivalent to the oxygen consumption in the media. C) Typical oxygen consumption curve observed when there is substrate cleavage. D) The curve observed in the absence of substrate cleavage, catechol was added at the end of the reaction as a control for activity.

Figure S5-3. Spectra of selected substrates and their metabolites as detected in negative mode by UPLC-MS. Black peaks represent the substrate while red peaks depict the metabolites. Structures of both the substrate and the metabolite are as shown with their mass to charge ratios. The DOG used in the assay is indicated in blue. A thunderbolt sign shows the orthocleavage position.



Figure S5-4. PCR amplicons of TuDOG7, TuDOG10 and TuDOG11 and their neighboring intron containing eukaryotic genes.



Figure S5-5. *in situ* localization of *T. urticae DOG* genes (*TuDOG11* and *TuDOG3*). A DIG-labelled antisense probe was used for hybridization and the signal was developed using anti-DIG-AP and FastRed as substrate. The reaction product is visible as a red signal (the cuticle got red staining in both antisense and sense treatment so we consider that as false signals) while the spider mite body shows green auto fluorescence. Abbreviations: C, cuticle; PME, posterior midgut epithelium; PM, posterior midgut; V, ventriculus; VE, ventricular epithelium; Scale bars: 100 µm.



Figure S5-6. Stain-free SDS-PAGE (panel a) and western blot (panel b) of purified recombinant *T. urticae* DOGs. The 6x His tagged proteins are between 25-37 kDa.

File S5-1. DOG sequences used for phylogenetic analysis. This file should be opened with programs that can read fasta files e.g. text pad, snap gene viewer or MEGA software.

File S5-2a. Alleles of *TuDOG7*, *TuDOG15* as picked from London strain of *T. urticae* cDNA and those of *PcDOG5* and *PcDOG6* as picked from Lahijan strain of *P. citri* cDNA. This file should be opened with programs that can read fasta files e.g. text pad, snap gene viewer or MEGA software.

File S5-2b. Codon optimized sequences of *TuDOG7*, *TuDOG15*, *PcDOG5* and *PcDOG6* as ordered from Genscript (The Netherlands) without the signal peptide sequence. This file should be opened with programs that can read fasta files e.g. text pad, snap gene viewer or MEGA software.

File S5-3. Coverage plots of 17 *T. urticae* DOG genes and their surrounding regions in the genome of *Tetranychus urticae*. Gene models of DOG genes and their neighboring genes are depicted as follows: large and small orange boxes represent coding sequences and untranslated regions, respectively, whereas introns are shown as connecting lines between the boxes. (+) and (-) represent the forward and reverse strand, respectively. Underneath the gene models of *TuDOG17*, *TuDOG10* and *TuDOG11*, the length and position of an amplicon obtained by PCR (Fig. S5-4) is indicated with a red line. Next, coverage plots of Illumina-reads (""Illu.") from adult *T. urticae* polyA selected RNA (Grbić et al., 2011) and from genomic DNA sequencing of the EtoxR (""Etox") and London(""Lon") strain of *T. urticae* (Grbić et al., 2011; Van Leeuwen et al., 2012) are shown below the gene models. The Illumina reads coverage plots are followed by a coverage plot of Sanger (""San.") reads from genomic DNA sequencing of the London strain (Grbić et al., 2011) and by an alignment of these Sanger reads with the *T. urticae* genome of the London strain. Paired-end Sanger reads for which both reads are mapped in or extend nearby the indicated region are denoted by thin lines to show pair connections (Grbić et al., 2011) (see Wybouw et al., (2014), for mapping details).

File S5-4. DNA alignment of *TuDOG7* with a neighboring eukaryotic gene (*tetur07g05920*) identified as guanylate kinase. This file should be opened with programs that can read fasta files e.g. text pad, snap gene viewer or MEGA software.

File S5-5. DNA alignment of *TuDOG10* with a neighboring eukaryotic gene (*tetur12g01070*) identified as glutaminyl peptide cyclotransferase. This file should be opened with programs that can read fasta files e.g. text pad, snap gene viewer or MEGA software.

File S5-6. DNA alignment of *TuDOG11* with a neighboring eukaryotic gene (*tetur13g03880*) identified as SSUH2 homolog isoform X2. This file should be opened with programs that can read fasta files e.g. text pad, snap gene viewer or MEGA software.

File S5-7. Detailed phylogenetic analysis of tetranychoid DOGs (rectangular representation). For abbreviation of species names and sequences see Table S3. This file should be opened with a web browser e.g. Mozilla Firefox or Google Chrome.

Table S5-1. Primers. Primers used in qPCR to quantify the transcriptional response of DOGs to tomato defense (sheet1-host transfer primers), Primers used to synthesize *in situ* probes (sheet 2-in situ primers), primers used in RNAi for dsRNA synthesis and qPCR (sheet 3-RNAi primers), primers used to amplify *TuDOG7*, *TuDOG15*, *pcDOG5* and *pcDOG6* gene alleles from a cDNA pool (sheet 4-DOG allele selection primers) and primers used for HGT verification of *TuDOG7*, *TuDOG10* and *TuDOG11*(sheet 5-DOG-HGT verification primers).

Table S5-2. Compounds used in the substrate screening assay by oxygen consumption using a Clark-type electrode. All substrates were purchased from Sigma Aldrich (Belgium) except Transclovamide (Cayman Chemical, Netherlands). 20 mM stocks were freshly prepared in either methanol or DMSO prior to the assays.

Table S5-3. Number of *DOG* genes identified in tetranychoid species and the number of hosts these tetranychoid species have been reported on.

 Table S5-4. Orthologous tetranychoid DOG clusters containing T. urticae DOG genes.

Table S5-5. log2CPM values of *T. urticae DOG* genes across different stages (Grbić et al., 2011) and in *T. urticae* males or females (Ngoc et al., 2016).

Table S5-6. log2FC of T. urticae DOG genes in diapausing females, host plant adapted/acclimatized lines and in acaricide resistant strains

Table S5-7. Fecundity, mortality and survival data collected from RNAi injections experiments.

Table S5-8. Enzyme characteristics. Protein yields were estimated from protein concentrations determined using Bradford assay. Iron content was estimated using ferrozine assay with a self-developed Fe³⁺ standard curve. All purified DOG proteins had good enough yields, with the lowest yields in PcDOG5 and PcDOG6. All proteins were able to bind varying amounts of iron. The errors in iron content represent standard deviation of the mean (n=4).

Authors' contributions

TVL and NW conceived the study. CN, WD, NW and TVL designed the experiments. CN, WX, SDR and

JA performed the experiments. CN, MK, BV, MC and WD analysed data. CN wrote the manuscript with

significant input from NW, BV and TVL. All authors read and approved the final manuscript.

Chapter 6: General Discussion

6.1 General overview

Resistance development is a good example of rapid evolution that poses daunting challenges for agriculture (Bras et al., 2022). Resistance generally evolves as a result of directional selection exerted by pesticide treatments on pest populations of herbivorous arthropods (Bras et al., 2022). Studies suggest that some species are pre-adapted to evolve pesticide resistance due to their evolutionary history and standing genetic variation (Dermauw et al., 2018; Hardy et al., 2018; Walsh et al., 2022). While mutations in the target site of a pesticide can result in high resistance as discussed in chapter two, such mutations can also cause a fitness cost. These fitness effects have implications in the absence of a selection pressure as seen in chapter three. Resistance cases can also encompass both target site mutations and increased detoxification, as demonstrated in chapter four. Interestingly, a plethora of novel genes with a potential role in host plant adaptation and/or resistance are being discovered in arthropod herbivores. For instance; lipocalins, major facilitator superfamily (MFS) and short chain dehydrogenases (SDRs) that were upregulated in the multi-resistant T. urticae population studied in chapter four, or the laterally acquired DOG gene family discussed in chapter five. In the current chapter, the findings from these four experimental chapters are discussed in the context of resistance management, while suggesting possible future experiments and research avenues. To begin with, the complex nature of resistance and its implication on pest management is highlighted (section 6.2), with subsequent sections further exploring the various methods that can be used in characterizing resistance in agricultural pests. Further, the common factors between host plant adaptation and resistance are discussed (section 6.3), and the potential role of other gene families uncovered alongside the classical detoxification gene families (section 6.4). The chapter ends with exploring potential applications of the laboratory findings in a field setting (section 6.5).

6.2 The complexity of resistance

As it is was long believed that monogenic resistance is favored under field selection regimes, one expected single mutations in single genes to cause resistance in field populations (Ffrench-Constant, 2013; Georghiou, 1983; Osakabe et al., 2009; Roush and McKenzie, 1987). However, empirical work shows that this is rarely the case. Complex multi-resistance cases are often observed that involve a combination of multiple resistance mutations in different target sites and metabolic detoxification. One such complex resistance case was characterized in chapter four of this thesis. In addition to resistance mutations in different target genes, metabolic resistance was also identified as an underlying mechanism in the multi-resistant field population, VR-BE. In such resistance cases, whether target site and metabolic resistance mechanisms work additively or synergistically to resistance levels is not always clear. A potential synergistic action between target site mutations and detoxification enzymes can be inferred from our study with the *T. urticae* VR-BE population. Here, very high

resistance ratios (reaching >5000-fold) were recorded with compounds such as complex I inhibitors (pyridaben, tebufenpyrad and fenpyroximate), abamectin and fenbutatin oxide which we attributed to resistance mutations uncovered at the target sites of these compounds (H92R, I321T and V89A) and overexpression of multiple detoxification genes especially cytochrome P450s. Validation of these target site mutations in previous studies revealed that H92R mutation alone contributes up to 22-fold resistance to fenpyroximate, 30-fold resistance to tebufenpyrad and 60-fold resistance to pyridaben (Bajda et al., 2017), while I321T contributes up to 50 fold resistance to abamectin (Xue et al., 2021) and V89A confers moderate resistance to fenbutatin oxide, reaching 13-fold (De Beer et al., 2022b). Therefore, although we did not quantify the relative contribution of metabolic detoxification genes to the resistant phenotype, it is clear that the two resistance mechanisms are most likely acting in synergy to achieve the high resistance ratios observed in VR-BE. This suggests that combining both target site mutations and metabolic resistance can result in very strong resistance phenotypes. Indeed, multiresistant pest populations such as VR-BE that are resistant to most of the commercially available compounds are often encountered under field conditions, and strikingly these pest populations also resist compounds with new modes of action that have never been used in the field (Khajehali et al., 2011; Papapostolou et al., 2021). The high resistance to compounds of different MoA groups is a course for concern for resistance management, as it can lead to crop failure as was the case with VR-BE. Therefore, effective resistance management is essential so as to slow down the emergence and spread of resistance. This can be achieved by combining multiple control strategies in an integrated pest management approach. Here, strategies such as the use of nonchemical control methods and use of resistant cultivars is encouraged, and when necessary to use pesticides, reduced number of applications, optimal spray timings and dose rates, alterations of different MoA groups and regular monitoring of resistance should be observed (Corkley et al., 2022).

The complex nature of resistance is also evident from the various ways in which a target site mutation can impact resistance. In some resistance cases, a single target site mutation such as I1017F in chitin synthase can result in very high resistance to multiple mite growth inhibitors, with a potential risk of field failure (Demaeght et al., 2014; Papapostolou et al., 2021; Riga et al., 2017; Van Leeuwen et al., 2012). In other cases, individual mutations confer moderate levels of resistance, while co-occurrence of such mutations results in very high resistance levels to several compounds of the same MoA group (De Rouck et al., 2023). Good exemplars are the mutations in succinate dehydrogenase, I260V and S56L. I260V and S56L, found in subunits B and C respectively, individually do not confer high resistance to complex II inhibitors. However, their co-occurrence results in very high resistance to several on byflubumide (Maeoka and Osakabe 2021). Also, a common observation is that although a target site mutation may confer resistance to several compounds, resistance levels to these compounds often

differs, as is the case with complex I inhibitors discussed above. This is probably caused by differences in binding modes of the compounds to the target site. Lastly, although very rare, negative cross resistance can also occur as was apparent in chapter two. The resistance mutation H258Y resulted in high cross-resistance to cyenopyrafen and pyflubumide, but increased the sensitivity to cyflumetofen. A similar case has been reported with SDH inhibitors in the fungus *Mycosphaerella graminicola*. The mutation H272Y (corresponding to H258Y in *T. urticae*) results in cross-resistance to boscalid, isopyrazam and bixafen, but causes hypersensitivity to fluopyram (Sierotzki and Scalliet 2013).

6.2.1 Resolving the genetic basis of resistance

In chapter two of this thesis, we used a classical genetic approach to resolve the resistance to cyenopyrafen and pyflubumide observed in a T. urticae strain carrying a resistance mutation, H258Y, in complex II of the mitochondria. Reciprocal crosses revealed that cyenopyrafen resistance inherited as a monogenic trait in this strain while pyflubumide resistance inherited as a polygenic trait. To better facilitate resistance management, it is important to identify the resistance locus and hence the genes possibly associated with resistance. Therefore, development of multi-locus analysis is of paramount importance. Genetic mapping in combination with next generation sequencing have revolutionized the identification of genetic loci associated with complex traits such as resistance. In the spider mite T. urticae and T. kanzawai, a bulked segregant analysis approach has been established (Van Leeuwen et al., 2012; Wybouw et al., 2019b). A BSA approach relies on crossing parental strains with contrasting phenotypes, bulking (pooling) the segregating progeny that display phenotypic extremes, and identifying genomic intervals with divergent allele frequencies across progeny bulks (Kurlovs et al., 2019). Using this approach, three quantitative trait loci (QTL) were previously linked to pyflubumide resistance in the T. urticae JPR-R strain that we used for our study in chapter 2. In their study, Fotoukkiaii et al., (2021) revealed that two QTLs were found on the first chromosome and centered on cytochrome P450 genes. Subsequent comparative transcriptomics showed that CYP392A16 and CYP392E8 were overexpressed in the resistant JPR-R strain. The third QTL coincided with a cytochrome P450 reductase (CPR), which is necessary for P450 based metabolism (Snoeck et al., 2019; Wybouw et al., 2019).

At the time of QTL mapping, the H258Y mutation was not present in the resistant JPR-R strain. At this time, JPR-R was already highly resistant to pyflubumide (LC₅₀ values reaching 2000 mg/L) as a result of metabolic detoxification by cytochrome P450 activity (Fotoukkiaii et al., 2021). During the current experiments, we screened the succinate dehydrogenase (SDH) subunits for target site mutations, and the LC₅₀ values had risen to >5000 mg/L, suggesting that H258Y evolved as an additional resistance mechanism (given the continuous selection pressure with pyflubumide). Whether the metabolic detoxification identified by Fotoukkiaii et al., (2021) was still functional in our current study and

contributed to the observed polygenic inheritance of pyflubumide resistance is not clear. A H258L substitution (at the same position as the mutation we studied) was recently identified in field populations from Turkey with a history of exposure to cyflumetofen (İnak et al., 2022b). Although H258L has not yet been functionally validated, *in silico* modelling suggests that just like H258Y, this new mutation might influence resistance to cyenopyrafen and pyflubumide but most likely not cyflumetofen (İnak et al., 2022b). These results confirm that pesticide resistance is a rapidly evolving trait in arthropods, as previously suggested (Hawkins et al., 2019; Sparks and Nauen, 2015). Therefore, regular surveys should be conducted following the launch of a pesticide to monitor the resistance status in pest populations.

6.2.2 Target site mutation versus metabolic detoxification: What is the relative importance?

Once resistance is determined as polygenic (i.e. caused by a combination of multiple factors) and the various associated loci identified, the overall resistance may be the sum of contributions made by each individual factor, although synergistic or antagonistic interactions between resistance loci also occur (Riga et al., 2017; Williams et al., 2005; Zhang et al., 2016). It is therefore important to determine the relative importance and phenotypic strength of each individual resistance locus to the complex resistance phenotype. Functional validation of resistance mutations has been carried out using either in vitro or in vivo methods. In chapter 2, we used in vitro inhibition assays as one of the methods to functionally validate the complex II mutation H258Y. Using mitochondria extracts from a pyflubumide resistant strain (carrying H258Y mutation at the subunit B of SDH) and extracts from a wildtype strain (without the mutation), we could functionally validate that H258Y contributes to high cross-resistance between cyenopyrafen and pyflubumide but not cyflumetofen. This is because the mutation dramatically decreased the inhibition potency of cyenopyrafen and the active metabolite of pyflubumide in the resistant strain (12.5-fold and 22.8-fold less respectively), but did not negatively impact the cyflumetofen metabolite. The technique has also been used in a previous study by De Beer et al., (2022b) to validate the V89A mutation that was identified in ATP synthase (ATPase) gene of VR-BE (chapter four). In their study, De Beer et al., (2022b) revealed that V89A mutation results in a 2-fold lower inhibition of ATPase activity by fenbutatin oxide. These assays however do not tell to what extent the resistance mutations determine the resistant phenotype in the field. A more precise way to determine the effect of a mutation in vivo is to introduce it to a defined susceptible genetic background (Riga et al., 2017). This can be done using genome editing techniques such as CRISPR/Cas9. In a proof of concept study, Dermauw et al., (2020a) recently demonstrated the feasibility of this technique in creating gene knock-outs in mites. However, the editing efficiency was low and needs further fine-tuning before it can be used as a functional genetic tool in spider mites. As such, most studies make use of the insect model *Drosophila melanogaster* as an alternative workhorse to assess the relative strength of spider mite mutations using CRISPR/Cas9. Although this technique is direct, rapid and convincing, it does not always yield the desired results for spider mite mutations, probably due to differences in biology between mites and insects. For instance, an attempt to introduce some spider mutations such as G314D and G326E in GluCl1 and GluCl3 respectively seemed to be lethal, and transgenic lines could not be generated (Xue et al., 2021). As such, it is important to assess the mutation in its original organism to obtain reliable results.

In spider mites, marker assisted back-crossing provides a relatively precise method to isolate the mutation of interest from other resistance factors (Riga et al., 2017). The relative contribution of the resistance mutation to the resistant phenotype is then analyzed by comparing the genetically identical strains that differ only in a small region of the chromosome which carries the resistant locus of interest (Bajda et al., 2017). Using this technique (see chapter 2), the H258Y mutation was introduced into a susceptible strain and toxicity bioassays on the congenic lines revealed that indeed the mutation confers very high cross-resistance to cyenopyrafen and pyflubumide but not cyflumetofen. Such information can be very useful in resistance management, since in the event of detection of H258Y mutation in the field, cyflumetofen can still be used as a viable option to manage the resistant population. Back-crossing has also been successfully used in previous studies to investigate the relative contribution of other spider mite target site mutations (Bajda et al., 2017; De Beer et al., 2022b; Riga et al., 2017; Xue et al., 2021). These studies have relied on the assumption that a high recombination rate in T. urticae, apparent from previous studies (Demaeght et al., 2014; Van Leeuwen et al., 2012; Wybouw et al., 2019), results in near-isogenic lines following introgression. As revealed by our study however, it is important to carry out additional reciprocal crosses after introgression to ensure that no residual resistance factors are present in the introgressed lines. Resistance should inherit as a monogenic trait after introgression as it is expected that other resistance factors are uncoupled during the repeated marker assisted backcrossing. However, this was not the case with our H258Y mutation studied in chapter 2. The resistance to pyflubumide was still polygenic after introgression. As we used a genetically diverse recipient population, the genetic variants segregating in such a population might result in unclear nuclear-mitochondria interactions. Oxidative phosphorylation, which is one of the most important biological functions for energy generation, depends on interactions between imported nuclear-encoded proteins and those encoded by mitochondrial DNA. Therefore, proteins from these two genomes must be highly compatible in order to maintain structural and biochemical properties (Wolff et al., 2014). A considerable level of nucleotide variation has been observed in the genes coding for mitochondrial subunits at the level of populations from the same species and even within individuals (Wolff et al, 2014), and accumulating evidence from both vertebrates and invertebrates

suggests that this sequence polymorphism is pervasively tied to considerable phenotypic effects (Ballard and Whitlock, 2004; Blier et al., 2001; Dowling et al., 2008; Galtier et al., 2009; Rand, 2001). Even so, the imported proteins and mitochondrial proteins are expected to be compatible in their native susceptible or resistant strains since the individuals in the strain have evolved together. However, introgression introduces a nuclear encoded protein from the resistant strain that must be imported into the mitochondria of a susceptible strain, and this could result in potential negative interactions that manifest in the introgressed phenotype, especially given that there were multiple segregating variants in the susceptible strain. The use of inbred lines as the recipient population eliminates genetic variation, thus could potentially minimize the occurrence of such interactions. It is also possible that allelic variants may fail to break apart if the resistance locus is in a low-recombination region of the genome. Testing for the distribution of recombination events can be carried out by following the protocol recently described by Ji et al., (2023). Briefly, virgin females of an inbred susceptible strain are crossed to males of an inbred resistant strain, generating F1 female heterozygotes. Since unfertilized eggs develop into haploid males, haploid F2 males are produced from the F1 virgin females. (Note: the haploid F2 males have recombined chromosomes as a result of meiotic crossovers in their hybrid F1 mothers). Subsequently, a number of F2 males are crossed individually to virgin females of the susceptible strain to generate F3 families. The females in each of the resulting F3 families are genetically identical because they arose from a cross between one isogenic haploid male and isogenic females of the susceptible strain. Therefore, only two genotypes (RS and SS) are possible for each locus in an F3 isogenic family (Figure 6-1, Ji et al., (2023). Next, an average number of F3 females are collected from each F3 family for RNA extraction and sequencing. To identify the recombination events, RNA-seq reads are aligned to the reference genome, then using RNA-seq reads at single nucleotide polymorphism (SNP) positions predicted from genomic sequencing data of the resistant and susceptible strains, the F3 families are genotyped and recombination breakpoints assigned as midpoints between the respective flanking junction SNP sites (i.e regions that run from SS to RS and viceversa in a sliding window analysis). For a detailed protocol, refer to Ji et al., (2023). From the study by Ji et al., (2023), the recombination events were not randomly distributed, and there were no large chromosomal regions of very low recombination. Rather, an average of 2 to 5 recombination events per chromosome were observed for T. urticae (Figure 6-1b). Although difficult to make comparisons with other organisms as most data is gathered in centimorgan per mega base, the recombination rate observed for T. urticae can be considered high as it compares to that of honey bees which are so far the only eukaryotes reported to have high recombination rate of about 5 recombination events per chromosome (Wallberg et al., 2015).



Figure 6-1. Experimental design for distribution of recombination events. a Making the genetic crosses to generate F3 families. b Histogram of the recombination events in three *T. urticae* chromosomes as determined with 458 F3 families using RNA-seq based genotypes. Note, a median of two recombination events were observed for each chromosome. c Distribution of the recombination events in F3 families as assessed with a sliding window analysis (orange line, left axis), and proportion of RS genotypes (black line, right axis). Note, there are no large chromosomal regions of very low recombination. Significant deviations from the expected 1:1 RS:SS genotype ratios are only observed on chromosome 2 (i.e an excess of RS genotypes, encircled). This figure is adapted from Ji et al., 2023.

Next to assessing the relative importance of resistance mutations, the contribution of detoxification genes to the resistant phenotype can be inferred from sequencing total RNA (RNAseq) followed by gene expression analysis. Using this technique in chapter four, we identified a great number of detoxification genes that were differentially expressed in the multi-resistant VR-BE population compared to a susceptible strain of *T. urticae*. The upregulation of multiple detoxification genes has been previously reported in studies with resistant *T. urticae* populations and strains adapted to various host plants (Dermauw et al., 2013; Snoeck et al., 2018; Wybouw et al., 2019a). This confirms that *T. urticae* recruits multiple genes from various detoxification enzyme families as a first line response to different chemical stresses, as previously suggested by Dermauw et al., (2013). While the regulatory mechanisms involved were unclear in these previous studies, Kurlovs et al., (2022) recently showed that gene regulation, especially in trans, affects the expression of detoxification genes in multi-resistant strains. An even more recent study pinned the large trans-driven differences in detoxification

gene expression in *T. urticae* resistant strains to a nuclear receptor HR96-related gene (Ji et al., 2023). Some genes might be upregulated as a result of co-regulation in a general stress response. Therefore, follow-up tests are required to narrow down on specific detoxification genes contributing to the resistant phenotype. These include synergism assays, which are a good first indicator of metabolic resistance, and whose results can help design resistance management strategies. The common synergists used are: piperonyl butoxide (PBO), diethyl maleate (DEM) and S,S,S-tributyl phosphorotrithiolate (DEF) which inhibit P450s, GSTs and CCEs respectively. In chapter four, synergism assays with PBO revealed increased metabolism by P450s as a potential resistance mechanism to the complex II inhibitors cyenopyrafen and cyflumetofen. As such combining these acaricides with PBO can hypothetically improve their efficacy in controlling the resistant population.

As earlier discussed, Quantitative trait locus (QTL) mapping based on bulked segregant analysis (BSA) can also been used to identify potential candidate genes contributing to resistance, as they will be located within the BSA peaks associated with resistance or in adjacent chromosomes falling within the QTL intervals. Important candidate genes contributing to resistance can also be inferred from the pool of differentially expressed genes by RNA interference (RNAi). For instance, CYP392A16 is a cytochrome P450 gene mostly found upregulated in resistant T. urticae strains. Silencing of this gene via RNAi in an abamectin resistant strain has been shown to reduce the resistance levels from 3400- to 1900-fold compared to a susceptible strain (Papapostolou et al., 2022). Similarly, the knockdown by RNAi of an HR96 gene confirmed its importance in trans-regulation of detoxification gene expression (Ji et al., 2023). Although a relatively fast method, RNAi has some drawbacks in that it is not always precise enough due to off target effects on related genes, and does not work equally well across different pest species, tissues or developmental stages (Cooper et al., 2019). Alternatively, CRISPR/Cas9 knockout of candidate detoxification genes can be carried out in species where this technique is applicable. The primary difference between RNAi and CRISPR/Cas9 is that RNAi results in gene knockdown, reducing gene expression at the mRNA level while CRISPR/Cas9 results in gene knockout, permanently silencing the gene at the DNA level. As such, CRIPSR/Cas9 generates stronger and consistent phenotypes (Tyagi et al., 2020). However, the practical use of CRISPR/Cas9 for gene knockout is limited to only a few species. Recently, CRISPR/Cas9 was optimized for use in T. urticae, but has not yet been used to knockout detoxification genes (De Rouck et al., 2024). The technique has however been successfully applied in insect species, for example, to validate the role of cytochrome P450 gene in detoxification of insecticides in the hemipteran Nilaparvata lugens (Zhang et al., 2023).

Where possible, identified candidate genes can be functionally validated by recombinant expression followed by metabolism assays. This *in vitro* technique involves cloning of the gene of interest in heterologous systems such as *Escehrichia coli*, baculovirus or yeast (Berger et al., 2004; Byrne et al.,

2005; Rosano and Ceccarelli, 2014). The expressed protein is then purified and investigated for the ability to metabolize pesticides. As highlighted in our study in chapter four, most candidate genes have not yet been functionally characterized, owing to the challenges faced with heterologous expression systems. These include: difficulties in finding the optimal conditions for the expression of some candidate genes; instability of the expressed protein and improper post-translational modifications such as glycosylation and improper folding that can lead to a non-functional protein. Besides, even if the protein is successfully expressed, it can end up in inclusion bodies which makes it difficult to purify. Some proteins also aggregate during purification, thus becoming inactive. Nevertheless, some candidate detoxification genes often found upregulated in resistant populations of *T. urticae* have been functionally characterized (reviewed in De Rouck et al., (2023), including CYP392A16 which can metabolize abamectin and pyflubumide *in vitro* (Fotoukkiaii et al., 2021; Riga et al., 2014), TuGST05 that can metabolize cyflumetofen (Pavlidi et al., 2017), UGT10 and UGT29 that can glycosylate abamectin (Xue et al., 2020)and CCEinc18 that can metabolize bifenthrin (De Beer et al., 2022a).

To conclude, both target site mutations and metabolic detoxification play an important role in complex resistance phenotypes as seen from the various methods. Based on the body of literature on spider mite pests, resistance almost always involves multiple underpinning mechanisms, and some data shows that metabolic resistance first arises, followed by target site resistance (for example pyflubumide resistance characterized in chapter two) (Fotoukkiaii et al., 2021, 2020). Under continuous selection pressure with a pesticide, combining both target site and metabolic resistance mechanisms gives a stronger resistance phenotype. But, resistance mechanisms might also carry a fitness cost. Therefore, with relaxation of pesticide use, selection against mechanisms that put a high functional constraint on the pesticide target is likely to occur, and therefore they will be lost in the population.

6.2.3 Pleiotropic fitness costs associated with resistance

Overexpression of a resistance conferring gene is associated with a fitness cost if reallocation of energy occurs at the expense of metabolic and developmental processes. Similarly as seen in chapter three, a target site modification can result in a fitness cost when the target site mutation results in inefficiency of the encoded gene product, especially if it is essential for viability of the pest. Admittedly, it is not straightforward to assess if a resistance mechanism is associated with a pleiotropic fitness cost. Nevertheless, multiple studies on the potential fitness costs of a variety of resistance mechanisms to chemical compounds of different MoA have been performed (see Bajda et al., (2018) and the review by Kliot and Ghanim, (2012). Unfortunately, the quality and reliability of some of these studies varies considerably due to the experimental setups and methodologies. As revealed by our study in chapter three, analysis of fertility life table parameters, which is the most frequently used method in assessing

the fitness costs associated with a resistance mutation is not always sufficient. This is because the method fails to put into consideration unfavorable conditions (such as high population density, suboptimal nutrition, fluctuating temperatures and presence of competing strains), during which fitness costs are most likely to appear. Additionally, the method is biased in controlling for variation in fertility and mating behaviour, given that only a few individuals are used to represent the entire pest population. We showed that a good experimental setup should also consider a population evolution approach, where the susceptible and resistant strains are kept in direct competition against one another for a period of time without pesticide selection. A population setup controls for non-optimal conditions such as food shortage, high rate of migration and high population density, therefore any fitness costs such as energy metabolism, that would only appear under unfavorable conditions is not missed out. After identifying the fitness status of a pest population, management strategies can take advantage of the presence of a fitness cost by extending periods between pesticide treatment. This creates an unsprayed refugia where susceptible individuals can thrive, thus facilitating their selection in the population. In the absence of a fitness cost, alternating pesticides with unrelated MoA which does not discriminate between susceptible and resistant individuals can slow down the spread of resistance (Leeper et al., 1986).

Comparing our study to a review by Ffrench-Constant and Bass, (2017), which outlines other basic criteria that should be followed when designing studies on fitness costs associated with pesticide resistance, there is another key point to note. According to the authors, it is more meaningful to study the pleiotropic fitness costs associated with a field evolved resistance mechanism because it has direct implications in management of resistance in the field. In my view however, although the resistant genotypes evolving from continuous selection in the laboratory may not be true representatives of genotypes found in the field, studying laboratory evolved resistance is nevertheless very much informative. In chapter two and three, we validated a laboratory evolved resistance mutation H258Y and its fitness costs. Resistance mutations on the same residue are widespread in fungi, where they result in cross-resistance to multiple fungicidal SDH inhibitors. In comparison to fungal SDH inhibitors which have been in the global market as early as 1966 (Sierotzki and Scalliet, 2013), acaricidal SDH inhibitors belong to one of the newest MoA groups, developed less than 15 years ago (Furuya et al., 2017; Sparks and Nauen, 2015) and thus not yet registered for use in most countries. This is probably the reason why resistance mutations to acaricidal SDH inhibitors are not yet widely studied and reported. However, prior knowledge on such mutations and their fitness implications, in combination with tailor made early detection strategies, will prove useful in the near future as acaricidal SDH inhibitors continue to be registered for use in many countries. In light of this, a mutation H258L on the same residue as H258Y was recently discovered at a low frequency in a field population from Turkey that was exposed to cyflumetofen (inak et al., 2022b). The mutation is predicted to affect binding of the complex II inhibitors pyflubumide and cyenopyrafen in a similar way as H258Y (inak et al., 2022b). Based on the knowledge from H258Y therefore, it is advisable to avoid the introduction of cyenopyrafen and pyflubumide, as this could accelerate selection of the mutation and hence development of resistance to these acaricides. In our fitness cost study (chapter three), we also designed and tested a highly sensitive detection strategy for H258Y mutation based on droplet digital PCR (ddPCR) technique recently described by Mavridis et al., (2021). Given the high sensitivity of ddPCR (detection limit of 1% i.e. one mutant allele in a background of 100 wild type alleles), early field screening and monitoring can be done in regions using SDH inhibitors for pest control, enabling appropriate intervention measures to avoid the spread of resistance in the field.

6.3. A link between host plant adaptation and resistance

The interpretation of metabolic resistance in generalist herbivores is complicated by the realization that similar gene expression responses evolve after both the development of pesticide resistance and adaptation to new host plants (Dermauw et al., 2013; Zhu et al., 2016). In a study with T. urticae strains, Dermauw et al., (2013) observed striking resemblance between the transcriptional profiles of multiacaricide resistant strains and mites maintained on tomato for five generations after transfer from bean. Most of the genes with altered expression in both the multi-resistant strains and the tomato adapted mites belonged to known detoxification enzyme families (CYPs, CCEs, GSTs and UGTs) (Dermauw et al., 2013). The authors also observed that the tomato adapted mites had a decreased susceptibility to some acaricides from different MoA groups, suggesting that host plant adaptation can influence pesticide resistance. These observations support an earlier proposed model whereby the ability to mount a broad response to diverse plant defense chemicals predisposes generalist arthropod herbivores to evolve resistance to pesticides (Alyokhin and Chen, 2017; Dermauw et al., 2013). Our study with the multi-resistant VR-BE population in chapter four provides additional evidence to support this 'pre-adaptation syndrome' by showing that the host plant influences gene expression in the generalist herbivore, T. urticae, and that these host specific changes in transcript level affect acaricide toxicity and resistance. This is supported experimentally by synergism assays, which reveal that treatment of the tomato population with a synergist that inhibits P450s restores the toxicity of cyflumetofen to similar levels with that of the bean population, suggesting that the 4-fold higher LC_{50} value recorded with the tomato population prior to treatment with PBO was due to increased metabolic detoxification by P450s in this population. Indeed, cytochrome P450s were among the most strikingly differentially expressed genes in the two populations, and are induced in response to phytochemicals (Vandenhole et al., 2021). Moreover, P450s can metabolize both acaricides and plant allelochemicals. For instance, the CYP6B enzymes in Lepidopteran species can metabolize plant defense furanocoumarins (Berenbaum et al., 2011), and heterologous expressed CYP6B enzymes have recently been shown to efficiently metabolize the pyrethroid esfenvalerate *in vitro* (Shi et al., 2021). Besides P450s, there are detoxification enzymes from other gene families that have also been shown to metabolize both natural and synthetic xenobiotics. In spider mites for example, recombinant *T. urticae* UGTs (UGT11, UGT59 and UGT66) could glycosylate a wide range of plant secondary metabolites from the flavonoid class as well as a broad spectrum of acaricides *in vitro* (Snoeck et al., 2019b). This functional redundancy of detoxification genes challenge resistance management and pest control strategies, as multiple genes are available to arthropod herbivores to cope with the natural and synthetic xenobiotic stresses they encounter. But, to what extent the detoxification routes for plant allelochemicals are co-opted for synthetic pesticides remains a topic of intense debate (Dermauw et al., 2018; Hardy et al., 2018).

To maximize the effectiveness of modern integrated pest management strategies, the important question then would be whether there are detoxification genes that evolved to detoxify plant allelochemicals, and whether these genes are also targets of selection for resistance to synthetic compounds. These are difficult questions to answer, and ones that will take a considerable amount of research, given that only a small percentage of detoxification genes have been functionally characterized. Besides, genomes of most pests are still unavailable, and for the pests such as T. urticae whose genomes have been annotated, new discoveries of genes with unknown functions continue to be made. Such genes include multiple genes acquired by horizontal gene transfer from microorganisms (Grbić et al., 2011; Wybouw et al., 2018), which have equipped arthropod herbivores with new metabolic capabilities (Wybouw et al., 2018, 2016). In a long term population study with T. urticae, Wybouw et al., (2019a), investigated whether the genomic responses to selection by a pesticide and host plant are similar, revealing that the two adaptation processes most likely have different genetic architectures. According to Wybouw et al., (2019a), host plant adaptation has a highly polygenic response, which is consistent with host plant adaptation observed with herbivorous insects (Jones, 1998; Oppenheim et al., 2015). So far, a few candidate genes for host plant adaptation are known, for example the CYP6CY3 gene that can efficiently detoxify nicotine, thus adapting aphids to feed on tobacco (Bass et al., 2013) and the laterally acquired β -cyanoalanine synthase gene that can detoxify cyanogenic glucosides, thus enabling spider mites to feed on cyanogenic plants (Wybouw et al., 2014). To add to this body of knowledge, a laterally acquired gene family in spider mites, the intradiol ring cleavage dioxygenases (DOGs), was functionally characterized in chapter five. Through heterologous expression in E. coli followed by metabolic assays with a collection of model substrates, plant secondary metabolites and pesticides, we show that DOGs are more involved in host plant adaptation than detoxification of anthropogenic pesticides. For example, recombinant TUDOG11 was able to
metabolize known tomato defense metabolites caffeic acid and chlorogenic acid, but could not metabolize abamectin, even though it was among the genes upregulated in abamectin resistant strains (Xue et al., 2020). This might be attributed to the fact that DOGs split between two hydroxyl groups (ortho cleavage), a structural feature that is common in aromatic compounds that constitute most plant allelochemicals, but one that lacks in synthetic acaricides and pesticides. As such, although *TuDOG11* was among the genes specifically upregulated in the VR-BE population on tomato and not in the VR-BE population on bean, it is probably acting in response to tomato defenses or probably upregulated as a result of co-regulation in a more general response to stress. Indeed Kurlovs et al., (2022) recently showed that *DOGs* are mainly highly upregulated as a result of trans-regulatory variation in multi-pesticide resistant strains, and an even more recent study identified a HR-96 related gene as the master regulator of this trans-driven expression of detoxification genes (among them *DOGs*) observed in resistant *T. urticae* strains (Ji et al., 2023). Still, the question of whether DOGs participate in pesticide detoxification downstream of phase I enzymes should be investigated in the future with intermediate products formed from hydroxylation steps by phase I enzymes.

6.4 Beyond the classics: Evolution of xenobiotic adaptation

Xenobiotic response has been widely studied in insects and mites (reviewed in Gao et al., (2022) and De Rouck et al., (2023), revealing similar results in general, that over hundreds or even thousands of genes are up- or downregulated in response to host plant chemicals or pesticides. Beyond the classical detoxification enzyme families (P450, UGTs, GST and CCE) that often appear on the list of differentially expressed genes in response to xenobiotic stress, genes such as *DOGs, lipocalins, MFS* and *SDRs*, that were previously not implicated with resistance (Dermauw et al., 2013; Snoeck et al., 2018; Wybouw et al., 2015), are also found upregulated, and will be briefly discussed in this section. Lipocalins, MFS and SDRs have not yet been functionally characterized, but their function is speculated based on observations from transcriptomic analysis.

The laterally acquired *DOG* genes characterized in chapter five are known to catalyze the oxygen mediated ring fission in aromatic rings by specifically cleaving between two adjacent hydroxyl groups (ortho-cleavage). The aromatic ring structure containing two adjacent hydroxyl groups is found in some plant secondary metabolites, therefore you would expect to find DOGs in other plant feeding herbivores, especially polyphagous insects. However, this is not the case as to date, DOGs have not yet been reported in insect genomes in spite of a rich repertoire of laterally acquired genes in insect species (Chen et al., 2016; Crisp et al., 2015; Li et al., 2011; Sun et al., 2013; Zhu et al., 2011). But, as noted earlier in chapter one, plant-herbivore interaction is a co-evolutionary adaptation, in which plants have evolved defense mechanisms that are tailor-made to the attacking herbivore and the herbivore is in turn adapted to feeding on specific groups of plants. Since DOGs offer a selective

advantage evident from their ability to degrade a wide variety of plant allelochemicals, spider mites might have acquired them to aid in the expansion of host plant range. It was earlier proposed that the horizontal gene transfer of DOGs occurred after the split of mite lineages, as DOGs have not been discovered in genomes of non-plant feeding Acari (Dermauw et al., 2013). Our phylogenetic analysis now suggests that the transfer event occurred before the split of Tenuipalpidae (false spider mites) and Tetranychidae (spider mites) families, and further reveals that lineage, genus or species specific expansions occurred within the Tetranychidae family following acquisition.

Gene expression analysis of the multi-resistant VR-BE population in chapter four revealed differential expression of lipocalins, which have only been marginally associated with arthropod detoxification (Dermauw et al., 2013). Lipocalins are a large group of small extracellular proteins with the ability to bind hydrophobic molecules (Ahnström et al., 2007; Flower et al., 2000). They are found in many species, and have diverse roles in metabolism, perception, reproduction, developmental processes and modulation of immune responses (Chudzinski-Tavassi et al., 2010). In *T. urticae*, 58 lipocalin genes have been identified, a number that exceeds by far those found in insects (*Drosophila melanogaster*,4; *Apis mellifera*, 4; *Rhodnius prolixus*, 22) and in humans (10) (Dermauw et al., 2013). Due to the hydrophobic ligand binding properties associated with lipocalins (Ahnström et al., 2007), *T. urticae* lipocalins may bind acaricides or plant toxins, resulting in sequestration of these normally hydrophobic molecules.

Next to lipocalins, genes belonging to the major facilitator superfamily (MFS) were also among the differentially expressed genes, especially in the tomato population. MFS are single polypeptide carriers capable of transporting small solutes in response to chemiosmotic ion gradients (Saier et al., 1999). Mechanistically, MFS transporters exhibit three distinct kinetic mechanisms: (i) there are uniporters which transport only one type of substrate and are energized solely by the substrate gradient; (ii) symporters which transport two molecules in the same direction simultaneously, making use of electrochemical gradient of one of the substrates as the driving force and (iii) antiporters which transport two or more substrates across the membrane but in opposite directions (Law et al., 2009). MFS transporters are well studied in prokaryotes, and have a defined role in transport of toxic substances in bacteria and fungi (Kretschmer et al., 2009; Kumar et al., 2020; Saidijam et al., 2006). In T. urticae, 109 genes that cluster into three OrthoMCL groups (10082,10032 and 10236) have been identified (Dermauw et al., 2013). Members of cluster 10236 are highly similar to anion/cation symporter (ACS) family while cluster 10082 are highly similar to sodium ion dependent glucose transporter family. On the other hand, most members of cluster 10032 exhibit similarities with bacterial tetracycline:H⁺ antiporters and their mammalian homologs the heme-carrier proteins/thymic-folate co-transporters (Dermauw et al., 2013; Shayeghi et al., 2005). Their upregulation in *T. urticae* might result in a higher efflux of acaricides/toxic plant metabolites out of spider mite cells as previously suggested by Dermauw et al., (2013).

Short chain dehydrogenases (SDRs) were also among the differentially expressed genes in the multiresistant population, VR-BE, and have also been found upregulated in *T. urticae* populations on different hosts (Snoeck et al., 2018). The SDR superfamily forms one of the largest and most highly diverse protein superfamilies found in all domains of life (Kallberg et al., 2010). They are associated with biosynthesis and metabolism of endogenous signaling molecules like steroid hormones and retinoids, as well as detoxification of xenobiotics (Hoffmann and Maser, 2007; Škarydová and Wsól, 2012). Alcohol dehydrogenases are some of the well characterized SDRs in insects (Figueroa-Teran et al., 2016; Mayoral et al., 2013; Zhang et al., 2004), and have been found in the saliva of aphids, white flies and thrips (Stafford-Banks et al., 2014; Su et al., 2012). In *T. urticae*, 88 full length SDRs have been identified, including several apparent species-specific expansions (Snoeck et al., 2018). Some of these expansions cluster together with a Drosophila SDR named sniffer, a carbonyl reductase involved in the prevention of oxidative stress-induced neurodegeneration (Martin et al., 2011). The same expansions were differentially expressed upon acclimation of *T. urticae* to different host plants (Snoeck et al., 2018), and might therefore play a protective role during spider mite feeding.

In addition to the genes described above, some of the genes with the most striking gene expression changes in the multi-resistant VR-BE population had an unknown function, and the encoded product was predicted to be secreted. Such genes included tetur11q05420, tetur11q05450 and tetur46q00020 which were overexpressed in the population on tomato, recording log₂ fold changes of 10, 13 and ~9 respectively. These same genes were upregulated by ~700 fold upon adaptation of T. urticae from bean to tomato in Dermauw et al., (2013). The three genes have also been identified in the salivary composition of T. urticae, and belong to a T. urticae specific OrthoMCL group (Tu MCL 21) (Jonckheere et al., 2016). To facilitate its polyphagous lifestyle, T. urticae has recently been shown to weaken or even suppress plant defenses through secretion of effector-like proteins (Blaazer et al., 2018; Villarroel et al., 2016). Admittedly, arthropod effectors are difficult to identify. However, they share some common characteristics such as: (i) the presence of an N-terminal signal peptide that directs them to the secretory route; (ii) they encode products showing rapid evolution such as poor sequence similarity with other genera, multiple gene copies and high rates of non-synonymous mutations; (iii) they are functionally highly specific, and (iv) their expression levels are dependent on the host plant (Jonckheere et al., 2016). These characteristics were also found in tetur11g05420, tetur11q05450 and tetur46q00020 (secreted gene product, high expression in tomato compared to bean VR-BE population and lack of homology to existing OrthoMCL groups), suggesting that they could function as effectors although further research is needed to ascertain their function in T. urticae.

6.4.1 Other potential players in xenobiotic stress response

Symbionts. Many arthropod species are colonized by microorganisms that influence their ecology and evolution (Berman et al., 2023; Perlmutter and Bordenstein, 2020). Interactions with bacterial symbionts have been studied extensively especially in insect species (Bras et al., 2022; Itoh et al., 2018; Liu and Guo, 2019), with recent advances revealing that microbial symbionts can facilitate xenobiotic resistance via direct detoxification or by activating the insects immune system to encapsulate the toxins (Bras et al., 2022). For instance, Riptortus pedestris, a serious insect pest of the leguminous crops, harbors a bacterial symbiont of the genus Burkholderia in its gut. These bacteria metabolize the organophosphorous insecticide fenitrothion to 3-methyl-4-nitrophenol, which is then excreted by the insect (Sato et al., 2021). As revealed by transcriptomic analysis and reverse genetics, fenitrothion degrading genes are highly expressed when the symbiont is cultured on minimal medium containing fenitrothion as the sole carbon source, and deletion of these genes via homologous-recombinationbased deletion method significantly reduces the survival rate of colonized insects upon treatment with fenitrothion (Sato et al., 2021). Similarly, *Bifidobacterium*, a symbiont of the honey bee, can metabolize amygdalin, a cyanogenic glycoside. As revealed by LC-MS analysis, the metabolic products prunasin and hydrogen cyanide were detected in the culture medium when bacterial isolates were grown in minimal medium containing amygdalin as the sole carbon source (Motta et al., 2022).

Unlike studies with bacterial symbionts, studies on arthropod viruses are very limited (Shi et al., 2016; Wu et al., 2020), and those that are available mainly focus on the antagonistic relationship between pathogenic viruses and their hosts, yet, a diverse range of non-pathogenic viruses have also been reported in arthropods (Bonning, 2019; Liu et al., 2015; Nouri et al., 2018; Shi et al., 2016). For instance, the false spider mite Brevipalpus yothersi is associated with the transmission of citrus leprosis virus C, an RNA virus that suppresses mite-induced plant defenses, resulting in increased mite performance and greater virus transmissibility (Arena et al., 2016). In our study with the multi-resistant VR-BE population (chapter four), several RNA viruses including picorna-like virus, aphis glycine virus and dicistrovirus were discovered in the VR-BE population on bean and the reference GSS strain, but whether their relationship with T. urticae is mutualistic (both the host and the virus benefit from each other) or commensal (only the virus benefits without harming the host), is not known. Nevertheless, potential virus-host interactions mechanisms can be inferred from a recent study exploring the molecular interactions between dicistrovirus and Drosophila, which revealed that just like other RNA viruses, the replication machinery of dicistrovirus is mediated by RNA dependent RNA polymerase (RdRp) in complex with or requiring host proteins and viral linked protein (VPg) (Warsaba et al., 2019). RdRp homologs have previously been identified in the genome of T. urticae, and associated with the amplification of RNA interference (RNAi) silencing signals (Grbić et al., 2011). In a study with T. urticae,

Niu et al., (2019) uncovered a high number of symmetrically distributed reads (>4000 reads) corresponding to dicistrovirus derived small RNAs, which also presented a 21bp peak, suggesting that the RNAi antiviral immunity is triggered. But whether the RdRp genes in *T. urticae* are involved in RNAi responses against viruses has not yet been determined (Niu et al., 2019). However, judging from the stable infection with the viruses maintained in the bean population of *T. urticae*, I speculate that the viruses are able to influence the host RNAi response and perhaps the general response to stress and fitness of the host. But to what extent and the mechanisms involved needs to be further elucidated. Additionally, *Cardinium* and *Wolbachia* symbionts are common in insects and mites and could also modulate viral infections (Cogni et al., 2021; Haghshenas-Gorgabi et al., 2023; Teixeira et al., 2008). Whether such symbionts were present in VR-BE populations and modulating infection with the various viruses should be further investigated.

6.5 Future perspectives: Transferring laboratory knowledge to the field

As discussed, resistance development is one of the major problems that comes with overreliance on chemicals for pest control, not to mention the challenges that come with development of new pesticides due to increasing costs for discovery, stringent regulatory requirements before a new product can be launched in the market and public concerns on environmental safety and human health (Van Leeuwen et al., 2020). These factors result in a decrease in the number of available pest control agents, and therefore widespread efforts should be made to safeguard the utility of the available pesticides. In order to make rational decisions on pesticide use, detailed knowledge of the resistance mechanisms is imperative, as such knowledge can for example be utilized in the development of diagnostic markers to monitor resistance development in crop pests. A good diagnostic marker for resistance should have a high contribution to the resistance phenotype, should be easily identified in a high-throughput manner and should cover a wide geographical region (Van Leeuwen et al., 2020). In chapter two of this thesis, a mutation of the highly conserved histidine residue H258Y was validated using marker assisted back-crossing, and found to confer high resistance to two complex II inhibitors. Additionally, the fitness cost of this resistance mutation was determined in chapter three, which further increases its value as a potential diagnostic marker. Although the mutation has not been reported in field populations of T. urticae, it can easily be detected in very low frequencies using the highly sensitive ddPCR technique as demonstrated in chapter three. Since the mutation occurs on a highly conserved histidine residue across species, such knowledge can also be transformed to other pests such as fungi, where mutations involving this residue are widespread in field populations, and confer high cross-resistance to fungal SDH inhibitors (Avenot and Michailides, 2010; Fraaije et al., 2012; Sierotzki and Scalliet, 2013). Identification and validation of multiple molecular markers, coupled with the development of a user-friendly database for the molecular markers could prove to be a valuable tool for rational guidance of resistance management programs (Van Leeuwen et al., 2020).

Next to safeguarding the available pesticides by monitoring resistance development, pesticide use needs to be reduced with 50% by 2030 according to a new EU guideline on sustainable use of pesticides (https://food.ec.europa.eu/plants/pesticides/sustainable-use-pesticides en). As such, other control methods will need to be developed to address/compensate the reduced use of pesticides. In light of this, RNAi has shown great potential in pest control (Niu et al., 2018), albeit with some limitations such as inefficient delivery of dsRNA to target cells and undesirable off target effects (Cooper et al., 2019; Suzuki et al., 2017). The potential of RNAi as a pest management tool is therefore dependent on the choice of the target gene, method of delivery and expression of dsRNA. Additionally, a good RNAi target gene should be lethal to the target pest but benign to beneficial organisms that live in the same ecological niche. Recently, HGT genes have been proposed as suitable targets for RNAi, with potential in mite pest control due to the important role they play in adaptation (Sun et al., 2023). DOGs for example have shown an important role in mitigating plant defenses, therefore facilitating adaptation to a new host plant (see chapter five). Furthermore, these genes have not been reported in insect genomes, and therefore silencing of DOGs may not be harmful to beneficial insects such as ladybugs and bees which might be found in the same ecological niche as spider mites. Additionally, the divergence between spider mites and their natural enemy the predatory mite, which is used as the major biological control of spider mites, suggests that these HGT genes might be uniquely appropriate as specific RNAi targets for pest spider mites (Sun et al., 2023). In a proof of concept study, Sun et al., (2023) evaluated the suitability of several HGT genes, among them UGTs, DOGs and genes involved in folate and carotenoid metabolism as potential candidates for RNAi in the citrus red mite Panonychus citri. By feeding mites with citrus leaves coated with dsRNA solution, the DOG genes were identified as the best candidates as they resulted in the highest silencing efficiency of 73-78% and a mortality of 56-68% when the dsRNA fed mites were kept on soybean or citrus leaves for 10 days (Sun et al., 2023). In our study in chapter five, injecting dsRNA of two T. urticae DOG genes identified as important for adaptation to tomato resulted in a silencing efficiency of 89-96% and a cumulative mortality of ~40% when dsRNA injected mites were kept on tomato leaves for three days. Although the results from both studies were significant in a laboratory setup, they are insufficient in the context of crop protection, especially the low mortality which suggests that the silencing effect was temporary. Since there are 17 DOG genes in T. urticae and 12 DOG genes in P. citri, it can be assumed that the low mortality was as a result of a compensatory effect from the rest of the DOG genes that were not silenced. Indeed, multiple substrates were shared between the DOG enzymes that we functionally characterized in chapter five. In the future, knocking down multiple DOG genes might improve persistence of the

silencing effect and potentially increase the mortality. Additionally, to overcome the current limitations with delivery, options such as coating the dsRNA with nanoparticles which can then be applied in foliar sprays, or using transgenic plants expressing the dsRNA of the target HGT gene can be explored (Wu et al., 2022).

References

- Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. J. Am. Mosq. Control Assoc. 18, 265–267. https://doi.org/10.1093/jee/18.2.265a
- Abe, H., Ohnishi, J., Narusaka, M., Seo, S., Narusaka, Y., Tsuda, S., Kobayashi, M., 2008. Function of Jasmonate in Response and Tolerance of Arabidopsis to Thrip Feeding. Plant Cell Physiol. 49, 68–80. https://doi.org/10.1093/pcp/pcm168
- Abe, H., Shimoda, T., Ohnishi, J., Kugimiya, S., Narusaka, M., Seo, S., Narusaka, Y., Tsuda, S., Kobayashi, M., 2009. Jasmonate-dependent plant defense restricts thrips performance and preference. BMC Plant Biol. 9, 97. https://doi.org/10.1186/1471-2229-9-97
- Agrawal, A.A., 2000. Host-Range Evolution: Adaptation and Trade-Offs in Fitness of Mites on Alternative Hosts. Ecology 81, 500–508.
- Ahn, S.-J., Dermauw, W., Wybouw, N., Heckel, D.G., Van Leeuwen, T., 2014. Bacterial origin of a diverse family of UDP-glycosyltransferase genes in the *Tetranychus urticae* genome. Insect Biochem. Mol. Biol. 50, 43–57. https://doi.org/10.1016/j.ibmb.2014.04.003
- Ahn, S.J., Vogel, H., Heckel, D.G., 2012. Comparative analysis of the UDP-glycosyltransferase multigene family in insects. Insect Biochem. Mol. Biol. 42, 133–147. https://doi.org/10.1016/j.ibmb.2011.11.006
- Ahnström, J., Faber, K., Axler, O., Dahlbäck, B., 2007. Hydrophobic ligand binding properties of the human lipocalin apolipoprotein M. J. Lipid Res. 48, 1754–1762. https://doi.org/10.1194/jlr.M700103-JLR200
- Ahrazem, O., Gómez-Gómez, L., Rodrigo, M.J., Avalos, J., Limón, M.C., 2016. Carotenoid cleavage oxygenases from microbes and photosynthetic organisms: Features and functions. Int. J. Mol. Sci. 17. https://doi.org/10.3390/ijms17111781
- Alavijeh, E.S., Khajehali, J., Snoeck, S., Panteleri, R., Ghadamyari, M., Jonckheere, W., Bajda, S., Saalwaechter, C., Geibel, S., Douris, V., Vontas, J., Van Leeuwen, T., Dermauw, W., 2020. Molecular and genetic analysis of resistance to METI-I acaricides in Iranian populations of the citrus red mite *Panonychus citri*. Pestic. Biochem. Physiol. 164, 73–84. https://doi.org/10.1016/j.pestbp.2019.12.009
- Alba, J.M., Schimmel, B.C.J., Glas, J.J., Ataide, L.M.S., Pappas, M.L., Villarroel, C.A., Schuurink, R.C., Sabelis, M.W., Kant, M.R., 2015. Spider mites suppress tomato defenses downstream of jasmonate and salicylate independently of hormonal crosstalk. New Phytol. 205, 828–840. https://doi.org/10.1111/nph.13075
- Albajes, R., Gullino, M.L., van Lenteren, J.C., Elad, Y., 1999. Integrated Pest and Disease Management in Greenhouse Crops, Developments in Plant Pathology. Kluwer Academic Publishers, Dordrecht, The Netherlands. https://doi.org/10.1007/0-306-47585-5
- Alberti, G., Crooker, A., 1985. Internal anatomy. In: Helle, W., and Sabelis, M. W., eds. Spider mites: their biology, natural enemies and control: vol. 1A. Elsevier Science Publishers B.V., Amsterdam, The Netherlands, pp. 29–62.
- Ali, J.G., Agrawal, A.A., 2012. Specialist versus generalist insect herbivores and plant defense. Trends Plant Sci. 17, 293–302. https://doi.org/10.1016/j.tplants.2012.02.006
- Almagro Armenteros, J.J., Tsirigos, K.D., Sønderby, C.K., Petersen, T.N., Winther, O., Brunak, S., von Heijne, G., Nielsen, H., 2019. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat. Biotechnol. 37, 420–423. https://doi.org/10.1038/s41587-019-0036-z
- Alyokhin, A., Chen, Y.H., 2017. Adaptation to toxic hosts as a factor in the evolution of insecticide resistance. Curr. Opin. Insect Sci. 21, 33–38. https://doi.org/10.1016/J.COIS.2017.04.006
- Anazawa, Y., Tomita, T., Aiki, Y., Kozaki, T., Kono, Y., 2003. Sequence of a cDNA encoding acetylcholinesterase from susceptible and resistant two-spotted spider mite, *Tetranychus urticae*. Insect Biochem. Mol. Biol. 33, 509–514. https://doi.org/10.1016/S0965-1748(03)00025-0

- Anders, S., Pyl, P.T., Huber, W., 2015. HTSeq-A Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169. https://doi.org/10.1093/bioinformatics/btu638
- Andow, D.A., Hidaka, K., 1989. Experimental natural history of sustainable agriculture: syndromes of production. Agric. Ecosyst. Environ. 27, 447–462. https://doi.org/10.1016/0167-8809(89)90105-9
- Andrews, S., 2010. FastQC: A Quality Control tool for High Throughput Sequence Data [WWW Document]. URL https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed 4.13.23).
- Arena, G.D., Ramos-González, P.L., Nunes, M.A., Ribeiro-Alves, M., Camargo, L.E.A., Kitajima, E.W., Machado, M.A., Freitas-Astúa, J., 2016. Citrus leprosis virus C infection results in hypersensitive-like response, suppression of the JA/ET plant defense pathway and promotion of the colonization of its mite vector. Front. Plant Sci. 7, 1–17. https://doi.org/10.3389/fpls.2016.01757
- Arora, R., Sandhu, S., 2017. Breeding insect resistant crops for sustainable agriculture, Breeding Insect Resistant Crops for Sustainable Agriculture. https://doi.org/10.1007/978-981-10-6056-4
- Avenot, H.F., Michailides, T.J., 2010. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. Crop Prot. 29, 643–651. https://doi.org/10.1016/j.cropro.2010.02.019
- Avenot, H.F., Michailides, T.J., 2007. Resistance to boscalid fungicide in Alternaria alternata isolates from Pistachio in California. Plant Dis. 91, 1345–1350. https://doi.org/10.1094/PDIS-91-10-1345
- Bajda, S., Dermauw, W., Greenhalgh, R., Nauen, R., Tirry, L., Clark, R.M., Van Leeuwen, T., 2015. Transcriptome profiling of a spirodiclofen susceptible and resistant strain of the European red mite *Panonychus ulmi* using strand-specific RNA-seq. BMC Genomics 16. https://doi.org/10.1186/s12864-015-2157-1
- Bajda, S., Dermauw, W., Panteleri, R., Sugimoto, N., Douris, V., Tirry, L., Osakabe, M., Vontas, J., Van Leeuwen, T., 2017. A mutation in the PSST homologue of complex I (NADH:ubiquinone oxidoreductase) from *Tetranychus urticae* is associated with resistance to METI acaricides. Insect Biochem. Mol. Biol. 80, 79–90. https://doi.org/10.1016/j.ibmb.2016.11.010
- Bajda, S., Riga, M., Wybouw, N., Papadaki, S., Ouranou, E., Fotoukkiaii, S.M., Vontas, J., Van Leeuwen, T., 2018. Fitness costs of key point mutations that underlie acaricide target-site resistance in the two-spotted spider mite *Tetranychus urticae*. Evol. Appl. 11, 1540–1553. https://doi.org/10.1111/eva.12643
- Balabanidou, V., Grigoraki, L., Vontas, J., 2018. Insect cuticle: a critical determinant of insecticide resistance. Curr. Opin. insect Sci. 27, 68–74. https://doi.org/10.1016/J.COIS.2018.03.001
- Ballard, J.W.O., Whitlock, M.C., 2004. The incomplete natural history of mitochondria. Mol Ecol 13, 729–744. https://doi.org/https://doi.org/10.1046/j.1365-294X.2003.02063.x
- Bass, C., Zimmer, C.T., Riveron, J.M., Wilding, C.S., Wondji, C.S., Kaussmann, M., Field, L.M., Williamson, M.S., Nauen, R., 2013. Gene amplification and microsatellite polymorphism underlie a recent insect host shift. Proc Natl Acad Sci U S A 110, 19460–19465. https://doi.org/10.1073/pnas.1314122110
- Bautista, M.A.M., Miyata, T., Miura, K., Tanaka, T., 2009. RNA interference-mediated knockdown of a cytochrome P450, CYP6BG1, from the diamondback moth, *Plutella xylostella*, reduces larval resistance to permethrin. Insect Biochem. Mol. Biol. 39, 38–46. https://doi.org/https://doi.org/10.1016/j.ibmb.2008.09.005
- BBC Future, 2020. The Biblical locust plagues of 2020 BBC Future [WWW Document]. URL https://www.bbc.com/future/article/20200806-the-biblical-east-african-locust-plagues-of-2020 (accessed 2.28.23).
- Bebber, D.P., Holmes, T., Gurr, S.J., 2014. The global spread of crop pests and pathogens. Glob. Ecol. Biogeogr. 23, 1398–1407. https://doi.org/10.1111/GEB.12214
- Behmer, S.T., 2008. Insect Herbivore Nutrient Regulation. Annu Rev Entomol. 54, 165-87. https://doi.org/10.1146/annurev.ento.54.110807.090537 54, 165–187.
- Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J. R. Stat. Soc. Ser. B 57, 289–300. https://doi.org/10.1111/J.2517-6161.1995.TB02031.X

- Bennett, R.N., Wallsgrove, R.M., 1994. Secondary metabolites in plant defence mechanisms. New Phytol. 127, 617–633. https://doi.org/10.1111/j.1469-8137.1994.tb02968.x
- Bensoussan, N., Santamaria, M.E., Zhurov, V., Diaz, I., Grbić, M., Grbić, V., 2016. Plant-Herbivore Interaction: Dissection of the Cellular Pattern of *Tetranychus urticae* Feeding on the Host Plant. Front. Plant Sci. 0, 1105. https://doi.org/10.3389/FPLS.2016.01105
- Bensoussan, N., Zhurov, V., Yamakawa, S., O'Neil, C.H., Suzuki, T., Grbić, M., Grbić, V., 2018. The Digestive System of the Two-Spotted Spider Mite, *Tetranychus urticae* Koch, in the Context of the Mite-Plant Interaction. Front. Plant Sci. 0, 1206. https://doi.org/10.3389/FPLS.2018.01206
- Berenbaum, M.R., Favret, C., Schuler, M. a, 2011. On Defining " Key Innovations " in an Adaptive Radiation : Cytochrome P450S and Papilionidae Author (s): May R. Berenbaum, Colin Favret, Mary A. Schuler Source : The American Naturalist, Vol. 148, Supplement (Nov., 1996), pp. S139-S155 Publis. Am. Nat. 148.
- Berger, I., Fitzgerald, D.J., Richmond, T.J., 2004. Baculovirus expression system for heterologous multiprotein complexes. Nat Biotechnol 22, 1583–1587. https://doi.org/10.1038/nbt1036
- Berman, T.S., Izraeli, Y., Lalzar, M., Mozes-Daube, N., Lepetit, D., Tabic, A., Varaldi, J., Zchori-Fein, E., 2023. RNA Viruses Are Prevalent and Active Tenants of the Predatory Mite *Phytoseiulus persimilis* (Acari: Phytoseiidae). Microb. Ecol. 86, 2060-2072. https://doi.org/10.1007/s00248-023-02210-0
- Bevan, M.W., Uauy, C., Wulff, B.B.H., Zhou, J., Krasileva, K., Clark, M.D., 2017. Genomic innovation for crop improvement. Nature 543, 346–354. https://doi.org/10.1038/nature22011
- Bianchetti, C.M., Harmann, C.H., Takasuka, T.E., Hura, G.L., Dyer, K., Fox, B.G., 2013. Fusion of dioxygenase and lignin-binding domains in a novel secreted enzyme from cellulolytic streptomyces sp. SIRexaa-e. J. Biol. Chem. 288, 18574–18587. https://doi.org/10.1074/jbc.M113.475848
- Blaazer, C.J.H., Villacis-perez, E.A., Chafi, R., Roberts, M.R., 2018. Why Do Herbivorous Mites Suppress Plant Defenses? Front. Plant Sci. 30, 1–16. https://doi.org/10.3389/fpls.2018.01057
- Bleeker, P.M., Mirabella, R., Diergaarde, P.J., VanDoorn, A., Tissier, A., Kant, M.R., Prins, M., De Vos, M., Haring, M.A., Schuurink, R.C., 2012. Improved herbivore resistance in cultivated tomato with the sesquiterpene biosynthetic pathway from a wild relative. Proc. Natl. Acad. Sci. U. S. A. 109, 20124–20129. https://doi.org/10.1073/pnas.1208756109
- Blier, P.U., Dufresne, F., Burton, R.S., 2001. Natural selection and the evolution of mtDNA-encoded peptides: evidence for intergenomic co-adaptation. Trends in Genetics 17, 400–406. https://doi.org/https://doi.org/10.1016/S0168-9525(01)02338-1
- Bock, K.W., 2016. The UDP-glycosyltransferase (UGT) superfamily expressed in humans, insects and plants: Animal-plant arms-race and co-evolution. Biochem. Pharmacol. 99, 11–17. https://doi.org/10.1016/j.bcp.2015.10.001
- Bonning, B., 2019. The Insect Virome: Opportunities and Challenges. Curr. Issues Mol. Biol 34, 1–12. https://doi.org/https://doi.org/10.21775/cimb.034.001
- Bourguet, D., Guillemaud, T., Chevillon, C., Raymond, M., 2004. Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipiens*. Evolution (N. Y). 58, 128–135. https://doi.org/10.1111/j.0014-3820.2004.tb01579.x
- Bras, A., Roy, A., Heckel, D.G., Anderson, P., Karlsson Green, K., 2022. Pesticide resistance in arthropods: Ecology matters too. Ecol. Lett. 25, 1746–1759. https://doi.org/10.1111/ele.14030
- Brattsten, L.B., 1988. Enzymic adaptations in leaf-feeding insects to host-plant allelochemicals. J. Chem. Ecol. 14, 1919–1939. https://doi.org/10.1007/BF01013486
- Bray, J.E., Marsden, B.D., Oppermann, U., 2009. The human short-chain dehydrogenase/reductase (SDR) superfamily: A bioinformatics summary. Chem. Biol. Interact. 178, 99–109. https://doi.org/https://doi.org/10.1016/j.cbi.2008.10.058

- Brevik, K., Bueno, E.M., McKay, S., Schoville, S.D., Chen, Y.H., 2021. Insecticide exposure affects intergenerational patterns of DNA methylation in the Colorado potato beetle, *Leptinotarsa decemlineata*. Evol. Appl. 14, 746–757. https://doi.org/10.1111/eva.13153
- Brito, L.P., Linss, J.G.B., Lima-Camara, T.N., Belinato, T.A., Peixoto, A.A., Lima, J.B.P., Valle, D., Martins, A.J., 2013. Assessing the Effects of Aedes aegypti kdr Mutations on Pyrethroid Resistance and Its Fitness Cost. PLoS One, 8, e60878. https://doi.org/10.1371/journal.pone.0060878
- Bruce, T.J.A., 2010. Tackling the threat to food security caused by crop pests in the new millennium. Food Secur. 2, 133–141. https://doi.org/10.1007/s12571-010-0061-8
- Bryon, A., Kurlovs, A.H., Dermauw, W., Greenhalgh, R., Riga, M., Grbic, M., Tirry, L., Osakabe, M., Vontas, J., Clark, R.M., Leeuwen, T. Van, 2017. Disruption of a horizontally transferred phytoene desaturase abolishes carotenoid accumulation and diapause in *Tetranychus urticae*. Proc. Natl. Acad. Sci. U. S. A. 114, E5871– E5880. https://doi.org/10.1073/pnas.1706865114
- Bryon, A., Wybouw, N., Dermauw, W., Tirry, L., Van Leeuwen, T., 2013. Genome wide gene-expression analysis of facultative reproductive diapause in the two-spotted spider mite *Tetranychus urticae*. BMC Genomics 14, 1–20. https://doi.org/10.1186/1471-2164-14-815
- Bugg, T.D.H., Winfield, C.J., 1998. Enzymatic cleavage of aromatic rings: Mechanistic aspects of the catechol dioxygenases and later enzymes of bacterial oxidative cleavage pathways. Nat. Prod. Rep. 15, 513–530. https://doi.org/10.1039/a815513y
- Bullock, D.G., 1992. Crop rotation. CRC. Crit. Rev. Plant Sci. 11, 309–326. https://doi.org/10.1080/07352689209382349
- Byrne, L.J., O'Callaghan, K.J., Tuite, M.F., 2005. Heterologous Gene Expression in Yeast, in: Smales, C.M., James, D.C. (Eds.), Therapeutic Proteins: Methods and Protocols. Humana Press, Totowa, NJ, pp. 51–64. https://doi.org/10.1385/1-59259-922-2:051
- Çağatay, N.S., Menault, P., Riga, M., Vontas, J., Ay, R., 2018. Identification and characterization of abamectin resistance in *Tetranychus urticae* Koch populations from greenhouses in Turkey. Crop Prot. 112, 112–117. https://doi.org/https://doi.org/10.1016/j.cropro.2018.05.016
- Castle, S.J., Prabhaker, N., Henneberry, T.J., Toscano, N.C., 2009. Host plant influence on susceptibility of *Bemisia tabaci* (Hemiptera: Aleyrodidae) to insecticides. Bull. Entomol. Res. 99, 263–273. https://doi.org/10.1017/S0007485308006329
- Cha, C.J., 2006. Catechol 1,2-Dioxygenase from *Rhodococcus rhodochrous* N75 Capable of Metabolizing Alkyl-Substituted Catechols. J. Microbiol. Biotechnol. 16, 778–785.
- Chaudhary, B., 2013. Plant domestication and resistance to herbivory. Int. J. Plant Genomics. 2013, 572784. https://doi.org/10.1155/2013/572784
- Chen, L.-H., Tsai, H.-C., Yu, P.-L., Chung, K.-R., 2017. A Major Facilitator Superfamily Transporter-Mediated Resistance to Oxidative Stress and Fungicides Requires Yap1, Skn7, and MAP Kinases in the Citrus Fungal Pathogen Alternaria alternata. PLoS One 12, e0169103.
- Chen, L., Wei, J., Liu, C., Zhang, W., Wang, B., Niu, L.L., Liang, G., 2018. Specific binding protein ABCC1 is associated with Cry2Ab toxicity in *Helicoverpa armigera*. Front. Physiol. 9, 1–11. https://doi.org/10.3389/fphys.2018.00745
- Chen, W., Hasegawa, D.K., Kaur, N., Kliot, A., Pinheiro, P.V., Luan, J., Stensmyr, M.C., Zheng, Y., Liu, W., Sun, H., Xu, Y., Luo, Y., Kruse, A., Yang, X., Kontsedalov, S., Lebedev, G., Fisher, T.W., Nelson, D.R., Hunter, W.B., Brown, J.K., Jander, G., Cilia, M., Douglas, A.E., Ghanim, M., Simmons, A.M., Wintermantel, W.M., Ling, K.S., Fei, Z., 2016. The draft genome of whitefly *Bemisia tabaci* MEAM1, a global crop pest, provides novel insights into virus transmission, host adaptation, and insecticide resistance. BMC Biol. 14,110. https://doi.org/10.1186/s12915-016-0321-y
- Chudzinski-Tavassi, A.M., Carrijo-Carvalho, L.C., Waismam, K., Farsky, S.H.P., Ramos, O.H.P., Reis, C. V., 2010. A lipocalin sequence signature modulates cell survival. FEBS Lett. 584, 2896–2900.

https://doi.org/10.1016/j.febslet.2010.05.008

- Cogni, R., Ding, S.D., Pimentel, A.C., Day, J.P., Jiggins, F.M., 2021. Wolbachia reduces virus infection in a natural population of Drosophila. Commun Biol 4, 1327. https://doi.org/10.1038/s42003-021-02838-z
- Constabel, C.P., Barbehenn, R., 2008. Defensive roles of polyphenol oxidase in plants. Induc. Plant Resist. to Herbiv. 253-270. https://doi.org/10.1007/978-1-4020-8182-8_12
- Cooper, A.M.W., Silver, K., Zhang, J., Park, Y., Zhu, K.Y., 2019. Molecular mechanisms influencing efficiency of RNA interference in insects. Pest Manag Sci. 75, 18–28. https://doi.org/https://doi.org/10.1002/ps.5126
- Corkley, I., Fraaije, B., Hawkins, N., 2022. Fungicide resistance management: Maximizing the effective life of plant protection products. Plant Pathol 71, 150–169. https://doi.org/https://doi.org/10.1111/ppa.13467
- Cornell, H. V., Hawkins, B.A., 2003. Herbivore Responses to Plant Secondary Compounds: A Test of Phytochemical Coevolution Theory. 161, 507-22. https://doi.org/10.1086/368346 161, 507–522. https://doi.org/10.1086/368346
- Crisp, A., Boschetti, C., Perry, M., Tunnacliffe, A., Micklem, G., 2015. Expression of multiple horizontally acquired genes is a hallmark of both vertebrate and invertebrate genomes. Genome Biol. 16,50. https://doi.org/10.1186/s13059-015-0607-3
- Croft, B.A., Van De Baan, H.E., 1988. Ecological and genetic factors influencing evolution of pesticide resistance in tetranychid and phytoseiid mites. Exp. Appl. Acarol. 4, 277–300. https://doi.org/10.1007/BF01196191
- Croom, E., 2012. Chapter Three Metabolism of Xenobiotics of Human Environments, in: Hodgson, E. (Ed.), Progress in Molecular Biology and Translational Science. Academic Press, pp. 31–88. https://doi.org/https://doi.org/10.1016/B978-0-12-415813-9.00003-9
- Crow, J.F., 1957. Genetics of Insect Resistance to Chemicals. Annu. Rev. Entomol. 2, 227–246. https://doi.org/10.1146/annurev.en.02.010157.001303
- Cruse, C., Moural, T., Zhu, F., 2023. Dynamic Roles of Insect Carboxyl/Cholinesterases in Chemical Adaptation. MDPI insects. 14, 194. https://doi.org/10.3390/insects14020194
- Dao, T.T.H., Linthorst, H.J.M., Verpoorte, R., 2011. Chalcone synthase and its functions in plant resistance. Phytochem. Rev. 10, 397–412. https://doi.org/10.1007/s11101-011-9211-7
- Davies, A.G., Game, A.Y., Chen, Z., Williams, T.J., Goodall, S., Yen, J.L., McKenzie, J.A., Batterham, P., 1996. Scalloped wings is the *Lucilia cuprina* Notch homologue and a candidate for the modifier of fitness and asymmetry of diazinon resistance. Genetics. 143, 1321–1337. https://doi.org/10.1093/genetics/143.3.1321
- De Beer, B., Vandenhole, M., Njiru, C., Spanoghe, P., Dermauw, W., Van Leeuwen, T., 2022a. High-Resolution Genetic Mapping Combined with Transcriptome Profiling Reveals That Both Target-Site Resistance and Increased Detoxification Confer Resistance to the Pyrethroid Bifenthrin in the Spider Mite Tetranychus urticae. Biology (Basel). 11, 1630. https://doi.org/10.3390/biology11111630
- De Beer, B., Villacis-Perez, E., Khalighi, M., Saalwaechter, C., Vandenhole, M., Jonckheere, W., Ismaeil, I., Geibel, S., Van Leeuwen, T., Dermauw, W., 2022b. QTL mapping suggests that both cytochrome P450-mediated detoxification and target-site resistance are involved in fenbutatin oxide resistance in *Tetranychus urticae*. Insect Biochem. Mol. Biol. 145, 103757. https://doi.org/10.1016/j.ibmb.2022.103757
- De Rouck, S., Mocchetti, A., Dermauw, W., Van Leeuwen, T., 2024. SYNCAS: Efficient CRISPR/Cas9 gene-editing in difficult to transform arthropods. Insect Biochem Mol Biol 165, 104068. https://doi.org/10.1016/j.ibmb.2023.104068
- De Rouck, S., Inak, E., Dermauw, W., Van Leeuwen, T., 2023. A review of the molecular mechanisms of acaricide resistance in mites and ticks. Insect Biochem. Mol. Biol. 159, 103981. https://doi.org/10.1016/j.ibmb.2023.103981
- Demaeght, P., Dermauw, W., Tsakireli, D., Khajehali, J., Nauen, R., Tirry, L., Vontas, J., Lümmen, P., Van Leeuwen, T., 2013. Molecular analysis of resistance to acaricidal spirocyclic tetronic acids in *Tetranychus urticae*:

CYP392E10 metabolizes spirodiclofen, but not its corresponding enol. Insect Biochem. Mol. Biol. 43, 544–554. https://doi.org/10.1016/j.ibmb.2013.03.007

- Demaeght, P., Osborne, E.J., Odman-Naresh, J., Grbić, M., Nauen, R., Merzendorfer, H., Clark, R.M., Van Leeuwen, T., 2014. High resolution genetic mapping uncovers chitin synthase-1 as the target-site of the structurally diverse mite growth inhibitors clofentezine, hexythiazox and etoxazole in *Tetranychus urticae*. Insect Biochem. Mol. Biol. 51, 52–61. https://doi.org/10.1016/j.ibmb.2014.05.004
- Denness, L., McKenna, J.F., Segonzac, C., Wormit, A., Madhou, P., Bennett, M., Mansfield, J., Zipfel, C., Hamann, T., 2011. Cell Wall Damage-Induced Lignin Biosynthesis Is Regulated by a Reactive Oxygen Species- and Jasmonic Acid-Dependent Process in Arabidopsis. Plant Physiol. 156, 1364. https://doi.org/10.1104/PP.111.175737
- Dermauw, W., Ilias, A., Riga, M., Tsagkarakou, A., Grbić, M., Tirry, L., Van Leeuwen, T., Vontas, J., 2012. The cysloop ligand-gated ion channel gene family of *Tetranychus urticae*: Implications for acaricide toxicology and a novel mutation associated with abamectin resistance. Insect Biochem. Mol. Biol. 42, 455–465. https://doi.org/10.1016/j.ibmb.2012.03.002
- Dermauw, W., Jonckheere, W., Riga, M., Livadaras, I., Vontas, J., Van Leeuwen, T., 2020a. Targeted mutagenesis using CRISPR-Cas9 in the chelicerate herbivore Tetranychus urticae. Insect Biochem. Mol. Biol. 120, 103347. https://doi.org/10.1016/j.ibmb.2020.103347
- Dermauw, W., Pym, A., Bass, C., Van Leeuwen, T., Feyereisen, R., 2018. Does host plant adaptation lead to pesticide resistance in generalist herbivores? Curr. Opin. Insect Sci. 26, 25–33. https://doi.org/10.1016/j.cois.2018.01.001
- Dermauw, W., Van Leeuwen, T., 2014. The ABC gene family in arthropods: Comparative genomics and role in insecticide transport and resistance. Insect Biochem. Mol. Biol. 45, 89–110. https://doi.org/10.1016/J.IBMB.2013.11.001
- Dermauw, W., Van Leeuwen, T., Feyereisen, R., 2020b. Diversity and evolution of the P450 family in arthropods. Insect Biochem. Mol. Biol. 127, 103490. https://doi.org/10.1016/J.IBMB.2020.103490
- Dermauw, W., Wybouw, N., Rombauts, S., Menten, B., Vontas, J., Grbić, M., Clark, R.M., Feyereisen, R., Van Leeuwen, T., 2013. A link between host plant adaptation and pesticide resistance in the polyphagous spider mite *Tetranychus urticae*. Proc. Natl. Acad. Sci. U. S. A. 110, 113–122. https://doi.org/10.1073/pnas.1213214110
- Després, L., David, J.-P., Gallet, C., 2007. The evolutionary ecology of insect resistance to plant chemicals. Trends Ecol. Evol. 22, 298–307. https://doi.org/https://doi.org/10.1016/j.tree.2007.02.010
- Devonshire, A.L., Field, L.M., 1991. Gene Amplification and Insecticide Resistance. Annu. Rev. Entomol. 36, 1–21. https://doi.org/10.1146/annurev.en.36.010191.000245
- Devonshire, A.L., Moores, G.D., 1982. A carboxylesterase with broad substrate specificity causes organophosphorus, carbamate and pyrethroid resistance in peach-potato aphids (*Myzus persicae*). Pestic. Biochem. Physiol. 18, 235–246. https://doi.org/https://doi.org/10.1016/0048-3575(82)90110-9
- Dixon, R.A., Xie, D.-Y., Sharma, S.B., 2004. Proanthocyanidins a final frontier in flavonoid research? New Phytol. 165, 9–28. https://doi.org/10.1111/j.1469-8137.2004.01217.x
- Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. Sequence analysis STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21. https://doi.org/10.1093/bioinformatics/bts635
- Dong, K., Du, Y., Rinkevich, F., Nomura, Y., Xu, P., Wang, L., Silver, K., Zhorov, B.S., 2014. Molecular biology of insect sodium channels and pyrethroid resistance. Insect Biochem. Mol. Biol. 50, 1–17. https://doi.org/https://doi.org/10.1016/j.ibmb.2014.03.012
- Douris, V., Steinbach, D., Panteleri, R., Livadaras, I., Pickett, J.A., Van Leeuwen, T., Nauen, R., Vontas, J., 2016. Resistance mutation conserved between insects and mites unravels the benzoylurea insecticide mode of action on chitin biosynthesis. Proc. Natl. Acad. Sci. U. S. A. 113, 14692–14697.

https://doi.org/10.1073/pnas.1618258113/-/dcsupplemental

- Dowling, D.K., Friberg, U., Lindell, J., 2008. Evolutionary implications of non-neutral mitochondrial genetic variation. Trends Ecol Evol 23, 546–554. https://doi.org/https://doi.org/10.1016/j.tree.2008.05.011
- Duffey, S.S., Stout, M.J., 1996. Antinutritive and Toxic Components of Plant Defense Against Insects. Arch.Insect Biochem. Physiol. 37, 3–37. https://doi.org/10.1002/(SICI)1520-6327(1996)32:1<3::AID-ARCH2>3.0.CO;2-1
- Dunley, J.E., Croft, B.A., 1992. Dispersal and gene flow of pesticide resistance traits in phytoseiid and tetranychid mites. Exp. Appl. Acarol. 14, 313–325. https://doi.org/10.1007/BF01200570
- Dussourd, D.E., 2017. Behavioral Sabotage of Plant Defenses by Insect Folivores. Annu. Rev. Entomol. 62, 15–34. https://doi.org/10.1146/annurev-ento-031616-035030
- Ehrlich, P.R., Raven, P.H., 1964. Butterflies and Plants : A Study in Coevolution. Evolution. 18, 586–608. https://doi.org/10.2307/2406212
- Elzinga, D.A., De Vos, M., Jander, G., 2014. Suppression of plant defenses by a Myzus persicae (green peach aphid) salivary effector protein. Molecular Plant-Microbe Interactions 27, 747–756. https://doi.org/10.1094/MPMI-01-14-0018-R
- Enayati, A.A., Ranson, H., Hemingway, J., 2005. Insect glutathione transferases and insecticide resistance. Insect Mol. Biol. 14, 3–8. https://doi.org/10.1111/j.1365-2583.2004.00529.x
- Erb, M., Kliebenstein, D.J., 2020. Plant Secondary Metabolites as Defenses, Regulators, and Primary Metabolites: The Blurred Functional Trichotomy. Plant Physiol. 184, 39. https://doi.org/10.1104/PP.20.00433
- Falguières, T., 2022. ABC Transporters in Human Diseases: Future Directions and Therapeutic Perspectives. Int. J. Mol. Sci. 23. https://doi.org/10.3390/ijms23084250
- FAO, 2018. Fall Armyworm an Emerging Food Security Global Threat International Plant Protection Convention [WWW Document]. URL https://www.ippc.int/en/news/fall-armyworm-an-emerging-food-securityglobal-threat/ (accessed 2.28.23).
- Ferraroni, M., Kolomytseva, M.P., Solyanikova, I.P., Scozzafava, A., Golovleva, L.A., Briganti, F., 2006. Crystal Structure of 3-Chlorocatechol 1,2-dioxygenase Key Enzyme of a New Modified Ortho-pathway from the Gram-positive *Rhodococcus opacus* 1CP Grown on 2-chlorophenol. J. Mol. Biol. 360, 788–799. https://doi.org/10.1016/j.jmb.2006.05.046
- Ferraroni, M., Seifert, J., Travkin, V.M., Thiel, M., Kaschabek, S., Scozzafava, A., Golovleva, L., Schlömann, M., Briganti, F., 2005. Crystal structure of the hydroxyquinol 1,2-dioxygenase from *Nocardioides simplex* 3E, a key enzyme involved in polychlorinated aromatics biodegradation. J. Biol. Chem. 280, 21144–21154. https://doi.org/10.1074/jbc.M500666200
- Fetzner, S., 2012. Ring-Cleaving Dioxygenases with a Cupin Fold. Appl. Environ. Microbiol. 78, 2505–14. https://doi.org/10.1128/AEM.07651-11
- Feyereisen, R., 2012. Insect CYP Genes and P450 Enzymes. Insect Mol. Biol. Biochem. 236–316. https://doi.org/10.1016/B978-0-12-384747-8.10008-X
- Feyereisen, R., 2005. 4.1 Insect Cytochrome P450, in: Gilbert, L.I.B.T.-C.M.I.S. (Ed.), Comprehensive Molecular Insect Science. Elsevier, Amsterdam, pp. 1–77. https://doi.org/https://doi.org/10.1016/B0-44-451924-6/00049-1
- Feyereisen, R., Dermauw, W., Van Leeuwen, T., 2015. Genotype to phenotype, the molecular and physiological dimensions of resistance in arthropods. Pestic. Biochem. Physiol. 121, 61–77. https://doi.org/10.1016/J.PESTBP.2015.01.004
- Ffrench-Constant, R.H., 2013. The molecular genetics of insecticide resistance. Genetics. 194, 807–815. https://doi.org/10.1534/genetics.112.141895

Ffrench-Constant, R.H., Bass, C., 2017. Does resistance really carry a fitness cost? Curr. Opin. Insect Sci. 21, 39-

46. https://doi.org/10.1016/j.cois.2017.04.011

- Figueroa-Teran, R., Pak, H., GJ, B., Tittiger, C., 2016. High substrate specificity of ipsdienol dehydrogenase (IDOLDH), a short-chain dehydrogenase from Ips pini bark beetles. J. Biochem. 160, 141–51. https://doi.org/10.1093/jb/mvw019
- Fisher, C., 2019. Phenol-Chloroform Extraction for dsRNA Purification. https://doi.org/10.17504/protocols.io.4pmgvk6
- Flores-Gutierrez, A.M., Mora, F., Avila-Cabadilla, L.D., Boege, K., del-Val, E., 2020. Assessing the cascading effects of management and landscape on the arthropod guilds occurring in papaya plantations. Agric. Ecosyst. Environ. 293, 106836. https://doi.org/10.1016/j.agee.2020.106836
- Flower, D.R., North, A.C.T., Sansom, C.E., 2000. The lipocalin protein family: structural and sequence overview. Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol. 1482, 9–24. https://doi.org/https://doi.org/10.1016/S0167-4838(00)00148-5
- Fotoukkiaii, S.M., Mermans, C., Wybouw, N., Van Leeuwen, T., 2020. Resistance risk assessment of the novel complex II inhibitor pyflubumide in the polyphagous pest *Tetranychus urticae*. J. Pest Sci. 93, 1085–1096. https://doi.org/10.1007/s10340-020-01213-x
- Fotoukkiaii, S.M., Tan, Z., Xue, W., Wybouw, N., Van Leeuwen, T., 2019. Identification and characterization of new mutations in mitochondrial cytochrome b that confer resistance to bifenazate and acequinocyl in the spider mite *Tetranychus urticae*. Pest Manag. Sci. 76, 1154–1163. https://doi.org/10.1002/PS.5628
- Fotoukkiaii, S.M., Wybouw, N., Kurlovs, A.H., Tsakireli, D., Pergantis, S.A., Clark, R.M., Vontas, J., Leeuwen, T. Van, 2021. High-resolution genetic mapping reveals cis-regulatory and copy number variation in loci associated with cytochrome P450-mediated detoxification in a generalist arthropod pest. PLoS Genet. 17. https://doi.org/10.1371/JOURNAL.PGEN.1009422
- Fraaije, B.A., Bayon, C., Atkins, S., Cools, H.J., Lucas, J.A., Fraaije, M.W., 2012. Risk assessment studies on succinate dehydrogenase inhibitors, the new weapons in the battle to control Septoria leaf blotch in wheat. Mol. Plant Pathol. 13, 263–275. https://doi.org/10.1111/j.1364-3703.2011.00746.x
- Froger, A., Hall, J.E., 2007. Transformation of Plasmid DNA into *E. Coli* using the heat shock method. J. Vis. Exp. 6. https://doi.org/10.3791/253
- Frost, C.J., Appel, H.M., Carlson, J.E., De Moraes, C.M., Mescher, M.C., Schultz, J.C., 2007. Within-plant signalling via volatiles overcomes vascular constraints on systemic signalling and primes responses against herbivores. Ecol. Lett. 10, 490–498. https://doi.org/10.1111/J.1461-0248.2007.01043.X
- Fry, J., 1989. Evolutionary adaptation to host plants in a laboratoty population of the phytophagous mite Tetranychus urticae Koch. Oecologia 83, 568. https://doi.org/10.1007/BF00317211
- Furuya, T., Machiya, K., Fujioka, S., Nakano, M., Inagaki, K., 2017. Development of a novel acaricide, pyflubumide. J. Pestic. Sci. 42, 132–136. https://doi.org/10.1584/JPESTICS.J17-02
- Futuyma, D.J., Agrawal, A.A., 2009. Macroevolution and the biological diversity of plants and herbivores. Proc. Natl. Acad. Sci. U. S. A. 106, 18054–18061. https://doi.org/10.1073/pnas.0904106106
- Galtier, N., Nabholz, B., Glemin, S., Hurst, G.D.D., 2009. Mitochondrial DNA as a marker of molecular diversity: a reappraisal. Mol Ecol 18, 4541–4550. https://doi.org/https://doi.org/10.1111/j.1365-294X.2009.04380.x
- Gao, L., Qiao, H., Wei, P., Moussian, B., Wang, Y., 2022. Xenobiotic responses in insects. Arch Insect Biochem Physiol. https://doi.org/10.1002/arch.21869
- Georghiou, G.P., 1983. Management of resistance in arthropods. In: Georghiou, G.P., Saito, T. (eds) Pest Resistance to Pesticides. Springer, Boston, MA. https://doi.org/10.1007/978-1-4684-4466-7_32
- Georghiou, G.P., 1969. Genetics of resistance to insecticides in houseflies and mosquitoes. Exp. Parasitol. 26,224-255. https://doi.org/10.1016/0014-4894(69)90116-7
- Georghiou, G.P., Taylor, C.E., 1977. Genetic and biological influences in the evolution of insecticide resistance. J.

Econ. Entomol. 70, 319-323. https://doi.org/10.1093/jee/70.3.319

- Giovani, B., Bellance, N., Jose, C., Rossignol, R., 2011. Relationships Between Mitochondrial Dynamics and Bioenergetics. In: Lu, B. (eds) Mitochondrial Dynamics and Neurodegeneration. Springer, Dordrecht. https://doi.org/10.1007/978-94-007-1291-1_2
- Goergen, G., Kumar, P.L., Sankung, S.B., Togola, A., Tamò, M., 2016. First report of outbreaks of the fall armyworm *Spodoptera frugiperda* (J E Smith) (Lepidoptera, Noctuidae), a new alien invasive pest in West and Central Africa. PLoS One. 11, 1–9. https://doi.org/10.1371/journal.pone.0165632
- Gotoh, T., Bruin, J., Sabelis, M.W., Menken, S.B.J., 1993. Host race formation in *Tetranychus urticae*: genetic differentiation, host plant preference, and mate choice in a tomato and a cucumber strain. Entomol. Exp. Appl. 68, 171–178. https://doi.org/10.1111/j.1570-7458.1993.tb01700.x
- Gould, F., Carrol, C.R., Futuyma, D.J., 1982. Cross-Resistance To Pesticides and Plant Defenses: a Study of the Two-Spotted Spider Mite. Entomol. Exp. Appl. 31, 175–180. https://doi.org/10.1111/j.1570-7458.1982.tb03132.x
- Grafton-Cardwell, E.E., Granett, J., Normington, S.M., 1991. Influence of dispersal from almonds on the population dynamics and acaricide resistance frequencies of spider mites infesting neighboring cotton. Exp. Appl. Acarol. 10, 187–212. https://doi.org/10.1007/BF01198650
- Grbić, M., Van Leeuwen, T., Clark, R.M., Rombauts, S., Rouzé, P., Grbić, V., Osborne, E.J., Dermauw, W., Ngoc, T., Cao, P., Ortego, F., Hernández-Crespo, P., Diaz, I., Martinez, M., Navajas, M., Sucena, É., Magalhães, S., Nagy, L., Pace, R.M., Djuranović, Y., 2011. The genome of *Tetranychus urticae* reveals herbivorous pest adaptations. Nature. 479, 487–492. https://doi.org/10.1038/nature10640
- Gu, Z., Eils, R., Schlesner, M., 2016. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics. 32, 2847–2849. https://doi.org/10.1093/bioinformatics/btw313
- Guo, J., Lemire, B.D., 2003. The Ubiquinone-binding Site of the Saccharomyces cerevisiae Succinate-Ubiquinone Oxidoreductase Is a Source of Superoxide. J. Biol. Chem. 278, 47629–47635. https://doi.org/10.1074/JBC.M306312200
- Guzik, U., Hupert-kocurek, K., 2013. Intradiol Dioxygenases The Key Enzymes in Xenobiotics Degradation. Biodegrad. Hazard. Spec. Prod. 7 129–153.
- Haghshenas-Gorgabi, N., Poorjavd, N., Khajehali, J., Wybouw, N., 2023. Cardinium symbionts are pervasive in Iranian populations of the spider mite Panonychus ulmi despite inducing an infection cost and no demonstrable reproductive phenotypes when Wolbachia is a symbiotic partner. Exp Appl Acarol 91, 369– 380. https://doi.org/10.1007/s10493-023-00840-0
- Halkier, B.A., Gershenzon, J., 2006. Biology and biochemistry of glucosinolates. Annu. Rev. Plant Biol. 57, 303– 333. https://doi.org/10.1146/annurev.arplant.57.032905.105228
- Hall, T., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows [WWW Document]. URL https://www.researchgate.net/publication/258565830_BioEdit_An_important_software_for_molecular_ biology (accessed 4.23.20).
- Harayamas, S., Rekik, M., 1989. Bacterial Aromatic Ring-cleavage Enzymes Are Classified into Two Different Gene Families*. J Biol Chem. 264, 15328-15333. https://www.jbc.org/article/S0021-9258(19)84830-5/pdf
- Hardstone, M.C., Lazzaro, B.P., Scott, J.G., 2009. The effect of three environmental conditions on the fitness of cytochrome P450 monooxygenase-mediated permethrin resistance in *Culex pipiens quinquefasciatus*. BMC Evol. Biol. 9, 1–13. https://doi.org/10.1186/1471-2148-9-42
- Hardy, N.B., Peterson, D.A., Ross, L., Rosenheim, J.A., 2018. Does a plant-eating insect's diet govern the evolution of insecticide resistance? Comparative tests of the pre-adaptation hypothesis. Evol Appl. 11, 739–747. https://doi.org/10.1111/eva.12579

Hatefi, Y., Stiggall, D.L., 1978. Preparation and properties of NADH: Cytochrome c oxidoreductase (complex I–III).

Methods Enzymol. 53, 5-10. https://doi.org/10.1016/S0076-6879(78)53005-X

- Hawkins, N.J., Bass, C., Dixon, A., Neve, P., 2019. The evolutionary origins of pesticide resistance. Biol. Rev. 94, 135–155. https://doi.org/10.1111/brv.12440
- Hayashi, N., Sasama, Y., Takahashi, N., Ikemi, N., 2013. Cyflumetofen, a novel acaricide Its mode of action and selectivity. Pest Manag. Sci. 69, 1080–1084. https://doi.org/10.1002/ps.3470
- Heckel, D.G., 2014. Insect Detoxification and Sequestration Strategies, in: Insect Detoxification and Sequestration

 Strategies.
 Annual
 Plant
 Reviews.
 Elsevier,
 pp.
 293–302.

 https://doi.org/https://doi.org/10.1002/9781118829783.ch3
- Heidel-Fischer, H.M., Kirsch, R., Reichelt, M., Ahn, S.-J., Wielsch, N., Baxter, S.W., Heckel, D.G., Vogel, H., Kroymann, J., 2019. An Insect Counteradaptation against Host Plant Defenses Evolved through Concerted Neofunctionalization. Mol. Biol. Evol. 36, 930–941. https://doi.org/10.1093/molbev/msz019
- Heidel-Fischer, H.M., Vogel, H., 2015. Molecular mechanisms of insect adaptation to plant secondary compounds. Curr. Opin. Insect Sci. 8, 8–14. https://doi.org/10.1016/J.COIS.2015.02.004
- Hoeks, J., Hesselink, M., Schrauwen, P., 2012. Mitochondrial Respiration, in: Encyclopedia of Exercise Medicine in Health and Disease. Springer Berlin Heidelberg, pp. 587–590. https://doi.org/10.1007/978-3-540-29807-6_136
- Hoffmann, F., Maser, E., 2007. Carbonyl Reductases and Pluripotent Hydroxysteroid Dehydrogenases of the Short-chain Dehydrogenase/reductase Superfamily. Drug Metab. Rev. 39, 87–144. https://doi.org/10.1080/03602530600969440
- Hogenhout, S.A., Bos, J.I.B., 2011. Effector proteins that modulate plant–insect interactions. Curr. Opin. Plant Biol. 14, 422–428. https://doi.org/10.1016/J.PBI.2011.05.003
- Hollingworth, R., Ahammadsahib, K.I., 1995. Inhibitors of respiratory complex I. Mechanisms, pesticidal actions and toxicology. Rev.Pestic.Toxicol. 3, 277–302.
- Horsefield, R., Yankovskaya, V., Sexton, G., Whittingham, W., Shiomi, K., Ömura, S., Byrne, B., Cecchini, G., Iwata, S., 2006. Structural and Computational Analysis of the Quinone-binding Site of Complex II (Succinate-Ubiquinone Oxidoreductase): A mechanism of electron transfer and proton conduction during ubiquinone reduction *. J. Biol. Chem. 281, 7309–7316. https://doi.org/10.1074/JBC.M508173200
- Hospital, F., 2001. Size of donor chromosome segments around introgressed loci and reduction of linkage drag in marker-assisted backcross programs. Genetics. 158, 1363–1379. https://doi.org/10.1093/genetics/158.3.1363
- Howe, G.A., Jander, G., 2008. Plant immunity to insect herbivores. Annu. Rev. Plant Biol. 59, 41–66. https://doi.org/10.1146/annurev.arplant.59.032607.092825
- Howe, G.A., Ryan, C.A., 1999. Suppressors of Systemin Signaling Identify Genes in the Tomato Wound Response Pathway. Genetics. 153,1411-1421. https://doi.org/10.1093/genetics/153.3.1411
- Huang, L. shar, Lümmen, P., Berry, E.A., 2021. Crystallographic investigation of the ubiquinone binding site of respiratory Complex II and its inhibitors. Biochim. Biophys. Acta - Proteins Proteomics 1869, 140679. https://doi.org/10.1016/J.BBAPAP.2021.140679
- Huang, L., Sun, G., Cobessi, D., Wang, A., Shen, J., Tung, E., Anderson, V., Berry, E., 2006. 3-nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme. J. Biol. Chem. 281, 5965–5972. https://doi.org/10.1074/JBC.M511270200
- Ilias, A., Vontas, J., Tsagkarakou, A., 2014. Global distribution and origin of target site insecticide resistance mutations in *Tetranychus urticae*. Insect Biochem. Mol. Biol. 48, 17–28. https://doi.org/https://doi.org/10.1016/j.ibmb.2014.02.006
- İnak, E., Alpkent, Y.N., Çobanoğlu, S., Dermauw, W., Van Leeuwen, T., 2019. Resistance incidence and presence of resistance mutations in populations of *Tetranychus urticae* from vegetable crops in Turkey. Exp. Appl.

Acarol. 78, 343-360. https://doi.org/10.1007/s10493-019-00398-w

- İnak, E., Alpkent, Y.N., Çobanoğlu, S., Toprak, U., Van Leeuwen, T., 2022a. Incidence of spiromesifen resistance and resistance mechanisms in *Tetranychus urticae* populations collected from strawberry production areas in Turkey. Crop Prot. 160, 106049. https://doi.org/10.1016/j.cropro.2022.106049
- İnak, E., Alpkent, Y.N., Saalwaechter, C., Albayrak, T., İnak, A., Dermauw, W., Geibel, S., Van Leeuwen, T., 2022b. Long-term survey and characterization of cyflumetofen resistance in *Tetranychus urticae* populations from Turkey. Pestic. Biochem. Physiol. 188, 105235. https://doi.org/10.1016/j.pestbp.2022.105235
- Inaoka, D.K., Shiba, T., Sato, D., Balogun, E.O., Sasaki, T., Nagahama, M., Oda, M., Matsuoka, S., Ohmori, J., Honma, T., Inoue, M., Kita, K., Harada, S., 2015. Structural Insights into the Molecular Design of Flutolanil Derivatives Targeted for Fumarate Respiration of Parasite Mitochondria. Int. J. Mol. Sci. 16,15287-15308. https://doi.org/10.3390/IJMS160715287
- International plant protection convention, 2021. A global challenge to prevent and mitigate plant pest risks in agriculture, forestry and ecosystems, in: Scientific Review of the Impact of Climate Change on Plant Pests. FAO on behalf of the IPPC Secretariat, Rome. https://doi.org/10.4060/cb4769en
- IRAC international MoA working group, 2022. IRAC Mode of Action Classification Scheme [WWW Document]. Insectic. Resist. Action Comm. URL https://irac-online.org/mode-of-action/classification-online/ (accessed 3.9.23).
- Ishii, N., Tsuda, M., Yasuda, K., Yanase, S., Suzuki, K., 1998. A mutation in succinate dehydrogenase cytochrome. Nat. Lett. 394, 694–697. https://doi.org/10.1038/29331
- Itoh, H., Tago, K., Hayatsu, M., Kikuchi, Y., 2018. Detoxifying symbiosis: Microbe-mediated detoxification of phytotoxins and pesticides in insects. Nat Prod Rep. https://doi.org/10.1039/c7np00051k
- Itoh, Y., Shimotsuma, Y., Jouraku, A., Dermauw, W., Van Leeuwen, T., Osakabe, M., 2021. Combination of target site mutation and associated CYPs confers high-level resistance to pyridaben in *Tetranychus urticae*. Pestic. Biochem. Physiol. 181, 105000. https://doi.org/10.1016/J.PESTBP.2021.105000
- Jancova, P., Anzenbacher, P., Anzenbacherova, E., 2010. Phase II drug metabolizing enzymes. Biomed. Pap. 154, 103–116. https://doi.org/10.5507/bp.2010.017
- Jeppson, L.R., Keifer, H.H., Baker, E.W., 1975. Mites Injurious to Economic Plants. University of California Press. https://doi.org/doi:10.1525/9780520335431
- Ji, M., Vandenhole, M., De Beer, B., De Rouck, S., Villacis-Perez, E., Feyereisen, R., Clark, R.M., Van Leeuwen, T., 2023. A nuclear receptor HR96-related gene underlies large trans-driven differences in detoxification gene expression in a generalist herbivore. Nat Commun. 14, 4990. https://doi.org/10.1038/s41467-023-40778w
- Jonckheere W., 2018. The salivary proteome of *Tetranychus urticae*: key to its polyphagous nature? PhD thesis, University of Amsterdam, The Netherlands.
- Jonckheere, W., Dermauw, W., Khalighi, M., Pavlidi, N., Reubens, W., Baggerman, G., Tirry, L., Menschaert, G., Kant, M.R., Vanholme, B., Van Leeuwen, T., 2017. A gene family coding for salivary proteins (SHOT) of the polyphagous spider mite *Tetranychus urticae* exhibits fast host-dependent transcriptional plasticity. Mol. Plant-Microbe Interact. 31, 112–124. https://doi.org/10.1094/MPMI-06-17-0139-R
- Jonckheere, W., Dermauw, W., Zhurov, V., Wybouw, N., Van Den Bulcke, J., Villarroel, C.A., Greenhalgh, R., Grbić, M., Schuurink, R.C., Tirry, L., Baggerman, G., Clark, R.M., Kant, M.R., Vanholme, B., Menschaert, G., Van Leeuwen, T., 2016. The salivary protein repertoire of the polyphagous spider mite *Tetranychus urticae*: A quest for effectors. Mol. Cell. Proteomics 15, 3594–3613. https://doi.org/10.1074/mcp.M116.058081
- Jones, C.D., 1998. The Genetic Basis of *Drosophila sechellia*'s Resistance to a Host Plant Toxin. Genetics. 149, 1899-908. doi: 10.1093/genetics/149.4.1899
- Joseph-Horne, T., Hollomon, D.W., Wood, P.M., 2001. Fungal respiration: a fusion of standard and alternative components. Biochim. Biophys. Acta - Bioenerg. 1504, 179–195. https://doi.org/10.1016/S0005-

2728(00)00251-6

- Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S.A.A., Ballard, A.J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A.W., Kavukcuoglu, K., Kohli, P., Hassabis, D., 2021. Highly accurate protein structure prediction with AlphaFold. Nat. 596, 583–589. https://doi.org/10.1038/s41586-021-03819-2
- Kallberg, Y., Oppermann, U., Persson, B., 2010. Classification of the short-chain dehydrogenase/reductase superfamily using hidden Markov models. FEBS J. 277, 2375–2386. https://doi.org/10.1111/j.1742-4658.2010.07656.x
- Kant, M.R., Ament, K., Sabelis, M.W., Haring, M.A., Schuurink, R.C., 2004. Differential timing of spider miteinduced direct and indirect defenses in tomato plants. Plant Physiol. 135, 483–495. https://doi.org/10.1104/pp.103.038315
- Kant, M.R., Jonckheere, W., Knegt, B., Lemos, F., Liu, J., Schimmel, B.C.J., Villarroel, C.A., Ataide, L.M.S., Dermauw,
 W., Glas, J.J., Egas, M., Janssen, A., Van Leeuwen, T., Schuurink, R.C., Sabelis, M.W., Alba, J.M., 2015.
 Mechanisms and ecological consequences of plant defence induction and suppression in herbivore communities. Ann. Bot. 115, 1015–1051. https://doi.org/10.1093/aob/mcv054
- Kant, M.R., Sabelis, M.W., Haring, M.A., Schuurink, R.C., 2008. Intraspecific variation in a generalist herbivore accounts for differential induction and impact of host plant defences. Proc. R. Soc. B Biol. Sci. 275, 443– 452. https://doi.org/10.1098/rspb.2007.1277
- Karatolos, N., Williamson, M., Denholm, I., Gorman, K., Ffrench-Constant, R., Nauen, R., 2012. Resistance to spiromesifen in *Trialeurodes vaporariorum* is associated with a single amino acid replacement in its target enzyme acetyl-coenzyme A carboxylase. Insect Mol. Biol. 21, 327–334. https://doi.org/10.1111/j.1365-2583.2012.01136.x
- Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. https://doi.org/10.1093/molbev/mst010
- Kaulmann, U., Kaschabek, S.R., And, §, Schlo⁻⁻mann, M., Schlo⁻⁻mann, S., 2001. Mechanism of Chloride Elimination from 3-Chloro-and 2,4-Dichloro-cis,cis-Muconate: New Insight Obtained from Analysis of Muconate Cycloisomerase Variant CatB-K169A ⁺. J. Bacteriol. 183, 4551–4561. https://doi.org/10.1128/JB.183.15.4551-4561.2001
- Kennedy, C.J., Tierney, K.B., 2012. Xenobiotic Protection protection /Resistance Mechanisms in Organisms. In: Meyers, R.A. (eds) Encyclopedia of Sustainability Science and Technology. Springer, New York, NY. https://doi.org/10.1007/978-1-4419-0851-3_51
- Keon, J., Antoniw, J., Carzaniga, R., Deller, S., Ward, J.L., Baker, J.M., Beale, M.H., Hammond-Kosack, K., Rudd, J.J., 2007. Transcriptional adaptation of *Mycosphaerella graminicola* to programmed cell death (PCD) of its susceptible wheat host. Mol. Plant-Microbe Interact. 20, 178–193. https://doi.org/10.1094/MPMI-20-2-0178
- Keskin, N., Kumral, N.A., 2015. Screening tomato varietal resistance against the two-spotted spider mite [*Tetranychus urticae* (Koch)]. Int. J. Acarol. 41, 300–309. https://doi.org/10.1080/01647954.2015.1028440
- Khajehali, J., van Leeuwen, T., Grispou, M., Morou, E., Alout, H., Weill, M., Tirry, L., Vontas, J., Tsagkarakou, A., 2010. Acetylcholinesterase point mutations in European strains of *Tetranychus urticae* (Acari: Tetranychidae) resistant to organophosphates. Pest Manag. Sci. 66, 220–228. https://doi.org/10.1002/ps.1884
- Khajehali, J., Van Nieuwenhuyse, P., Demaeght, P., Tirry, L., Van Leeuwen, T., 2011. Acaricide resistance and resistance mechanisms in *Tetranychus urticae* populations from rose greenhouses in the Netherlands. Pest Manag. Sci. 67, 1424–1433. https://doi.org/10.1002/ps.2191
- Khalighi, M., Dermauw, W., Wybouw, N., Bajda, S., Osakabe, M., Tirry, L., Leeuwen, T. Van, 2015. Molecular analysis of cyenopyrafen resistance in the two-spotted spider mite *Tetranychus urticae*. Pest Manag. Sci.

72, 103-112. https://doi.org/10.1002/PS.4071

- Khalighi, M., Tirry, L., Thomas, V.L., 2014. Cross-resistance risk of the novel complex II inhibitors cyenopyrafen and cyflumetofen in resistant strains of the two-spotted spider mite *Tetranychus urticae*. Pest Manag. Sci. 70, 365–368. https://doi.org/10.1002/PS.3641
- Khan, M., Seto, D., Subramaniam, R., Desveaux, D., 2018. Oh, the places they'll go! A survey of phytopathogen effectors and their host targets. Plant J. 93, 651–663. https://doi.org/10.1111/TPJ.13780
- Kielkiewicz, M., 2002. Influence of carmine spider mite *Tetranychus cinnabarinus Boisd*. (Acarida: Tetranychidae) feeding on ethylene production and the activity of oxidative enzymes in damaged tomato plants, in: Acarid Phylogeny and Evolution: Adaptation in Mites and Ticks. Springer Netherlands, pp. 389–392. https://doi.org/10.1007/978-94-017-0611-7 39
- Kim, Y.K., Xiao, C.L., 2011. Stability and fitness of pyraclostrobin-and boscalid-resistant phenotypes in field isolates of *Botrytis cinerea* from apple. Phytopathology. 101, 1385–1391. https://doi.org/10.1094/PHYTO-04-11-0123
- Kliot, A., Ghanim, M., 2012. Fitness costs associated with insecticide resistance. Pest Manag. Sci. 68, 1431–1437. https://doi.org/10.1002/ps.3395
- Koramutla, M.K., Kaur, A., Negi, M., Venkatachalam, P., Bhattacharya, R., 2014. Elicitation of jasmonate-mediated host defense in *Brassica juncea* (L.) attenuates population growth of mustard aphid *Lipaphis erysimi* (Kalt.). Planta. 240, 177–194. https://doi.org/10.1007/s00425-014-2073-7
- Kortbeek, R.W.J., van der Gragt, M., Bleeker, P.M., 2019. Endogenous plant metabolites against insects. Eur. J. Plant Pathol. 154, 67–90. https://doi.org/10.1007/s10658-018-1540-6
- Krainacker, D.A., Carey, J.R., 1990. Male demographic constraints to extreme sex ratio in the twospotted spider mite. Oecologia. 82, 417–423. https://doi.org/10.1007/BF00317492
- Kretschmer, M., Leroch, M., Mosbach, A., Walker, A.S., Fillinger, S., Mernke, D., Schoonbeek, H.J., Pradier, J.M., Leroux, P., De Waard, M.A., Hahn, M., 2009. Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus *Botrytis cinerea*. PLoS Pathog. 5. https://doi.org/10.1371/journal.ppat.1000696
- Kumar, A., Trefault, N., Olaniran, A.O., 2016. Microbial degradation of 2,4-dichlorophenoxyacetic acid: Insight into the enzymes and catabolic genes involved, their regulation and biotechnological implications. Crit. Rev. Microbiol. 42, 194–208. https://doi.org/10.3109/1040841X.2014.917068
- Kumar, S., Lekshmi, M., Parvathi, A., Ojha, M., Wenzel, N., Varela, M.F., 2020. Functional and structural roles of the major facilitator superfamily bacterial multidrug efflux pumps. Microorganisms. 8, 266. https://doi.org/10.3390/microorganisms8020266
- Kundu, A., Vadassery, J., 2019. Chlorogenic acid-mediated chemical defence of plants against insect herbivores. Plant Biol. 21, 185–189. https://doi.org/10.1111/plb.12947
- Kurlovs, A.H., De Beer, B., Ji, M., Vandenhole, M., De Meyer, T., Feyereisen, R., Clark, R.M., Van Leeuwen, T., 2022. Trans-driven variation in expression is common among detoxification genes in the extreme generalist herbivore *Tetranychus urticae*. PLoS Genet. 18, 1–33. https://doi.org/10.1371/journal.pgen.1010333
- Kurlovs, A.H., Snoeck, S., Kosterlitz, O., Van Leeuwen, T., Clark, R.M., 2019. Trait mapping in diverse arthropods by bulked segregant analysis. Curr Opin Insect Sci. 36, 57–65. https://doi.org/https://doi.org/10.1016/j.cois.2019.08.004
- Kwon, D., Yoon, K., Clark, J., Lee, S., 2010. A point mutation in a glutamate-gated chloride channel confers abamectin resistance in the two-spotted spider mite, *Tetranychus urticae* Koch. Insect Mol. Biol. 19, 583– 591. https://doi.org/10.1111/J.1365-2583.2010.01017.X
- Kwon, D.H., Choi, J.Y., Je, Y.H., Lee, S.H., 2012. The overexpression of acetylcholinesterase compensates for the reduced catalytic activity caused by resistance-conferring mutations in *Tetranychus urticae*. Insect Biochem. Mol. Biol. 42, 212–219. https://doi.org/10.1016/j.ibmb.2011.12.003

- Kwon, D.H., Clark, J.M., Lee, S.H., 2015. Toxicodynamic mechanisms and monitoring of acaricide resistance in the two-spotted spider mite. Pestic. Biochem. Physiol. 121, 97–101. https://doi.org/10.1016/J.PESTBP.2014.12.011
- Kwon, D.H., Clark, J.M., Lee, S.H., 2010a. Cloning of a sodium channel gene and identification of mutations putatively associated with fenpropathrin resistance in *Tetranychus urticae*. Pestic. Biochem. Physiol. 97, 93–100. https://doi.org/10.1016/j.pestbp.2009.07.009
- Kwon, D.H., Im, J.S., Ahn, J.J., Lee, J.H., Marshall Clark, J., Lee, S.H., 2010b. Acetylcholinesterase point mutations putatively associated with monocrotophos resistance in the two-spotted spider mite. Pestic. Biochem. Physiol. 96, 36–42. https://doi.org/10.1016/J.PESTBP.2009.08.013
- Labandeira, C.C., 2006. The four phases of plant-arthropod associations in deep time. Geol. Acta 4, 409–438.
- Labbé, P., Berthomieu, A., Berticat, C., Alout, H., Raymond, M., Lenormand, T., Weill, M., 2007. Independent duplications of the acetylcholinesterase gene conferring insecticide resistance in the mosquito *Culex pipiens*. Mol. Biol. Evol. 24, 1056–1067. https://doi.org/10.1093/molbev/msm025
- Labbé, P., Sidos, N., Raymond, M., Lenormand, T., 2009. Resistance gene replacement in the mosquito *Culex pipiens*: Fitness estimation from long-term cline series. Genetics. 182, 303–312. https://doi.org/10.1534/genetics.109.101444
- Lalève, A., Gamet, S., Walker, A.S., Debieu, D., Toquin, V., Fillinger, S., 2014. Site-directed mutagenesis of the P225, N230 and H272 residues of succinate dehydrogenase subunit B from *Botrytis cinerea* highlights different roles in enzyme activity and inhibitor binding. Environ. Microbiol. 16, 2253–2266. https://doi.org/10.1111/1462-2920.12282
- Law, C.J., Maloney, P.C., Wang, D., 2009. MFS family. Annu Rev Microbiol. 289–305. https://doi.org/10.1146/annurev.micro.61.080706.093329.Ins
- Le Gall, M., Behmer, S.T., 2014. Effects of Protein and Carbohydrate on an Insect Herbivore: The Vista from a Fitness Landscape. Integr. Comp. Biol. 54, 942–954. https://doi.org/10.1093/icb/icu102
- Lee, S.H., Kim, Y.H., Kwon, D.H., Cha, D.J., Kim, J.H., 2015. Mutation and duplication of arthropod acetylcholinesterase: Implications for pesticide resistance and tolerance. Pestic. Biochem. Physiol. 120, 118–124. https://doi.org/https://doi.org/10.1016/j.pestbp.2014.11.004
- Leeper, J.R., Roush, R.T., Reynolds, H.T., 1986. Preventing or man- aging resistance in arthropods. Pesticide resistance: Strategies and tac- tics for management. The National Academies Press, Washington, DC. https://doi.org/https://doi.org/10.17226/619
- Lenfant, N., Hotelier, T., Velluet, E., Bourne, Y., Marchot, P., Chatonnet, A., 2013. ESTHER, the database of the α/β-hydrolase fold superfamily of proteins: tools to explore diversity of functions. Nucleic Acids Res. 423– 429. https://doi.org/10.1093/nar/gks1154
- Lewis, J.A., Kandala, P., Penley, M.J., Morran, L.T., 2023. Gene flow accelerates adaptation to a parasite. Evolution (N. Y). 77, 1468–1478. https://doi.org/10.1093/evolut/qpad048
- Li, B., Yu, H., Luo, Y., Wu, H., 2016. The synthesis and acaricidal activity of SYP-9625. Mod Agrochem. 15, 15–16. https://doi.org/10.3969/j.issn.1671-5284.2016.06.004
- Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., Project, G., Subgroup, D.P., 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 25, 2078–2079. https://doi.org/10.1093/bioinformatics/btp352
- Li, H., Zhang, H., Guan, R., Miao, X., 2013. Identification of differential expression genes associated with host selection and adaptation between two sibling insect species by transcriptional profile analysis. BMC Genomics. 14, 582. https://doi.org/10.1186/1471-2164-14-582
- Li, W., Godzik, A., 2006. Cd-hit: A fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 22, 1658–1659. https://doi.org/10.1093/bioinformatics/bt1158
- Li, X., Schuler, M.A., Berenbaum, M.R., 2007. Molecular mechanisms of metabolic resistance to synthetic and

natural	xenobiotics.	Annu.	Rev.	Entomol.	52,	231–253.
https://doi.o	org/10.1146/annurev	.ento.51.11010	04.151104			

- Li, Z.W., Shen, Y.H., Xiang, Z.H., Zhang, Z., 2011. Pathogen-origin horizontally transferred genes contribute to the evolution of Lepidopteran insects. BMC Evol Biol. 11. https://doi.org/10.1186/1471-2148-11-356
- Liang, P., Jian-Zhou Cui, Yang, X.-Q., Gao, X.-W., 2007. Effects of host plants on insecticide susceptibility and carboxylesterase activity in *Bemisia tabaci* biotype B and greenhouse whitefly, *Trialeurodes vaporariorum*. Pest Manag. Sci. 63, 365–371. https://doi.org/10.1002/ps.1346
- Lindquist, E.E., 1998. REVIEW Evolution of phytophagy in trombidiform mites. Exp. Appl. Acarol. 22, 81–100. https://doi.org/10.1023/A:1006041609774
- Litskas, V.D., Migeon, A., Navajas, M., Tixier, M.S., Stavrinides, M.C., 2019. Impacts of climate change on tomato, a notorious pest and its natural enemy: Small scale agriculture at higher risk. Environ. Res. Lett. 14, 8. https://doi.org/10.1088/1748-9326/ab3313
- Liu, Q., Gao, T., Liu, W., Liu, Yusong, Zhao, Y., Liu, Yuerong, Li, W., Ding, K., Ma, F., Li, C., 2020. Functions of dopamine in plants: a review. Plant Signal. Behav. 15, 1827782. https://doi.org/10.1080/15592324.2020.1827782
- Liu, S., Chen, Y., Bonning, B.C., 2015. RNA virus discovery in insects. Curr. Opin. Insect Sci. 8, 54–61. https://doi.org/https://doi.org/10.1016/j.cois.2014.12.005
- Liu, X.-D., Guo, H.-F., 2019. Importance of endosymbionts Wolbachia and Rickettsia in insect resistance development. Curr Opin Insect Sci. 33, 84–90. https://doi.org/https://doi.org/10.1016/j.cois.2019.05.003
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2–ΔΔCT Method. Methods. 25, 402–408. https://doi.org/10.1006/METH.2001.1262
- Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. https://doi.org/10.1186/s13059-014-0550-8
- Lu, X., Vandenhole, M., Tsakireli, D., Pergantis, S.A., Vontas, J., Jonckheere, W., Van Leeuwen, T., 2023. Increased metabolism in combination with the novel cytochrome b target-site mutation L258F confers crossresistance between the Qo inhibitors acequinocyl and bifenazate in *Tetranychus urticae*. Pestic. Biochem. Physiol. 192, 105411. https://doi.org/https://doi.org/10.1016/j.pestbp.2023.105411
- Luckenbach, T., Fischer, S., Sturm, A., 2014. Current advances on ABC drug transporters in fish. Comp. Biochem.

 Physiol.
 Part
 C
 Toxicol.
 Pharmacol.
 165,
 28–52.

 https://doi.org/https://doi.org/10.1016/j.cbpc.2014.05.002
- Lueke, B., Douris, V., Hopkinson, J.E., Maiwald, F., Hertlein, G., Papapostolou, K.-M., Bielza, P., Tsagkarakou, A., Van Leeuwen, T., Bass, C., Vontas, J., Nauen, R., 2020. Identification and functional characterization of a novel acetyl-CoA carboxylase mutation associated with ketoenol resistance in *Bemisia tabaci*. Pestic. Biochem. Physiol. 166, 104583. https://doi.org/https://doi.org/10.1016/j.pestbp.2020.104583
- Lümmen, P., 1998. Complex I inhibitors as insecticides and acaricides. Biochim. Biophys. Acta Bioenerg. 1364, 287–296. https://doi.org/10.1016/S0005-2728(98)00034-6
- Lussey-Lepoutre, C., Hollinshead, K.E.R., Ludwig, C., Menara, M., Morin, A., Castro-Vega, L.-J., Parker, S.J., Janin, M., Martinelli, C., Ottolenghi, C., Metallo, C., Gimenez-Roqueplo, A.-P., Favier, J., Tennant, D.A., 2015. Loss of succinate dehydrogenase activity results in dependency on pyruvate carboxylation for cellular anabolism. Nat. Commun. 6, 8784. https://doi.org/10.1038/ncomms9784
- Ma, C.-S., Zhang, W., Peng, Y., Zhao, F., Chang, X.-Q., Xing, K., Zhu, L., Ma, G., Yang, H.-P., Rudolf, V.H.W., 2021. Climate warming promotes pesticide resistance through expanding overwintering range of a global pest. Nat. Commun. 12, 5351. https://doi.org/10.1038/s41467-021-25505-7
- Macke, E., Magalhães, S., Khan, H.D.T., Luciano, A., Frantz, A., Facon, B., Olivieri, I., 2011. Sex allocation in haplodiploids is mediated by egg size: Evidence in the spider mite *Tetranychus urticae* Koch. Proc. R. Soc. B Biol. Sci. 278, 1054–1063. https://doi.org/10.1098/rspb.2010.1706

- MacLean, A.M., MacPherson, G., Aneja, P., Finan, T.M., 2006. Characterization of the β-ketoadipate pathway in Sinorhizobium meliloti. Appl. Environ. Microbiol. 72, 5403–5413. https://doi.org/10.1128/AEM.00580-06
- Maeoka, A., Osakabe, M., 2021. Co-occurrence of subunit B and C mutations in respiratory complex II confers high resistance levels to pyflubumide and cyenopyrafen in the two-spotted spider mite *Tetranychus urticae* (Acari: Tetranychidae). Pest Manag. Sci. 77, 5149-5157. https://doi.org/10.1002/ps.6555
- Magalhães, S., Fayard, J., Janssen, A., Carbonell, D., Olivieri, I., 2007. Adaptation in a spider mite population after long-term evolution on a single host plant. J. Evol. Biol. 20, 2016–2027. https://doi.org/10.1111/j.1420-9101.2007.01365.x
- Maia, A.D.H.N., Luiz, A.J.B., Campanhola, C., 2000. Statistical inference on associated fertility life table parameters using jackknife technique: Computational aspects. J. Econ. Entomol. 93, 511–518. https://doi.org/10.1603/0022-0493-93.2.511
- Maia, A.D.H.N., Pazianotto, R.A.D.A., Luiz, A.J.B., Marinho-Prado, J.S., Pervez, A., 2014. Inference on arthropod demographic parameters: Computational advances using R. J. Econ. Entomol. 107, 432–439. https://doi.org/10.1603/EC13222
- Mannervik, B., 1985. The isoenzymes of glutathione transferase. Adv Enzym. Relat Areas Mol Biol. 57, 357–417. https://doi.org/10.1002/9780470123034.ch5.
- Martel, C., Zhurov, V., Navarro, M., Martinez, M., Cazaux, M., Auger, P., Migeon, A., Santamaria, M.E., Wybouw, N., Diaz, I., Leeuwen, T. Van, Navajas, M., Grbic, M., Grbic, V., 2015. Tomato Whole Genome Transcriptional Response to *Tetranychus urticae* Identifies Divergence of Spider Mite-Induced Responses Between Tomato and Arabidopsis. IS-MPMI. 28, 343–361.
- Martin, H.-J., Ziemba, M., Kisiela, M., Botella, J.A., Schneuwly, S., Maser, E., 2011. The Drosophila carbonyl reductase sniffer is an efficient 4-oxonon-2-enal (4ONE) reductase. Chem. Biol. Interact. 191, 48–54. https://doi.org/https://doi.org/10.1016/j.cbi.2010.12.006
- Matera, I., Ferraroni, M., Kolomytseva, M., Golovleva, L., Scozzafava, A., Briganti, F., 2010. Catechol 1,2dioxygenase from the Gram-positive *Rhodococcus opacus* 1CP: Quantitative structure/activity relationship and the crystal structures of native enzyme and catechols adducts. J. Struct. Biol. 170, 548–564. https://doi.org/10.1016/j.jsb.2009.12.023
- Matsuda, T., Kozaki, T., Ishii, K., Gotoh, T., 2018. Phylogeny of the spider mite sub-family Tetranychinae (Acari: Tetranychidae) inferred from RNA-Seq data. PLoS One. 13, e0203136. https://doi.org/10.1371/journal.pone.0203136
- Mavridis, K., Papapostolou, K.M., Riga, M., Ilias, A., Michaelidou, K., Bass, C., Van Leeuwen, T., Tsagkarakou, A., Vontas, J., 2021. Multiple TaqMan qPCR and droplet digital PCR (ddPCR) diagnostics for pesticide resistance monitoring and management, in the major agricultural pest *Tetranychus urticae*. Pest Manag. 78,263-273. Sci. https://doi.org/10.1002/ps.6632
- Mayoral, J., Leonard, K., Nouzova, M., Noriega, F., Defelipe, L., Turjanski, A., 2013. Functional analysis of a mosquito short-chain dehydrogenase cluster. Arch Insect Biochem Physiol. 82, 96–115. https://doi.org/10.1002/arch.21078
- Mazid, M., Ta, K., Mohammad, F., 2011. Role of secondary metabolites in defense mechanisms of plants, Review Article Biology and Medicine. 3,232-249
- McKenzie, J.A., 1996. Ecological and evolutionary aspects of insecticide resistance. Genetics Research. 68, 183-184.
- McKenzie, J.A., Clarke, G.M., 1988. Diazinon resistance, fluctuating asymmetry and fitness in the Australian sheep blowfly, *Lucilia cuprina*. Genetics. 120, 213–220. https://doi.org/10.1093/genetics/120.1.213
- Medja, F., Allouche, S., Frachon, P., Jardel, C., Malgat, M., de Camaret, B.M., Slama, A., Lunardi, J., Mazat, J.P., Lombès, A., 2009. Development and implementation of standardized respiratory chain spectrophotometric assays for clinical diagnosis. Mitochondrion. 9, 331–339. https://doi.org/10.1016/j.mito.2009.05.001

- Meng, N., Wei, Y., Gao, Y., Yu, K., Cheng, J., Li, X.-Y., Duan, C.-Q., Pan, Q.-H., 2020. Characterization of Transcriptional Expression and Regulation of Carotenoid Cleavage Dioxygenase 4b in Grapes. Front. Plant Sci. 0, 483. https://doi.org/10.3389/FPLS.2020.00483
- Menozzi, P., Shi, M.A., Lougarre, A., Tang, Z.H., Fournier, D., 2004. Mutations of acetylcholinesterase which confer insecticide resistance in *Drosophila melanogaster* populations. BMC Evol. Biol. 4, 1–7. https://doi.org/10.1186/1471-2148-4-4
- Mermans, C., Dermauw, W., Leeuwen, T. Van, 2017. A G326E substitution in the glutamate-gated chloride channel 3 (GluCl3) of the two-spotted spider mite *Tetranychus urticae* abolishes the agonistic activity of macrocyclic lactones. Pest Manag Sci. 73, 2413-2418. https://doi.org/10.1002/ps.4677
- Merzendorfer, H., 2014. Chapter One ABC Transporters and Their Role in Protecting Insects from Pesticides and Their Metabolites, in: Cohen, E.B.T.-A. in I.P. (Ed.), Target Receptors in the Control of Insect Pests: Part II. Academic Press, pp. 1–72. https://doi.org/https://doi.org/10.1016/B978-0-12-417010-0.00001-X
- Meyer, J.S., Ingersoll, C.G., McDonald, L.L., Boyce, M.S., 1986. Estimating Uncertainty in Population Growth Rates: Jackknife vs. Bootstrap Techniques. Ecol. Soc. Am. 67, 1156–1166. https://doi.org/https://doi.org/10.2307/1938671
- Migeon, A., Elodie, N., Dorkeld, F., 2010. Spider Mites Web: A comprehensive database for the Tetranychidae, in: Trends in Acarology. Springer Netherlands, pp. 557–560. https://doi.org/10.1007/978-90-481-9837-5_96
- Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees, in: 2010 Gateway Computing Environments Workshop, GCE 2010. https://doi.org/10.1109/GCE.2010.5676129
- Miller, R.W., Croft, B.A., Nelson, R.D., 1985. Effects of Early Season Immigration on Cyhexatin and Formetanate Resistance of *Tetranychus urticae* (Acari: Tetranychidae) on Strawberry in Central California. J. Econ. Entomol. 78, 1379–1388. https://doi.org/10.1093/jee/78.6.1379
- Misra, J.R., Horner, M.A., Lam, G., Thummel, C.S., 2011. Transcriptional regulation of xenobiotic detoxification in Drosophila. Genes Dev. 25, 1796–1806. https://doi.org/10.1101/gad.17280911
- Misra, P., Pandey, A., Tiwari, M., Chandrashekar, K., Sidhu, O.P., Asif, M.H., Chakrabarty, D., Singh, P.K., Trivedi, P.K., Nath, P., Tuli, R., 2010. Modulation of transcriptome and metabolome of tobacco by Arabidopsis transcription factor, AtMYB12, leads to insect resistance. Plant Physiol. 152, 2258–2268. https://doi.org/10.1104/pp.109.150979
- Mohammed AlJabr, A., Hussain, A., Rizwan-ul-Haq, M., Al-Ayedh, H., 2017. Toxicity of plant secondary metabolites modulating detoxification genes expression for natural red palm weevil pesticide development. Molecules. 22, 1–12. https://doi.org/10.3390/molecules22010169
- Montella, I.R., Schama, R., Valle, D., 2012. The classification of esterases: an important gene family involved in insecticide resistance - A review. Mem. Inst. Oswaldo Cruz. 107, 437–449. https://doi.org/10.1590/S0074-02762012000400001
- Moore, R.M., Harrison, A.O., McAllister, S.M., Polson, S.W., Wommack, K.E., 2020. Iroki: automatic customization and visualization of phylogenetic trees. PeerJ. 8, e8584. https://doi.org/10.7717/peerj.8584
- Mota-Sanchez, D., Wise, J.C., 2023. Arthropod Pesticide Resistance Database | Michigan State University [WWW Document]. URL https://www.pesticideresistance.org/ (accessed 4.18.23).
- Motta, E.V.S., Gage, A., Smith, T.E., Blake, K.J., Kwong, W.K., Riddington, I.M., Moran, N.A., 2022. Hostmicrobiome metabolism of a plant toxin in bees. Elife. 11, e82595. https://doi.org/10.7554/ELIFE.82595
- Moura, J.C.M.S., Bonine, C.A.V., de Oliveira Fernandes Viana, J., Dornelas, M.C., Mazzafera, P., 2010. Abiotic and biotic stresses and changes in the lignin content and composition in plants. J. Integr. Plant Biol. 52, 360– 376. https://doi.org/10.1111/J.1744-7909.2010.00892.X
- Movva, V., Pathipati, U.R., 2017. Feeding-induced phenol production in Capsicum annuum L. influences

Spodoptera litura F. larval growth and physiology. Arch. Insect Biochem. Physiol. 95, e21387. https://doi.org/10.1002/ARCH.21387

- Mu, C., Ma, B., Zhang, C., Geng, G., Zhang, X., Chen, L., Wang, M., Li, J., Zhao, T., Cheng, H., Zhang, Q., Ma, K., Luo, Q., Chang, R., Liu, Q., Wu, H., Liu, L., Wang, X., Wang, J., Zhang, Y., Zhao, Y., Wen, L., Chen, Q., Zhu, Y., 2018. Loss of SDHB reprograms energy metabolisms and inhibits high fat diet induced metabolic syndromes. bioRxiv. 259226. https://doi.org/10.1101/259226
- Naalden, D., van Kleeff, P.J.M., Dangol, S., Mastop, M., Corkill, R., Hogenhout, S.A., Kant, M.R., Schuurink, R.C., 2021. Spotlight on the Roles of Whitefly Effectors in Insect–Plant Interactions. Front Plant Sci. https://doi.org/10.3389/fpls.2021.661141
- Nakahira, K., 2011. Strategy for discovery of a novel miticide Cyenopyrafen which is one of electron transport chain inhibitors. J. Pestic. Sci. 36, 511–515. https://doi.org/10.1584/jpestics.w11-34
- Nakano, M., Yasokawa, N., Suwa, A., Fujioka, S., Furuya, T., Sakata, K., 2015. Mode of action of novel acaricide pyflubumide: Effects on the mitochondrial respiratory chain. J. Pestic. Sci 40, 19–24. https://doi.org/10.1584/jpestics.D14-086
- Nauen, R., Bass, C., Feyereisen, R., Vontas, J., 2021. The role of cytochrome P450s in insect toxicology and resistance. Annu. Rev. Entomol. 67, 105–124. https://doi.org/10.1146/annurev-ento-070621
- Navia, D., Novelli, V.M., Rombauts, S., Freitas-Astúa, J., Santos de Mendonça, R., Nunes, M.A., Machado, M.A., Lin, Y.-C., Le, P., Zhang, Z., Grbić, M., Wybouw, N., Breeuwer, J.A.J., Van Leeuwen, T., Van de Peer, Y., 2019. Draft Genome Assembly of the False Spider Mite *Brevipalpus yothersi*. Microbiol. Resour. Announc. 8, e01563-18. https://doi.org/10.1128/mra.01563-18
- Neish, A.C., 1960. Biosynthetic pathways of aromatic compounds. Annu. Rev. Plant Biol. 11, 55-80.
- Ngoc, P.C.T., Greenhalgh, R., Dermauw, W., Rombauts, S., Bajda, S., Zhurov, V., Grbić, M., Van de Peer, Y., Van Leeuwen, T., Rouzé, P., Clark, R.M., 2016. Complex Evolutionary Dynamics of Massively Expanded Chemosensory Receptor Families in an Extreme Generalist Chelicerate Herbivore. Genome Biol. Evol. 8, 3323–3339. https://doi.org/10.1093/gbe/evw249
- Nieuwenhuyse, P. Van, Leeuwen, T. Van, Khajehali, J., Vanholme, B., Tirry, L., 2009. Mutations in the mitochondrial cytochrome b of *Tetranychus urticae* Koch (Acari: Tetranychidae) confer cross-resistance between bifenazate and acequinocyl. Pest Manag. Sci. 65, 404–412. https://doi.org/10.1002/PS.1705
- Niu, J., Shen, G., Christiaens, O., Smagghe, G., He, L., Wang, J., 2018. Beyond insects: current status and achievements of RNA interference in mite pests and future perspectives. Pest Manag. Sci. 74, 2680–2687. https://doi.org/10.1002/ps.5071
- Niu, J., Zhang, W., Sun, Q.Z., Wang, J.J., 2019. Three novel RNA viruses in the spider mite *Tetranychus urticae* and their possible interactions with the host RNA interference response. J. Invertebr. Pathol. 166, 107228. https://doi.org/10.1016/j.jip.2019.107228
- Nouri, S., Matsumura, E.E., Kuo, Y.-W., Falk, B.W., 2018. Insect-specific viruses: from discovery to potential translational applications. Curr. Opin. Virol. 33, 33–41. https://doi.org/https://doi.org/10.1016/j.coviro.2018.07.006
- Oakeshott, J.G., Claudianos, C., Campbell, P.M., Newcomb, R.D., Russell, R.J., 2005. Biochemical genetics and genomics of insect esterases In: Gilbert LI, Iatrou K, Gill SS, (Eds). Comprehensive Molecular Insect Science. pp 1-73. Elsevier, Netherlands.
- Oerke, E.C., Dehne, H.W., 2004. Safeguarding production Losses in major crops and the role of crop protection. Crop Prot. 23, 275–285. https://doi.org/10.1016/j.cropro.2003.10.001
- Okonechnikov, K., Golosova, O., Fursov, M., the UGENE team, 2012. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics. 28, 1166–1167. https://doi.org/10.1093/bioinformatics/bts091
- Oliveira, C.M., Auad, A.M., Mendes, S.M., Frizzas, M.R., 2014. Crop losses and the economic impact of insect pests on Brazilian agriculture. Crop Prot. 56, 50–54. https://doi.org/10.1016/J.CROPRO.2013.10.022

- Onkokesung, N., Reichelt, M., Van Doorn, A., Schuurink, R.C., Van Loon, J.J.A., Dicke, M., 2014. Modulation of flavonoid metabolites in *Arabidopsis thaliana* through overexpression of the MYB75 transcription factor: Role of kaempferol-3,7- dirhamnoside in resistance to the specialist insect herbivore *Pieris brassicae*. J. Exp. Bot. 65, 2203–2217. https://doi.org/10.1093/jxb/eru096
- Oppenheim, S.J., Baker, R.H., Simon, S., Desalle, R., 2015. We can't all be supermodels: The value of comparative transcriptomics to the study of non-model insects. Insect Mol Biol. 24, 139-54. https://doi.org/10.1111/imb.12154
- Orelle, C., Mathieu, K., Jault, J.-M., 2019. Multidrug ABC transporters in bacteria. Res. Microbiol. 170, 381–391. https://doi.org/https://doi.org/10.1016/j.resmic.2019.06.001
- Orville, A.M., Lipscomb, J.D., Ohlendorf, D.H., 1997. Crystal structures of substrate and substrate analog complexes of protocatechuate 3,4-dioxygenase: Endogenous Fe3+ ligand displacement in response to substrate binding. Biochemistry. 36, 10052–10066. https://doi.org/10.1021/bi970469f
- Osakabe, M., Uesugi, R., Goka, K., 2009. Evolutionary aspects of acaricide-resistance development in spider mites. Psyche (London). 2009. https://doi.org/10.1155/2009/947439
- Papapostolou, K.M., Riga, M., Samantsidis, G.-R., Skoufa, E., Balabanidou, V., Van Leeuwen, T., Vontas, J., 2022.

 Over-expression in cis of the midgut P450 CYP392A16 contributes to abamectin resistance in *Tetranychus urticae*.

 Insect
 Biochem
 Mol
 Biol.
 142,
 103709.

 https://doi.org/https://doi.org/10.1016/j.ibmb.2021.103709
- Papapostolou, K.M., Riga, M., Charamis, J., Skoufa, E., Souchlas, V., Ilias, A., Dermauw, W., Ioannidis, P., Van Leeuwen, T., Vontas, J., 2021. Identification and characterization of striking multiple-insecticide resistance in a *Tetranychus urticae* field population from Greece. Pest Manag. Sci. 77, 666–676. https://doi.org/10.1002/ps.6136
- Pardo-López, L., Soberón, M., Bravo, A., 2013. Bacillus thuringiensis insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbiol. Rev. 37, 3–22. https://doi.org/10.1111/j.1574-6976.2012.00341.x
- Pascussi, J.-M., Gerbal-Chaloin, S., Duret, C., Daujat-Chavanieu, M., Vilarem, M.-J., Maurel, P., 2008. The Tangle of Nuclear Receptors that Controls Xenobiotic Metabolism and Transport: Crosstalk and Consequences. Annu. Rev. Pharmacol. Toxicol. 48, 1–32. https://doi.org/10.1146/annurev.pharmtox.47.120505.105349
- Paudel Timilsena, B., Niassy, S., Kimathi, E., Abdel-Rahman, E.M., Seidl-Adams, I., Wamalwa, M., Tonnang, H.E.Z., Ekesi, S., Hughes, D.P., Rajotte, E.G., Subramanian, S., 2022. Potential distribution of fall armyworm in Africa and beyond, considering climate change and irrigation patterns. Sci. Rep. 12, 1–15. https://doi.org/10.1038/s41598-021-04369-3
- Pavlidi, N., Khalighi, M., Myridakis, A., Dermauw, W., Wybouw, N., Tsakireli, D., Stephanou, E.G., Labrou, N.E., Vontas, J., Van Leeuwen, T., 2017. A glutathione-S-transferase (TuGSTd05) associated with acaricide resistance in *Tetranychus urticae* directly metabolizes the complex II inhibitor cyflumetofen. Insect Biochem. Mol. Biol. 80, 101–115. https://doi.org/10.1016/j.ibmb.2016.12.003
- Pavlidi, N., Vontas, J., Van Leeuwen, T., 2018. The role of glutathione S-transferases (GSTs) in insecticide resistance in crop pests and disease vectors. Curr. Opin. Insect Sci. 27, 97–102. https://doi.org/https://doi.org/10.1016/j.cois.2018.04.007
- Pedra, J.H.F., Mcintyre, L.M., Scharf, M.E., Pittendrigh, B.R., 2004. Genome-wide transcription profile of field-and laboratory-selected dichlorodiphenyltrichloroethane (DDT)-resistant Drosophila.
- Perlmutter, J.I., Bordenstein, S.R., 2020. Microorganisms in the reproductive tissues of arthropods. Nat. Rev. Microbiol. 18, 97–111. https://doi.org/10.1038/s41579-019-0309-z
- Peshin, R., 2014. Integrated pest management: Pesticide problems, vol.3. Integr. Pest Manag. Pestic. Probl. Vol.3 1–474. https://doi.org/10.1007/978-94-007-7796-5
- Pieterse, C.M.J., Van Der Does, D., Zamioudis, C., Leon-Reyes, A., Van Wees, S.C.M., 2012. Hormonal modulation of plant immunity. Annu. Rev. Cell Dev. Biol. 28, 489–521. https://doi.org/10.1146/annurev-cellbio-

092910-154055

Pimentel, D., Peshin, R., 2014. Integrated Pest Management. Springer, Dordrecht.

- Pimviriyakul, P., Wongnate, T., Tinikul, R., Chaiyen, P., 2020. Microbial degradation of halogenated aromatics: molecular mechanisms and enzymatic reactions. Microb. Biotechnol. 13, 67–86. https://doi.org/10.1111/1751-7915.13488
- Plapp, F.W., Campanhola, C., Bagwell, R.D., McCutchen, B.F., 1990. Management of Pyrethroid-resistant Tobacco Budworms on Cotton in the United States. Pestic. Resist. Arthropods. 237–260. https://doi.org/10.1007/978-1-4684-6429-0_9
- Popp, J., Pető, K., Nagy, J., 2013. Pesticide productivity and food security. A review. Agron. Sustain. Dev. 33, 243– 255. https://doi.org/10.1007/s13593-012-0105-x
- Poulton, J.E., 1990. Cyanogenesis in Plants1. Plant Physiol. 94, 401–405. doi: 10.1104/pp.94.2.401
- Prasad, A., Chirom, O., Prasad, M., 2021. Insect herbivores benefit from horizontal gene transfer. Trends Plant Sci. 26, 1096–1097. https://doi.org/10.1016/j.tplants.2021.07.012
- Price, D.R.G., Gatehouse, J.A., 2008. RNAi-mediated crop protection against insects. Trends Biotechnol. 26, 393– 400. https://doi.org/https://doi.org/10.1016/j.tibtech.2008.04.004
- Prout, T., 1971. The Relation Between Fitness Components and Population Prediction in Drosophila. II: Population Prediction. Genetics. 68, 151–167. https://doi.org/10.1093/genetics/68.1.151
- Putman, Wm.L., 1970. Occurrence and transmission of a virus disease of the european red mite, *Panonychus ulmi*. Can Entomol 102, 305–321. https://doi.org/DOI: 10.4039/Ent102305-3
- Pym, A., Singh, K.S., Nordgren, Å., Davies, T.G.E., Zimmer, C.T., Elias, J., Slater, R., Bass, C., 2019. Host plant adaptation in the polyphagous whitefly, *Trialeurodes vaporariorum*, is associated with transcriptional plasticity and altered sensitivity to insecticides. BMC Genomics. 20, 1–19. https://doi.org/10.1186/s12864-019-6397-3
- Quenouille, M.H., 1956. Notes on Bias in Estimation. Biometrika. 43, 353. https://doi.org/10.2307/2332914
- R Core Team, 2021. R: a language and environment for statistical computing. R Foundation for Statistical Computing , Vienna, Austria [WWW Document].
- Rand, D.M., 2001. The Units of Selection on Mitochondrial DNA. Annu Rev Ecol Syst 32, 415–448. https://doi.org/10.1146/annurev.ecolsys.32.081501.114109
- Rao, M.J., Xu, Y., Huang, Y., Tang, X., Deng, X., Xu, Q., 2019. Ectopic expression of citrus UDP-glucosyl transferase gene enhances anthocyanin and proanthocyanidins contents and confers high light tolerance in Arabidopsis. BMC Plant Biol. 19, 603. https://doi.org/10.1186/s12870-019-2212-1
- Raven, P.H., Wagner, D.L., 2021. Agricultural intensification and climate change are rapidly decreasing insect biodiversity. Proc. Natl. Acad. Sci. U. S. A. 118, 1–6. https://doi.org/10.1073/PNAS.2002548117
- Reddy, K.V.S., Zehr, U.B., 2004. Novel strategies for overcoming pests and diseases in India. Biology.
- Reed, D.K., Desjardins, P.R., 1982. Morphology of a non-occluded virus isolated from citrus red mite, Panonychus citri. Experientia 38, 468–469. https://doi.org/10.1007/BF01952642
- Rice, P., Longden, L., Bleasby, A., 2000. EMBOSS: The European Molecular Biology Open Software Suite. Trends Genet. 16, 276-277. https://doi.org/10.1016/S0168-9525(00)02024-2
- Richter, C., Dirks, M.E., Gronover, C.S., Prüfer, D., Moerschbacher, B.M., 2012. Silencing and Heterologous Expression of ppo-2 Indicate a Specific Function of a Single Polyphenol Oxidase Isoform in Resistance of Dandelion (*Taraxacum officinale*) Against *Pseudomonas syringae* pv. tomato. Mol Plant Microbe Interact. 25, 200–210. doi: 10.1094/MPMI-04-11-0082
- Riga, M., Bajda, S., Themistokleous, C., Papadaki, S., Palzewicz, M., Dermauw, W., Vontas, J., Leeuwen, T. Van, 2017. The relative contribution of target-site mutations in complex acaricide resistant phenotypes as

assessed by marker assisted backcrossing in *Tetranychus urticae*. Sci. Rep. 7, 1–12. https://doi.org/10.1038/s41598-017-09054-y

- Riga, M., Myridakis, A., Tsakireli, D., Morou, E., Stephanou, E.G., Nauen, R., Van Leeuwen, T., Douris, V., Vontas, J., 2015. Functional characterization of the *Tetranychus urticae* CYP392A11, a cytochrome P450 that hydroxylates the METI acaricides cyenopyrafen and fenpyroximate. Insect Biochem. Mol. Biol. 65, 91–99. https://doi.org/10.1016/j.ibmb.2015.09.004
- Riga, M., Tsakireli, D., Ilias, A., Morou, E., Myridakis, A., Stephanou, E.G., Nauen, R., Dermauw, W., Van Leeuwen, T., Paine, M., Vontas, J., 2014. Abamectin is metabolized by CYP392A16, a cytochrome P450 associated with high levels of acaricide resistance in *Tetranychus urticae*. Insect Biochem. Mol. Biol. 46, 43–53. https://doi.org/10.1016/j.ibmb.2014.01.006
- Ring, H., Gao, Z., Klein, N.D., Garwood, M., Bischof, J.C., Haynes, C.L., 2018. Ferrozine Assay for Simple and Cheap Iron Analysis of Silica-Coated Iron Oxide Nanoparticles: Ferrozine Assay for Simple and Cheap Iron Analysis of Silica-Coated Iron Oxide. ChemRxiv Preprint. https://doi.org/10.26434/chemrxiv.6815672.v1
- Rinkevich, F., Du, Y.F., Dong, K., 2013. Diversity and Convergence of Sodium Channel Mutations Involved in Resistance to Pyrethroids. Pestic Biochem Physiol. 106, 93–100. https://doi.org/10.1016/j.pestbp.2013.02.007
- Rioja, C., Zhurov, V., Bruinsma, K., Grbic, M., Grbic, V., 2017. Plant-herbivore interactions: A case of an extreme generalist, the two-spotted spider mite *Tetranychus urticae*. Mol. Plant-Microbe Interact. 30, 935–945. https://doi.org/10.1094/MPMI-07-17-0168-CR
- Robertson, J.L., Jones, M.M., Olguin, E., Alberts, B., 2017. Bioassays with Athropods, Third Edition. CRC Press, Boca Raton, FL.
- Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26, 139–140. https://doi.org/10.1093/bioinformatics/btp616
- Rode, N.O., Holtz, Y., Loridon, K., Santoni, S., Ronfort, J., Gay, L., 2018. How to optimize the precision of allele and haplotype frequency estimates using pooled-sequencing data. Mol. Ecol. Resour. 18, 194–203. https://doi.org/10.1111/1755-0998.12723
- Roopesh, K., Guyot, S., Sabu, A., Haridas, M., Isabelle, P.G., Roussos, S., Augur, C., 2010. Biotransformation of procyanidins by a purified fungal dioxygenase: Identification and characterization of the products using mass spectrometry. Process Biochem. 45, 904–913. https://doi.org/10.1016/J.PROCBIO.2010.02.019
- Root, R.B., Kareiva, P.M., 1984. The Search for Resources by Cabbage Butterflies (*Pieris Rapae*): Ecological Consequences and Adaptive Significance of Markovian Movements in a Patchy Environment. Ecol. Soc. 65, 147–165. https://doi.org/10.2307/1939467
- Rosano, G.L., Ceccarelli, E.A., 2014. Recombinant protein expression in *Escherichia coli*: advances and challenges. Front Microbiol. 5, 172. https://doi.org/10.3389/fmicb.2014.00172
- Roush, R.T., Daly, J.C., 1990. The Role of Population Genetics in Resistance Research and Management. Pestic. Resist. Arthropods. 97–152. https://doi.org/10.1007/978-1-4684-6429-0_5
- Roush, R.T., McKenzie, J.A., 1987. Ecological genetics of insecticide and acaricide resistance. Annu. Rev. Entomol. 32, 361–380. https://doi.org/https://doi.org/10.1146/annurev.en.32.010187.002045
- Ruprecht, J., Yankovskaya, V., Maklashina, E., Iwata, S., Cecchini, G., 2009. Structure of *Escherichia coli* Succinate:Quinone Oxidoreductase with an Occupied and Empty Quinone-binding Site. J. Biol. Chem. 284, 29836. https://doi.org/10.1074/JBC.M109.010058
- Saidijam, M., Benedetti, G., Ren, Q., Xu, Z., Hoyle, C., Palmer, S., Ward, A., Bettaney, K., Szakonyi, G., Meuller, J., Morrison, S., Pos, M., Butaye, P., Walravens, K., Langton, K., Herbert, R., Skurray, R., Paulsen, I., O'reilly, J., Rutherford, N., Brown, M., Bill, R., Henderson, P., 2006. Microbial drug efflux proteins of the major facilitator superfamily. Curr Drug Targets. 7, 793–811. https://doi.org/10.2174/138945006777709575

- Saier, M.H., Beatty, J.T., Goffeau, A., Harley, K.T., Heijne, W.H.M., Huang, S.C., Jack, D.L., Jähn, P.S., Lew, K., Liu, J., Pao, S.S., Paulsen, I.T., Tseng, T.T., Virk, P.S., 1999. The major facilitator superfamily. J. Mol. Microbiol. Biotechnol. 1, 257–279.
- Sami, A., -Bayati, A.L., 2019. Breeding for Tomato Resistance to Spider Mite *Tetranychus urticae* Koch (Acari: Tetranychidae).Plant. Soil Sci. https://doi.org/https://doi.org/10.13023/etd.2019.105
- Santamaria, M.E., Arnaiz, A., Gonzalez-Melendi, P., Martinez, M., Diaz, I., 2018. Plant perception and short-term responses to phytophagous insects and mites. Int. J. Mol. Sci. 19, 1356. https://doi.org/10.3390/ijms19051356
- Sato, Y., Jang, S., Takeshita, K., Itoh, H., Koike, H., Tago, K., Hayatsu, M., Hori, T., Kikuchi, Y., 2021. Insecticide resistance by a host-symbiont reciprocal detoxification. Nat Commun 12. 6432. https://doi.org/10.1038/s41467-021-26649-2
- Scalliet, G., Bowler, J., Luksch, T., Kirchhofer-Allan, L., Steinhauer, D., Ward, K., Niklaus, M., Verras, A., Csukai, M., Daina, A., Fonné-Pfister, R., 2012. Mutagenesis and functional studies with succinate dehydrogenase inhibitors in the wheat pathogen *Mycosphaerella graminicola*. PLoS One. 7, e35429. https://doi.org/10.1371/journal.pone.0035429
- Schägger, H., von Jagow, G., 1991. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal. Biochem. 199, 223–231. https://doi.org/10.1016/0003-2697(91)90094-A
- Schlachter, C.R., Daneshian, L., Amaya, J., Klapper, V., Wybouw, N., Borowski, T., Van Leeuwen, T., Grbic, V., Grbic, M., Makris, T.M., Chruszcz, M., 2019. Structural and functional characterization of an intradiol ringcleavage dioxygenase from the polyphagous spider mite herbivore *Tetranychus urticae* Koch. Insect Biochem. Mol. Biol. 107, 19–30. https://doi.org/10.1016/j.ibmb.2018.12.001
- Schoonhoven, L.M., Van Loon, J.J.A., Dicke, M., 2006. Insect-Plant Biology. Oxford University Press, Great Clarendon street, Oxford, UK.
- Schuman, M., Baldwin, I., 2016. The Layers of Plant Responses to Insect Herbivores. Annu Rev Entomol. 61, 373– 394. https://doi.org/10.1146/annurev-ento-010715-023851
- Semana, P., Powlowski, J., 2019. Four Aromatic Intradiol Ring Cleavage Dioxygenases from Aspergillus niger. Appl. Environ. Microbiol. 85, 1–18. https://doi.org/10.1128/AEM.01786-19
- Shayeghi, M., Latunde-Dada, G.O., Oakhill, J.S., Laftah, A.H., Takeuchi, K., Halliday, N., Khan, Y., Warley, A., McCann, F.E., Hider, R.C., Frazer, D.M., Anderson, G.J., Vulpe, C.D., Simpson, R.J., McKie, A.T., 2005. Identification of an Intestinal Heme Transporter. Cell. 122, 789–801. https://doi.org/https://doi.org/10.1016/j.cell.2005.06.025
- Sherr, C.J., 1994. The ins and outs of ring cleavage dioxygenases. Trends Cell Biol. 4, 15–18. https://doi.org/10.1016/j.tcb.2004.07.007
- Shi, M., Lin, X.-D., Tian, J.-H., Chen, L.-J., Chen, X., Li, C.-X., Qin, X.-C., Li, J., Cao, J.-P., Eden, J.-S., Buchmann, J., Wang, W., Xu, J., Holmes, E.C., Zhang, Y.-Z., 2016. Redefining the invertebrate RNA virosphere. Nature. 540, 539–543. https://doi.org/10.1038/nature20167
- Shi, M.A., Lougarre, A., Alies, C., Frémaux, I., Tang, Z.H., Stojan, J., Fournier, D., 2004. Acetylcholinesterase alterations reveal the fitness cost of mutations conferring insecticide resistance. BMC Evol. Biol. 8, 1–8. https://doi.org/doi: 10.1186/1471-2148-4-5
- Shi, Y., Jiang, Q., Yang, Y., Feyereisen, R., Wu, Y., 2021. Pyrethroid metabolism by eleven *Helicoverpa armigera* P450s from the CYP6B and CYP9A subfamilies. Insect Biochem. Mol. Biol. 135, 103597. https://doi.org/https://doi.org/10.1016/j.ibmb.2021.103597
- Shih, P.-Y., Sugio, A., Simon, J.-C., 2022. Annual Review of Entomology Molecular Mechanisms Underlying Host Plant Specificity in Aphids. https://doi.org/10.1146/annurev-ento-120220
- Showler, A.T., 2018. The Desert Locust in Africa and Western Asia: Complexities of War, Politics, Perilous Terrain, and Development | Radcliffe's IPM World Textbook, in: Omkar (Ed.), Pests and Their Management.

Springer Nature Singapore Pte Ltd., Singapore, pp. 825–869. https://doi.org/10.1007/978-981-10-8687-8

- Sierotzki, H., Scalliet, G., 2013. A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. Phytopathology. 103, 880–887. https://doi.org/10.1094/PHYTO-01-13-0009-RVW
- Simma, E.A., Hailu, B., Jonckheere, W., Rogiers, C., Duchateau, L., Dermauw, W., Van Leeuwen, T., 2020. Acaricide resistance status and identification of resistance mutations in populations of the two-spotted spider mite *Tetranychus urticae* from Ethiopia. Exp. Appl. Acarol. 82, 475–491. https://doi.org/10.1007/s10493-020-00567-2
- Simon, J.-C., d'Alençon, E., Guy, E., Jacquin-Joly, E., Jaquiéry, J., Nouhaud, P., Peccoud, J., Sugio, A., Streiff, R., 2015. Genomics of adaptation to host-plants in herbivorous insects. Brief. Funct. Genomics. 14, 413–423. https://doi.org/10.1093/bfgp/elv015
- Škarydová, L., Wsól, V., 2012. Human microsomal carbonyl reducing enzymes in the metabolism of xenobiotics: Well-known and promising members of the SDR superfamily. Drug Metab. Rev. 44, 173–191. https://doi.org/10.3109/03602532.2011.638304
- Skendži'c, S.S., Zovko, M., Pajač, I., Živkovi'c, Ž., Leši'c, V.L., Lemi'c, D.L., 2021. The Impact of Climate Change on Agricultural Insect Pests. Insects. 12, 440. https://doi.org/10.3390/insects12050440
- Smissaert, H., 1964. Cholinesterase Inhibition in Spider Mites Susceptible and Resistant to Organophosphate. Science. 143, 129–131. https://doi.org/10.1126/science.143.3602.129
- Smith, K.M., Hills, G.J., Munger, F., Gilmore, J.E., 1959. A Suspected Virus Disease of the Citrus Red Mite Panonychus citri (McG.). Nature 184, 70. https://doi.org/10.1038/184070a0
- Snoeck, S., Kurlovs, A.H., Bajda, S., Feyereisen, R., Greenhalgh, R., Villacis-Perez, E., Kosterlitz, O., Dermauw, W., Clark, R.M., Van Leeuwen, T., 2019a. High-resolution QTL mapping in *Tetranychus urticae* reveals acaricidespecific responses and common target-site resistance after selection by different METI-I acaricides. Insect Biochem. Mol. Biol. 110, 19–33. https://doi.org/10.1016/J.IBMB.2019.04.011
- Snoeck, S., Pavlidi, N., Pipini, D., Vontas, J., Dermauw, W., Van Leeuwen, T., 2019b. Substrate specificity and promiscuity of horizontally transferred UDP-glycosyltransferases in the generalist herbivore *Tetranychus urticae*. Insect Biochem. Mol. Biol. https://doi.org/10.1016/J.IBMB.2019.04.010
- Snoeck, S., Wybouw, N., Leeuwen, T. Van, Dermauw, W., 2018. Transcriptomic Plasticity in the Arthropod Generalist *Tetranychus urticae* Upon Long-Term Acclimation to Different Host Plants. G3 Genes | Genomes | Genetics. 8, 3865. https://doi.org/10.1534/G3.118.200585
- Sparks, T.C., Nauen, R., 2015. IRAC: Mode of action classification and insecticide resistance management. Pestic. Biochem. Physiol. 121, 122–128. https://doi.org/10.1016/J.PESTBP.2014.11.014
- Spinazzi, M., Casarin, A., Pertegato, V., Salviati, L., Angelini, C., 2012. Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. Nat. Protoc. 7, 1235–1246. https://doi.org/10.1038/nprot.2012.058
- Stafford-Banks, C.A., Rotenberg, D., Johnson, B.R., Whitfield, A.E., Ullman, D.E., 2014. Analysis of the Salivary Gland Transcriptome of *Frankliniella occidentalis*. PLoS One. 9, e94447.
- Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 30, 1312–1313. https://doi.org/10.1093/bioinformatics/btu033
- Sterck, L., Billiau, K., Abeel, T., Rouzé, P., Van de Peer, Y., 2012. ORCAE: online resource for community annotation of eukaryotes. Nat. Methods. 9, 1041. https://doi.org/10.1038/nmeth.2242
- Sterkel, M., Oliveira, P.L., 2017. Developmental roles of tyrosine metabolism enzymes in the blood-sucking insect Rhodnius prolixus. Proc. Biol. Sci. 284, 20162607. https://doi.org/10.1098/rspb.2016.2607
- Stone, B.F., 1968. A formula for determining degree of dominance in cases of monofactorial inheritance of resistance to chemicals. Bull. World Health Organ. 38, 325–326.

- Stumpf, N., Zebitz, C.P.W., Kraus, W., Moores, G.D., Nauen, R., 2001. Resistance to Organophosphates and Biochemical Genotyping of Acetylcholinesterases in *Tetranychus urticae* (Acari: Tetranychidae). Pestic. Biochem. Physiol. 69, 131–142. https://doi.org/10.1006/PEST.2000.2516
- Su, Q., Peng, Z., Tong, H., Xie, W., Wang, S., Wu, Q., Zhang, J., Li, C., Zhang, Y., 2019. A salivary ferritin in the whitefly suppresses plant defenses and facilitates host exploitation. J Exp Bot 70, 3343–3355. https://doi.org/10.1093/jxb/erz152
- Su, Y.-L., Li, J.-M., Li, M., Luan, J.-B., Ye, X.-D., Wang, X.-W., Liu, S.-S., 2012. Transcriptomic Analysis of the Salivary Glands of an Invasive Whitefly. PLoS One. 7, e39303.
- Sudo, M., Osakabe, M., 2022. freqpcr: Estimation of population allele frequency using qPCR ΔΔCq measures from bulk samples. Mol. Ecol. Resour. 22, 1380–1393. https://doi.org/10.1111/1755-0998.13554
- Sugimoto, N., Osakabe, M., 2019. Mechanism of acequinocyl resistance and cross-resistance to bifenazate in the two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae). Appl. Entomol. Zool. 54, 421–427. https://doi.org/10.1007/s13355-019-00638-w
- Sugimoto, N., Osakabe, M., 2014. Cross-resistance between cyenopyrafen and pyridaben in the twospotted spider mite *Tetranychus urticae* (Acari: Tetranychidae). Pest Manag. Sci. 70, 1090–1096. https://doi.org/10.1002/ps.3652
- Sugimoto, N., Takahashi, A., Ihara, R., Itoh, Y., Jouraku, A., Van Leeuwen, T., Osakabe, M., 2020. QTL mapping using microsatellite linkage reveals target-site mutations associated with high levels of resistance against three mitochondrial complex II inhibitors in *Tetranychus urticae*. Insect Biochem. Mol. Biol. 123, 103410. https://doi.org/10.1016/j.ibmb.2020.103410
- Sun, B.F., Xiao, J.H., He, S.M., Liu, L., Murphy, R.W., Huang, D.W., 2013. Multiple ancient horizontal gene transfers and duplications in lepidopteran species. Insect Mol Biol. 22, 72–87. https://doi.org/10.1111/imb.12004
- Sun, F., Huo, X., Zhai, Y., Wang, A., Xu, J., Su, D., Bartlam, M., Rao, Z., 2005. Crystal structure of mitochondrial respiratory membrane protein complex II. Cell. 121, 1043–1057. https://doi.org/10.1016/J.CELL.2005.05.025
- Sun, J., Li, C., Jiang, J., Song, C., Wang, C., Feng, K., Wei, P., He, L., 2022. Cross resistance, inheritance and fitness advantage of cyetpyrafen resistance in two-spotted spider mite, *Tetranychus urticae*. Pestic. Biochem. Physiol. 183, 105062. https://doi.org/10.1016/J.PESTBP.2022.105062
- Sun, Q., Yang, W., Shi, Y., Chai, W., Chen, R., Jiang, Y., Zhang, Y., Wang, ZG, Wang, ZJ, Niu, J., Wang, J., 2023. Horizontally transferred genes as natural and specific RNAi targets in a pest spider mite (*Panonychus citri*). Entomol. Gen. 43, 99–107. https://doi.org/10.1127/entomologia/2022/1788
- Sun, X., Hua, W., Wang, K., Song, J., Zhu, B., Gao, X., Liang, P., 2023. A novel V263I mutation in the glutamategated chloride channel of *Plutella xylostella* (L.) confers a high level of resistance to abamectin. Int. J. Biol. Macromol. 230, 123389. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2023.123389
- Suzuki, T., Nunes, M.A., España, M.U., Namin, H.H., Jin, P., Bensoussan, N., Zhurov, V., Rahman, T., De Clercq, R., Hilson, P., Grbic, V., Grbic, M., 2017. RNAi-based reverse genetics in the chelicerate model *Tetranychus urticae*: A comparative analysis of five methods for gene silencing. PLoS One.12, e0180654. https://doi.org/10.1371/journal.pone.0180654
- Suzuki, Y., Shiotsuki, T., Jouraku, A., Miura, K., Minakuchi, C., 2017. Benzoylurea resistance in western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae): The presence of a point mutation in chitin synthase 1. J. Pestic. Sci. 42, 93–96. https://doi.org/10.1584/jpestics.D17-023
- Swetha, V.P., Phale, P.S., 2005. Metabolism of carbaryl via 1,2-dihydroxynaphthalene by soil isolates *Pseudomonas sp.* strains C4, C5, and C6. Appl. Environ. Microbiol. 71, 5951–5956. https://doi.org/10.1128/AEM.71.10.5951-5956.2005
- Szeto, S.S.W., Reinke, S.N., Sykes, B.D., Lemire, B.D., 2010. Mutations in the Saccharomyces cerevisiae succinate dehydrogenase result in distinct metabolic phenotypes revealed through 1H NMR-based metabolic footprinting. J. Proteome Res. 9, 6729–6739. https://doi.org/10.1021/pr100880y

- Szeto, S.S.W., Reinke, S.N., Sykes, B.D., Lemire, B.D., 2007. Ubiquinone-binding Site Mutations in the Saccharomyces cerevisiae Succinate Dehydrogenase Generate Superoxide and Lead to the Accumulation of Succinate. J. Biol. Chem. 282, 27518–27526. https://doi.org/10.1074/JBC.M700601200
- Tabashnik, B.E., Mota-Sanchez, D., Whalon, M.E., Hollingworth, R.M., Carrière, Y., 2014. Defining terms for proactive management of resistance to Bt crops and pesticides. J Econ Entomol 107, 496–507. https://doi.org/10.1603/EC13458
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729. https://doi.org/10.1093/molbev/mst197
- Tello, J., Mammerler, R., Čajić, M., Forneck, A., 2019. Major Outbreaks in the Nineteenth Century Shaped Grape Phylloxera Contemporary Genetic Structure in Europe. Sci. Rep. 9, 17540. https://doi.org/10.1038/s41598-019-54122-0
- Thaler, J.S., Humphrey, P.T., Whiteman, N.K., 2012. Evolution of jasmonate and salicylate signal crosstalk. Trends Plant Sci. 17, 260–270. https://doi.org/https://doi.org/10.1016/j.tplants.2012.02.010
- Teixeira, L., Ferreira, Á., Ashburner, M., 2008. The Bacterial Symbiont Wolbachia Induces Resistance to RNA Viral Infections in Drosophila melanogaster. PLoS Biol 6, e1000002.
- Tian, T., Wu, Mingmei, Zhang, Y., Xu, D., Wu, Mingyue, Xie, W., Su, Q., Wang, S., 2022. Pesticide Resistance and Related Mutation Frequencies of *Tetranychus urticae* in Hainan, China. Horticulturae. 8, 590. https://doi.org/10.3390/horticulturae8070590
- Trifinopoulos, J., Nguyen, L.T., von Haeseler, A., Minh, B.Q., 2016. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res. 44, W232–W235. https://doi.org/10.1093/nar/gkw256
- Tsai, S.C., Li, Y.K., 2007. Purification and characterization of a catechol 1,2-dioxygenase from a phenol degrading *Candida albicans* TL3. Arch. Microbiol. 187, 199–206. https://doi.org/10.1007/s00203-006-0187-4
- Tyagi, S., Kesiraju, K., Saakre, M., Rathinam, M., Raman, V., Pattanayak, D., Sreevathsa, R., 2020. Genome Editing for Resistance to Insect Pests: An Emerging Tool for Crop Improvement. ACS Omega 5, 20674–20683. https://doi.org/10.1021/acsomega.0c01435
- Umina, P., Bass, C., van Rooyen, A., Chirgwin, E., Arthur, A., Pym, A., Mackisack, J., Mathews, A., Kirkland, L., 2022. Spirotetramat resistance in *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) and its association with the presence of the A2666V mutation. pest Manag. Sci. 78, 4822–4831. https://doi.org/10.1002/ps.7103.
- Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res. 40, e115. https://doi.org/10.1093/NAR/GKS596
- Van Laecke, K., Degheele, D., 1993. Effect of insecticide—synergist combinations on the survival of Spodoptera exigua. Pestic. Sci. 37, 283–288. https://doi.org/10.1002/PS.2780370308
- Van Leeuwen, T., Demaeght, P., Osborne, E., Dermauw, W., Gohlke, S., Nauen, R., Grbic, M., Tirry, L., Merzendorfer, H., Clark, R., 2012. Population bulk segregant mapping uncovers resistance mutations and the mode of action of a chitin synthesis inhibitor in arthropods. Proc. Natl. Acad. Sci. U. S. A. 109, 4407– 4412. https://doi.org/10.1073/PNAS.1200068109
- Van Leeuwen, T., Dermauw, W., 2016. The Molecular Evolution of Xenobiotic Metabolism and Resistance in Chelicerate Mites. Annu. Rev. Entomol. 61, 475–498. https://doi.org/10.1146/annurev-ento-010715-023907
- Van Leeuwen, T., Dermauw, W., Mavridis, K., Vontas, J., 2020. Significance and interpretation of molecular diagnostics for insecticide resistance management of agricultural pests. Curr. Opin. Insect Sci. 39, 69-76. https://doi.org/10.1016/j.cois.2020.03.006
- Van Leeuwen, T., Stillatus, V., Tirry, L., 2004. Genetic analysis and cross-resistance spectrum of a laboratoryselected chlorfenapyr resistant strain of two-spotted spider mite (Acari: Tetranychidae). Exp. Appl. Acarol. 32, 249–261. https://doi.org/10.1023/B:APPA.0000023240.01937.6d

Van Leeuwen, T., Tirry, L., Yamamoto, A., Nauen, R., Dermauw, W., 2015. The economic importance of acaricides

in the control of phytophagous mites and an update on recent acaricide mode of action research. Pestic. Biochem. Physiol. 121, 12–21. https://doi.org/10.1016/j.pestbp.2014.12.009

- Van Leeuwen, T., Vanholme, B., Van Pottelberge, S., Van Nieuwenhuyse, P., Nauen, R., Tirry, L., Denholm, I., 2008. Mitochondrial heteroplasmy and the evolution of insecticide resistance: Non-Mendelian inheritance in action. Proc. Natl. Acad. Sci. U. S. A. 105, 5980–5985. https://doi.org/10.1073/pnas.0802224105
- Van Leeuwen, T., Vontas, J., Tsagkarakou, A., Dermauw, W., Tirry, L., 2010. Acaricide resistance mechanisms in the two-spotted spider mite *Tetranychus urticae* and other important Acari: A review. Insect Biochem. Mol. Biol. 40, 563–572. https://doi.org/10.1016/J.IBMB.2010.05.008
- Van Pottelberge, Steven, Khajehali, J., Van Leeuwen, T., Tirry, L., 2009a. Effects of spirodiclofen on reproduction in a susceptible and resistant strain of *Tetranychus urticae* (Acari: Tetranychidae). Exp. Appl. Acarol. 47, 301–309. https://doi.org/10.1007/s10493-008-9226-y
- Van Pottelberge, Steven, Van Leeuwen, T., Khajehali, J., Tirry, L., 2009b. Genetic and biochemical analysis of a laboratory-selected spirodiclofen-resistant strain of *Tetranychus urticae* Koch (Acari: Tetranychidae). Pest Manag. Sci. 65, 358–366. https://doi.org/10.1002/ps.1698
- Van Pottelberge, S, Van Leeuwen, T., Nauen, R., Tirry, L., 2009. Resistance mechanisms to mitochondrial electron transport inhibitors in a field-collected strain of *Tetranychus urticae* Koch (Acari: Tetranychidae). Bull. Entomol. Res. 99, 23–31. https://doi.org/10.1017/S0007485308006081
- Vandenhole, M., Dermauw, W., Van Leeuwen, T., 2021. Short term transcriptional responses of P450s to phytochemicals in insects and mites. Curr. Opin. Insect Sci. 43, 117–127. https://doi.org/10.1016/j.cois.2020.12.002
- Vanholme, R., Sundin, L., Seetso, K.C., Kim, H., Liu, X., Li, J., De Meester, B., Hoengenaert, L., Goeminne, G., Morreel, K., Haustraete, J., Tsai, H.-H., Schmidt, W., Vanholme, B., Ralph, J., Boerjan, W., 2019. COSY catalyses trans–cis isomerization and lactonization in the biosynthesis of coumarins. Nat. Plants. 5, 1066– 1075. https://doi.org/10.1038/s41477-019-0510-0
- Vetting, M.W., Ohlendorf, D.H., 2000. The 1.8 Å crystal structure of catechol 1,2-dioxygenase reveals a novel hydrophobic helical zipper as a subunit linker. Structure. 8, 429–440. https://doi.org/10.1016/S0969-2126(00)00122-2
- Vijverberg, H.P.M., Van der Zalm, J.M., van den Bercken, J., 1982. Similar mode of action of pyrethroids and DDT on sodium channel gating in myelinated nerves. Nature. 295, 601–603. https://doi.org/10.1038/295601a0
- Villacis-Perez, E., Xue, W., Vandenhole, M., De Beer, B., Dermauw, W., Van Leeuwen, T., 2022. Intraspecific diversity in the mechanisms underlying abamectin resistance in a cosmopolitan pest. bioRxiv 2022.11.25.517948. https://doi.org/10.1101/2022.11.25.517948
- Villarroel, C.A., Jonckheere, W., Alba, J.M., Glas, J.J., Dermauw, W., Haring, M.A., Van Leeuwen, T., Schuurink, R.C., Kant, M.R., 2016. Salivary proteins of spider mites suppress defenses in *Nicotiana benthamiana* and promote mite reproduction. Plant J. 86, 119–131. https://doi.org/10.1111/tpj.13152
- Von Lintig, J., Wyss, A., 2001. Molecular Analysis of Vitamin A Formation: Cloning and Characterization of β-Carotene 15,15'-Dioxygenases. Arch. Biochem. Biophys. 385, 47–52. https://doi.org/10.1006/ABBI.2000.2096
- Wadke, N., Kandasamy, D., Vogel, H., Lah, L., Wingfield, B.D., Paetz, C., Wright, L.P., Gershenzon, J., Hammerbacher, A., 2016. The Bark-Beetle-Associated Fungus, *Endoconidiophora polonica*, utilizes the Phenolic Defense Compounds of Its Host as a Carbon Source. Plant Physiol. 171, 914–931. https://doi.org/10.1104/PP.15.01916
- Wallberg, A., Glémin, S., Webster, M.T., 2015. Extreme Recombination Frequencies Shape Genome Variation and Evolution in the Honeybee, *Apis mellifera*. PLoS Genet 11, e1005189-.
- Wan, Y., Wang, M., Chan, E.W.C., Chen, S., 2021. Membrane Transporters of the Major Facilitator Superfamily Are Essential for Long-Term Maintenance of Phenotypic Tolerance to Multiple Antibiotics in *E. coli*. Microbiol. Spectr. 9, e0184621. https://doi.org/10.1128/spectrum.01846-21

- Wang, C., Hawthorne, D., Qin, Y., Pan, X., Li, Z., Zhu, S., 2017. Impact of climate and host availability on future distribution of Colorado potato beetle. Sci. Rep. 7, 4489. https://doi.org/10.1038/s41598-017-04607-7
- Wang, C., Wang, X., Jin, Z., Müller, C., Pugh, T.A.M., Chen, A., Wang, T., Huang, L., Zhang, Y., Li, L.X.Z., Piao, S., 2022. Occurrence of crop pests and diseases has largely increased in China since 1970. Nat. Food. 3, 57– 65. https://doi.org/10.1038/s43016-021-00428-0
- Warsaba, R., Sadasivan, J., Jan, E., 2019. Dicistrovirus Host Molecular Interactions. Curr. Issues Mol. Biol. 34, 83–112. https://doi.org/https://doi.org/10.21775/cimb.034.083
- Wei, P., Chen, M., Nan, C., Feng, K., Shen, G., Cheng, J., He, L., 2019a. Downregulation of carboxylesterase contributes to cyflumetofen resistance in *Tetranychus cinnabarinus* (Boisduval). Pest Manag. Sci. 75, 2166– 2173. https://doi.org/10.1002/ps.5339
- Wei, P., Demaeght, P., De Schutter, K., Grigoraki, L., Labropoulou, V., Riga, M., Vontas, J., Nauen, R., Dermauw, W., Van Leeuwen, T., 2020. Overexpression of an alternative allele of carboxyl/choline esterase 4 (CCE04) of *Tetranychus urticae* is associated with high levels of resistance to the keto-enol acaricide spirodiclofen. Pest Manag. Sci. 76, 1142–1153. https://doi.org/10.1002/ps.5627
- Wei, P., Li, J., Liu, X., Nan, C., Shi, L., Zhang, Y., Li, C., He, L., 2019b. Functional analysis of four upregulated carboxylesterase genes associated with fenpropathrin resistance in *Tetranychus cinnabarinus* (Boisduval). Pest Manag. Sci. 75, 252–261. https://doi.org/10.1002/ps.5109
- Wells, T., Ragauskas, A.J., 2012. Biotechnological opportunities with the β-ketoadipate pathway. Trends Biotechnol. Trends. Biotechnol. 30, 627-37. https://doi.org/10.1016/j.tibtech.2012.09.008
- Wheelock, C.E., Shan, G., Ottea, J., 2005. Overview of carboxylesterases and their role in the metabolism of insecticides. J. Pestic. Sci. 30, 75–83. https://doi.org/10.1584/jpestics.30.75
- Wickham, H., 2009. Ggplot2. Elegant Graphics for Data Analysis. https://doi.org/10.1007/978-0-387-98141-3
- Williams, T.N., Mwangi, T.W., Wambua, S., Peto, T.E.A., Weatherall, D.J., Gupta, S., Recker, M., Penman, B.S., Uyoga, S., Macharia, A., Mwacharo, J.K., Snow, R.W., Marsh, K., 2005. Negative epistasis between the malaria-protective effects of α+-thalassemia and the sickle cell trait. Nat. Genet. 37, 1253–1257. https://doi.org/10.1038/ng1660
- WHO 1957. Expert Committee on Insecticides: seventh report. Switzerland. Retrieved from https://policycommons.net/artifacts/573805/expert-committee-on-insecticides/1552363/ on 06 Feb 2024. CID: 20.500.12592/tf0scs.
- Wolff, J.N., Ladoukakis, E.D., Enríquez, J.A., Dowling, D.K., 2014. Mitonuclear interactions: Evolutionary consequences over multiple biological scales. Philosophical Transactions of the Royal Society B: Biological Sciences. https://doi.org/10.1098/rstb.2013.0443
- Wood, P.M., Hollomon, D.W., 2003. A critical evaluation of the role of alternative oxidase in the performance of strobilurin and related fungicides acting at the Qo site of complex III. Pest Manag. Sci. 59, 499–511. https://doi.org/10.1002/ps.655
- Woolhouse, M.E.J., Webster, J.P., Domingo, E., Charlesworth, B., Levin, B.R., 2002. Biological and biomedical implications of the co-evolution of pathogens and their hosts. Nat. Genet. 32, 569–577. https://doi.org/10.1038/ng1202-569
- Wu, H., Pang, R., Cheng, T., Xue, L., Zeng, H., Lei, T., Chen, M., Wu, S., Ding, Y., Zhang, J., Shi, M., Wu, Q., 2020. Abundant and Diverse RNA Viruses in Insects Revealed by RNA-Seq Analysis: Ecological and Evolutionary Implications. Am. Soc. Microbiol. 5, 1–14. https://doi.org/10.1128/msystems.00039-20
- Wu, M., Adesanya, A.W., Morales, M.A., Walsh, D.B., Lavine, L.C., Lavine, M.D., Zhu, F., 2019. Multiple acaricide resistance and underlying mechanisms in *Tetranychus urticae* on hops. J. Pest Sci. (2004). 92, 543–555. https://doi.org/10.1007/s10340-018-1050-5
- Wu, M., Dong, Y., Zhang, Q., Li, S., Chang, L., Vanessa Loiacono, F., Ruf, S., Zhang, J., Bock, R., 2022. Efficient control of western flower thrips by plastid-mediated RNA interference. Proc. Natl. Acad. Sci. U. S. A. 119,

1-9. https://doi.org/10.1073/pnas.2120081119

- Wybouw N., 2015. The role of horizontally transferred genes in the xenobiotic adaptations of the spider mite *Tetranychus urticae*. PhD thesis, University of Amsterdam, The Netherlands.
- Wybouw, N., Zhurov, V., Martel, C., Bruinsma, K.A., Hendrickx, F., Grbic, V., Van Leeuwen, T., 2015. Adaptation of a polyphagous herbivore to a novel host plant extensively shapes the transcriptome of herbivore and host. Mol Ecol. 24, 4647–4663. https://doi.org/10.1111/mec.13330
- Wybouw, N., Dermauw, W., Tirry, L., Stevens, C., Grbic, M., Feyereisen, R., Van Leeuwen, T., 2014. A gene horizontally transferred from bacteria protects arthropods from host plant cyanide poisoning. Elife. 2014, 1–17. https://doi.org/10.7554/eLife.02365
- Wybouw, N., Kosterlitz, O., Kurlovs, A.H., Bajda, S., Greenhalgh, R., Snoeck, S., Bui, H., Bryon, A., Dermauw, W., Van Leeuwen, T., Clark, R.M., Leeuwen, T. Van, Clark, R.M., 2019a. Long-Term Population Studies Uncover the Genome Structure and Genetic Basis of Xenobiotic and Host Plant Adaptation in the Herbivore *Tetranychus urticae*. Genetics. 211, 1409–1427. https://doi.org/10.1534/geneticS.118.301803
- Wybouw, N., Kurlovs, A.H., Greenhalgh, R., Bryon, A., Kosterlitz, O., Manabe, Y., Osakabe, M., Vontas, J., Clark, R.M., Van Leeuwen, T., 2019b. Convergent evolution of cytochrome P450s underlies independent origins of keto-carotenoid pigmentation in animals. Proc. R. Soc. B. 286. https://doi.org/10.1098/rspb.2019.1039
- Wybouw, N., Pauchet, Y., Heckel, D.G., Leeuwen, T. Van, 2016. Horizontal gene transfer contributes to the evolution of arthropod herbivory. Genome Biol. Evol. 8, 1785–1801. https://doi.org/10.1093/gbe/evw119
- Wybouw, N., Van Leeuwen, T., Dermauw, W., 2018. A massive incorporation of microbial genes into the genome of *Tetranychus urticae*, a polyphagous arthropod herbivore. Insect Mol. Biol. 27, 333–351. https://doi.org/10.1111/imb.12374
- Wybouw, N., Zhurov, V., Martel, C., Bruinsma, K.A., Hendrickx, F., Grbic, V., Van Leeuwen, T., 2015. Adaptation of a polyphagous herbivore to a novel host plant extensively shapes the transcriptome of herbivore and host. Mol. Ecol. 24, 4647–4663. https://doi.org/10.1111/mec.13330
- Xiao, Y., Zhang, T., Liu, C., Heckel, D.G., Li, X., Tabashnik, B.E., Wu, K., 2014. Mis-splicing of the ABCC2 gene linked with Bt toxin resistance in *Helicoverpa armigera*. Sci. Rep. 4, 6184. https://doi.org/10.1038/srep06184
- Xie, M., Zhang, J., Tschaplinski, T.J., Tuskan, G.A., Chen, J.-G., Muchero, W., 2018. Regulation of Lignin Biosynthesis and Its Role in Growth-Defense Tradeoffs. Front. Plant Sci. 0, 1427. https://doi.org/10.3389/FPLS.2018.01427
- Xiong, L., Li, H., Jiang, L.-N., Ge, J.-M., Yang, W.-C., Zhu, X.L., Yang, G.-F., 2017. Structure-Based Discovery of Potential Fungicides as Succinate Ubiquinone Oxidoreductase Inhibitors. J Agric Food Chem. 65, 1021-1029. https://doi.org/10.1021/acs.jafc.6b05134
- Xiong, L., Shen, Y.Q., Jiang, L.N., Zhu, X.L., Yang, W.C., Huang, W., Yang, G.F., 2015. Succinate dehydrogenase: An ideal target for fungicide discovery. ACS Symp. Ser. 1204, 175–194. https://doi.org/10.1021/bk-2015-1204.ch013
- Xu, D., He, Y., Zhang, Y., Xie, W., Wu, Q., Wang, S., 2018. Status of pesticide resistance and associated mutations in the two-spotted spider mite, *Tetranychus urticae*, in China. Pestic. Biochem. Physiol. 150, 89–96. https://doi.org/10.1016/J.PESTBP.2018.07.008
- Xu, X., Chen, J., Xu, H., Li, D., 2014. Role of a major facilitator superfamily transporter in adaptation capacity of *Penicillium funiculosum* under extreme acidic stress. Fungal Genet. Biol. 69, 75–83. https://doi.org/https://doi.org/10.1016/j.fgb.2014.06.002
- Xue, W., Lu, X., Mavridis, K., Vontas, J., Jonckheere, W., Van Leeuwen, T., 2022. The H92R substitution in PSST is a reliable diagnostic biomarker for predicting resistance to mitochondrial electron transport inhibitors of complex I in European populations of *Tetranychus urticae*. Pest Manag. Sci. 78, 3644–3653. https://doi.org/10.1002/ps.7007

Xue, W., Mermans, C., Papapostolou, K.M., Lamprousi, M., Christou, I.K., Inak, E., Douris, V., Vontas, J., Dermauw,

W., Van Leeuwen, T., 2021. Untangling a Gordian knot: the role of a GluCl3 I321T mutation in abamectin resistance in *Tetranychus urticae*. Pest Manag. Sci. 77, 1581–1593. https://doi.org/10.1002/ps.6215

- Xue, W., Snoeck, S., Njiru, C., Inak, E., Dermauw, W., Van Leeuwen, T., 2020. Geographical distribution and molecular insights into abamectin and milbemectin cross-resistance in European field populations of *Tetranychus urticae*. Pest Manag. Sci. 76, 2569–2581. https://doi.org/10.1002/ps.5831
- Yang, C., Pan, H., Liu, Y., Zhou, X., 2015. Stably Expressed Housekeeping Genes across Developmental Stages in the Two-Spotted Spider Mite, *Tetranychus urticae*. PLoS One. 10, e0120833. https://doi.org/10.1371/journal.pone.0120833
- Yang, F., Zhang, Y., Huang, Q., Yin, G., Pennerman, K.K., Yu, J., Liu, Z., Li, D., Guo, A., 2015. Analysis of key genes of jasmonic acid mediated signal pathway for defense against insect damages by comparative transcriptome sequencing. Sci. Rep. 5, 16500. https://doi.org/10.1038/srep16500
- Yang, X., Margolies, D.C., Zhu, K.Y., Buschman, L.L., 2001. Host plant-induced changes in detoxification enzymes and susceptibility to pesticides in the twospotted spider mite (acari: tetranychidae). J. Econ. Entomol. 94, 381–387. https://doi.org/10.1603/0022-0493-94.2.381
- Yu, S.J., 1986. Molecular Aspects of Insect-Plant Associations. Springer US. DOI:10.1007/978-1-4613-1865-1
- Zhang, C.Q., Yuan, S.K., Sun, H.Y., Qi, Z.Q., Zhou, M.G., Zhu, G.N., 2007. Sensitivity of *Botrytis cinerea* from vegetable greenhouses to boscalid. Plant Pathol. 56, 646–653. https://doi.org/10.1111/j.1365-3059.2007.01611.x
- Zhang, H., Tian, W., Zhao, J., Jin, L., Yang, J., Liu, C., Yang, Y., Wu, S., Wu, K., Cui, J., Tabashnik, B.E., Wu, Y., 2012. Diverse genetic basis of field-evolved resistance to Bt cotton in cotton bollworm from China. Proc. Natl. Acad. Sci. U. S. A. 109, 10275–10280. https://doi.org/10.1073/pnas.1200156109
- Zhang, J., Dean, A.M., Brunet, F., Long, M., 2004. Evolving protein functional diversity in new genes of *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 101, 16246–16250. https://doi.org/10.1073/pnas.0407066101
- Zhang, Y., Gao, Y., Ye, W., Peng, Y., Zhu, K.Y., Gao, C., 2023. CRISPR/Cas9-mediated knockout of NICYP6CS1 gene reveals its role in detoxification of insecticides in *Nilaparvata lugens* (Hemiptera: Delphacidae). Pest Manag Sci. 79,2239-2246
- Zhang, Y., Meng, X., Yang, Y., Li, H., Wang, X., Yang, B., Zhang, J., Li, C., Millar, N.S., Liu, Z., 2016. Synergistic and compensatory effects of two point mutations conferring target-site resistance to fipronil in the insect GABA receptor RDL. Sci. Rep. 6, 32335. https://doi.org/10.1038/srep32335
- Zhang, Yan, Xu, D., Zhang, Youjun, Wu, Q., Xie, W., Guo, Z., Wang, S., 2021. Frequencies and mechanisms of pesticide resistance in *Tetranychus urticae* field populations in China. Insect Sci. 29, 827-839. https://doi.org/10.1111/1744-7917.12957
- Zhou, C., Cheng, J., Beadle, R., Earley, F.G., Li, Z., Maienfisch, P., 2020. Design, synthesis and acaricidal activities of Cyflumetofen analogues based on carbon-silicon isosteric replacement. Bioorganic Med. Chem. 28, 115509. https://doi.org/10.1016/j.bmc.2020.115509
- Zhu, B., Lou, M.M., Xie, G.L., Zhang, G.Q., Zhou, X.P., Li, B., Jin, G.L., 2011. Horizontal gene transfer in silkworm, Bombyx mori. BMC Genomics. 12, 248. https://doi.org/10.1186/1471-2164-12-248
- Zhu, F., Moural, T.W., Nelson, D.R., Palli, S.R., 2016. A specialist herbivore pest adaptation to xenobiotics through up-regulation of multiple Cytochrome P450s. Sci. Rep. 6, 20421. https://doi.org/10.1038/srep20421
- Zhu, F., Parthasarathy, R., Bai, H., Woithe, K., Kaussmann, M., Nauen, R., Harrison, D.A., Palli, S.R., 2010. A brainspecific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of *Tribolium castaneum*. Proc. Natl. Acad. Sci. U. S. A. 107, 8557–8562. https://doi.org/10.1073/pnas.1000059107
- Zhurov, V., Navarro, M., Bruinsma, K.A., Arbona, V., Santamaria, M.E., Cazaux, M., Wybouw, N., Osborne, E.J., Ens, C., Rioja, C., Vermeirssen, V., Rubio-Somoza, I., Krishna, P., Diaz, I., Schmid, M., Gómez-Cadenas, A., Van de Peer, Y., Grbić, M., Clark, R.M., Van Leeuwen, T., Grbić, V., 2014. Reciprocal Responses in the
Interaction between Arabidopsis and the Cell-Content-Feeding Chelicerate Herbivore Spider Mite. Plant Physiol. 164, 384–399. https://doi.org/10.1104/pp.113.231555

Zimmer, C.T., Garrood, W.T., Puinean, A.M., Eckel-Zimmer, M., Williamson, M.S., Davies, T.G.E., Bass, C., 2016. A CRISPR/Cas9 mediated point mutation in the alpha 6 subunit of the nicotinic acetylcholine receptor confers resistance to spinosad in *Drosophila melanogaster*. Insect Biochem. Mol. Biol. 73, 62. https://doi.org/10.1016/J.IBMB.2016.04.007

Summary

Crop pests continue to pose a threat to global food security due to the damage they cause on cultivated crops. Therefore, **In chapter 1**, I briefly outlined how agricultural intensification, selection of elite cultivars, pesticide use, international trade and climate change have increased vulnerability of modern agro-ecosystems to pests. I then focus on arthropod herbivores, which are the second most damaging pests after fungi, briefly exploring the feeding modes of various arthropod herbivores, their host plant utilization and interaction with the host plant. The use of chemicals, which is the major control method for arthropod pests is then introduced, together with the resistance problem that comes along with pesticide use. After exploring the various resistance mechanisms to plant allelochemicals and pesticides (collectively called xenobiotics), the pest of focus is introduced. The two spotted spider mite *Tetranychus urticae* is one of the most polyphagous members of Tetranychidae family (Arthropoda: Chelicerata: Acariformes) and also ranks among the top ten most resistant arthropod herbivores. This PhD was focused on understanding the molecular genetics of *T. urticae* that underlie resistance to xenobiotics.

In **chapter 2**, we focused on resistance to one of the newest MoA, mitochondrial complex II inhibitors (succinate dehydrogenase (SDH) inhibitors, IRAC Group 25). We screened the subunits of succinate dehydrogenase gene in a *T. urticae* laboratory strain resistant to pyflubumide, and uncovered a non-synonymous mutation H258Y. This mutation is located in a highly conserved region of the ubiquinone binding site. Using various techniques such as back-crossing to a susceptible *T. urticae* strain, toxicity bioassays, *in vitro* assays with mitochondria extracts, homology modeling and ligand docking, we were able to characterize H258Y and validate its involvement in resistance to SDH inhibitors. Our study revealed that H258Y results in loss of an important H-bond required for cyenopyrafen and pyflubumide binding, thus causing high cross-resistance to these two acaricides. The same mutation however enhances cyflumetofen binding by increasing hydrophobic interactions with the introduced tyrosine in the binding pocket, thus increasing cyflumetofen toxicity in the resistant strain.

In **chapter 3**, we combined various approaches to investigate whether H258Y mutation (described in chapter 2) results in any fitness costs of the resistant populations. Although the classical fertility life table analysis did not reveal any fitness costs, experimental evolution of 50:50 mixed populations of susceptible and resistant populations revealed that allele frequency of the resistant genotype (Y258) decreases steadily over a period of six months without acaricide selection, suggesting a fitness cost. In vitro assays confirmed presence of a fitness cost, revealing that H258Y significantly decreases the catalytic activity of complex II.

In **chapter 4**, resistance mechanisms in a resistant field population of *T. urticae* were investigated. The population, VR-BE, was collected from a tomato green house in Belgium where the grower had reported very low efficacy after treatment with various acaricides, resulting in crop failure. We first confirmed resistance to 12 compounds of different MoA groups. Screening for target site mutations revealed three fixed mutations (I1017F in the chitin synthase I gene, H92R in the PSST homologue of mitochondria complex I and V89A in the ATP synthase gene) and a segregating mutation (I321T in Glutamate gated chloride channel subunit 3). Further, lower toxicity values were recorded with some acaricides on the VR-BE population maintained on its original host tomato compared to the population maintained on the standard laboratory host bean, suggesting that the host plant influences acaricide toxicity. This hypothesis was supported by gene expression analysis on the populations on both hosts, which revealed a remarkable differential expression of detoxification genes, especially P450s. Synergism assays further pointed to increased metabolic detoxification as an additional resistance mechanism, and for complex II inhibitors, the differences in resistance levels on bean and tomato is associated with P450 metabolism.

In **chapter 5**, we used a multi-disciplinary approach to functionally characterize laterally acquired *T*. *urticae DOG* genes. First, we carried out a phylogenetic reconstruction to understand the evolutionary history of spider mite *DOGs*. Then, using three strains of *T*. *urticae* that respond differently to jasmonic acid (JA) defense (a JA inducer-resistant strain, a JA suppressor strain and a JA susceptible strain), we established that *DOG* expression is induced in response to jasmonic acid signaling. By *in situ* hybridization, we showed that the expression of DOGs is localized to the gut, suggesting a role in detoxification of xenobiotics. The presumed role in detoxification was further supported by reduced mite survival rate on tomato upon silencing of some *DOG* genes found upregulated upon *T*. *urticae*'s adaptation to tomato. Interestingly, recombinant DOGs were able to cleave a wide array of aromatic plant metabolites, including known tomato defense compounds such as caffeic acid and chlorogenic acid.

In **chapter 6**, the findings from the four experimental chapters were integrated and discussed in the light of advances made in understanding complex resistance mechanisms, exploring the fitness costs associated with resistance and the connection between host plant adaptation and pesticide resistance. Potential resistance and pest management strategies were also explored.

Samenvatting

Gewasplagen blijven een bedreiging vormen voor de wereldwijde voedselzekerheid vanwege de schade die ze aanrichten aan gecultiveerde gewassen. Daarom heb ik **in hoofdstuk 1** kort geschetst hoe intensivering van de landbouw, selectie van elite cultivars, gebruik van pesticiden, internationale handel en klimaatverandering de kwetsbaarheid van moderne agro-ecosystemen voor ongedierte hebben vergroot. Vervolgens concentreer ik me op herbivoren van geleedpotigen, de op een na meest schadelijke plagen na schimmels, en onderzoek ik kort de voedingswijzen van verschillende herbivoren van geleedpotigen, hun waardplantgebruik en interactie met de waardplant. Het gebruik van chemicaliën, de belangrijkste bestrijdingsmethode voor geleedpotige plagen, wordt vervolgens geïntroduceerd, samen met het resistentieprobleem dat gepaard gaat met het gebruik van pesticiden. Na het verkennen van de verschillende resistentiemechanismen tegen plant-allelochemicaliën en pesticiden (gezamenlijk xenobiotica genoemd), wordt de plaag van focus geïntroduceerd. De spintmijt *Tetranychus urticae* is een van de meest polyfage leden van de Tetranychidae-familie (Arthropoda: Chelicerata: Acariformes) en behoort ook tot de top tien van meest resistente geleedpotige herbivoren. Dit doctoraat was gericht op het begrijpen van de moleculaire genetica van *T. urticae* die ten grondslag ligt aan resistentie tegen xenobiotica.

In **hoofdstuk 2** hebben we ons gericht op resistentie tegen een van de nieuwste MoA, mitochondriaal complex II-remmers (succinaatdehydrogenase (SDH)-remmers, IRAC Groep 25). We hebben de subeenheden van het succinaatdehydrogenasegen gescreend in een laboratoriumstam van *T. urticae* die resistent is tegen pyflubumide, en hebben een niet-synonieme mutatie H258Y ontdekt. Deze mutatie bevindt zich in een sterk geconserveerd gebied van de ubiquinon-bindingsplaats. Met behulp van verschillende technieken, zoals terugkruising naar een vatbare *T. urticae*- stam, toxiciteitsbioassays, *in vitro* assays met mitochondria-extracten, homologiemodellering en ligand-docking, waren we in staat om H258Y te karakteriseren en zijn betrokkenheid bij resistentie tegen SDH-remmers te valideren. Onze studie onthulde dat H258Y resulteert in het verlies van een belangrijke H-binding die nodig is voor de binding van cyenopyrafen en pyflubumide, waardoor een hoge kruisresistentie tegen deze twee acariciden ontstaat. Dezelfde mutatie versterkt echter de binding van cyflumetofen door de hydrofobe interacties met het geïntroduceerde tyrosine in de bindingsholte te verhogen, waardoor de toxiciteit van cyflumetofen in de resistente stam toeneemt.

In **hoofdstuk 3** hebben we verschillende benaderingen gecombineerd om te onderzoeken of H258Ymutatie (beschreven in hoofdstuk 2) resulteert in fitnesskosten van de resistente populaties. Hoewel de klassieke analyse van de overlevingstafel voor vruchtbaarheid geen fitnesskosten aan het licht bracht, onthulde experimentele evolutie van 50:50 gemengde populaties van vatbare en resistente populaties dat de allelfrequentie van het resistente genotype (Y258) gestaag afneemt over een periode van zes maanden zonder acaricideselectie, suggereert fitnesskosten. In-vitrotesten bevestigden de aanwezigheid van fitnesskosten, waaruit bleek dat H258Y de katalytische activiteit van complex II aanzienlijk verlaagt.

In **hoofdstuk 4** werden resistentiemechanismen in een resistente veldpopulatie van *T. urticae* onderzocht. De populatie, VR-BE, werd verzameld uit een tomatenkas in België waar de teler een zeer lage werkzaamheid had gemeld na behandeling met verschillende acariciden, resulterend in mislukte oogsten. We hebben eerst de resistentie tegen 12 verbindingen van verschillende MoA-groepen bevestigd. Screening op doelplaatsmutaties onthulde drie vaste mutaties (I1017F in het chitinesynthase I-gen, H92R in de PSST-homoloog van mitochondriëncomplex I en V89A in het ATP-synthasegen) en een segregerende mutatie (I321T in glutamaat-gated chloridekanaalsubeenheid 3). Verder werden lagere toxiciteitswaarden geregistreerd met sommige acariciden op de VR-BE-populatie die op de oorspronkelijke gastheertomaat werd gehouden in vergelijking met de populatie die op de standaard laboratoriumgastboon werd gehouden, wat suggereert dat de waardplant de acaricidetoxiciteit beïnvloedt. Deze hypothese werd ondersteund door genexpressie-analyse van de populaties op beide gastheren, die een opmerkelijke differentiële expressie van ontgiftingsgenen onthulde, vooral P450's. Synergisme-assays wezen verder op verhoogde metabole ontgifting als een bijkomend resistentiemechanisme, en voor complex II-remmers zijn de verschillen in resistentieniveaus op bonen en tomaten geassocieerd met het P450-metabolisme.

In **hoofdstuk 5** gebruikten we een multidisciplinaire aanpak om lateraal verworven *T. urticae* functioneel te karakteriseren. *DOG*- genen. Eerst voerden we een fylogenetische reconstructie uit om de evolutionaire geschiedenis van spintmijthonden te begrijpen . Vervolgens hebben we met behulp van drie stammen van *T. urticae* die verschillend reageren op jasmonzuur (JA) verdediging (een JA-inductor-resistente stam, een JA-suppressorstam en een JA-gevoelige stam) vastgesteld dat DOG*expressie* wordt geïnduceerd als reactie op jasmonzuur. signalering. Door *in situ* hybridisatie hebben we aangetoond dat de expressie van DOG's gelokaliseerd is in de darm, wat een rol suggereert in de ontgifting van xenobiotica. De veronderstelde rol bij ontgifting werd verder ondersteund door een verminderde overlevingskans van mijten op tomaat na het uitschakelen van sommige *DOG-* genen waarvan werd vastgesteld dat ze opgereguleerd waren na aanpassing van *T. urticae aan tomaat*. Interessant is dat recombinante DOG's in staat waren een breed scala aan metabolieten van aromatische planten te splitsen, waaronder bekende tomatenafweerverbindingen zoals cafeïnezuur en chlorogeenzuur.

In **hoofdstuk 6** werden de bevindingen van de vier experimentele hoofdstukken geïntegreerd en besproken in het licht van de vooruitgang die is geboekt in het begrijpen van complexe resistentiemechanismen, het onderzoeken van de fitnesskosten die samenhangen met resistentie en het verband tussen waardplantadaptatie en resistentie tegen pesticiden. Potentiële resistentie- en plaagbestrijdingsstrategieën werden ook onderzocht.

Acknowledgements

My PhD journey which began five years ago is coming to an end. It has been a journey filled with bitter sweet moments, and one that has seen me achieve immense growth, both personally and professionally. It is now time to recognize the people that have made it memorable and worthwhile.

First and foremost, I would like to express my immense gratitude to my promoter Prof. Thomas Van Leeuwen for accepting me as his student and tirelessly guiding me throughout my PhD study. I have benefited a lot from your invaluable guidance, support and advice throughout these years. I would also like to sincerely thank my co-promoter Prof. Nicky Wybouw for his unfailing support, encouragement and contributions throughout my PhD. The both of you have really influenced and helped to shape me as a scientist. I truly appreciate you.

A warm appreciation to all the members of my thesis examination committee for the time and effort they dedicated to reading and assessing this thesis .

Special thanks to members of the technical staff, both past (Rik and Agnes) and present (Bjorn, Leen, Stephanie, Lieselot and Machiel). I greatly appreciate your kindness, support and willingness to help throughout the years.

To my dear colleagues: Wannes, Andre, Simon, Sabina, Catherine, Lore, Berdien, Xueping, Wenxin, Peng, Marilou, Laura, Antonio, Sander, Wim and all members of the acarology lab. Thank you very much for your friendliness, cheer and collaborative spirit. I will always cherish the memories of a warm and relaxed working environment that you created. To my awesome office mates Rohit, Berdien and 'lunch buddies' Antonio, Clauvis, Xueping, Trin and Ericmar. You always made the office and lunch breaks so lively with our different cultures. Thank you for all the scientific and casual talks and laughs. I'm looking forward to a reunion sometime in the future.

I also greatly appreciate my international friends outside UGent, with whom we made a home away from home. Dearest Ernest and Suvi, Kate and family, Sheila and family, Albert and family, Peter, Rhoda, Esther, Buju and many more who animated my PhD journey. Thank you for the happy moments away from work. They helped me maintain a healthy work-life balance. Because of you, Belgium has always felt like a second home.

To my family back in Kenya. My dearest dad and mum, thank you for being my role models, and for the sacrifices over the years. Without you, I wouldn't be here today. Thank you for your encouragement, unwavering support and prayers. My siblings (Boniface, Kennedy and Ann Beatrice). I can't begin to imagine how hard it always has been for you to see me off at the airport each time I came for holidays and had to travel back after a few weeks. Thank you for the long phone calls in the evenings and weekends that kept me up to date with family affairs. Now that my PhD is over, we will travel more often for family reunions.

Finally to my love, Daniel. I'm so glad that I met you. Thank you for being my biggest cheerleader and support system. Thank you for always stepping up when I had to work late in the evening or during the weekends. Thank you for the encouragement during those days I felt overwhelmed, for the stability you offered, providing me with the grounding I needed to accomplish this goal. I look forward to creating more memories and accomplishing more goals with you. To our boys (Jayden, Nathan and Adrian). Thank you for believing in mama! Your gentle love, warm hugs, cuddles and giggles always cheered me up after a long day at work. You give me hope and the courage to face anything in this life, and this PhD was just one of those things.

Curriculum vitae

Personal information

Full name: Christine Wakuthii Njiru

Address: Sprietestraat 12, 8792 Desselgem

Date of Birth and place: 24 December 1990, Kirinyaga, Kenya

Telephone: +32465112245

Email: tinahnjiru@gmail.com



Education

PhD Bioscience Engineering (Ghent University, Belgium)
Research topic: Molecular basis and fitness costs of xenobiotic resistance in the polyphagous pest <i>Tetranychus urticae</i>
Inter-university master program of Molecular Biology (KU Leuven, VUB &Universiteit Antwerp)
Research topic: CRISPR/Cas9 Targeted Mutagenesis of Raffinose synthase gene in <i>Phaseolus vulgaris</i>

2010-2013 Bachelor of Technology, Industrial Microbiology and Biotechnology (Technical University of Mombasa, Kenya)

Research topic: Bioprospecting macro-algae for potential industrial dyes

Publications

*Njiru, C, *Vandenhole, M., Jonckheere, W., Wybouw, N., Van Leeuwen, T., 2023. The host plant strongly modulates acaricide resistance levels to mitochondrial complex II inhibitors in a multi-resistant field population of Tetranychus urticae. Pesticide Biochemistry and Physiology. 196:105591.

Njiru, C., Saalwaechter, C., Mavridis, K., Vontas, J., Geibel, S., Wybouw, N., Van Leeuwen, T., 2023. The complex II resistance mutation H258Y in succinate dehydrogenase subunit B causes fitness penalties associated with mitochondrial respiratory deficiency. Pest Management Science. 02 July 2023.

De Beer, B., Vandenhole, M., Njiru, C., Spanoghe, P., Dermauw, W., Van Leeuwen, T., 2022. High-Resolution Genetic Mapping Combined with Transcriptome Profiling Reveals That Both Target-Site Resistance and Increased Detoxification Confer Resistance to the Pyrethroid Bifenthrin in the Spider Mite Tetranychus urticae. Biology (Basel). 11: 1630.

Njiru, C., Saalwaechter, C., Gutbrod, O., Geibel, S., Wybouw, N., Van Leeuwen, T., 2022. A H258Y mutation in subunit B of the succinate dehydrogenase complex of the spider mite Tetranychus urticae confers resistance to cyenopyrafen and pyflubumide, but likely reinforces cyflumetofen binding and toxicity. Insect Biochemistry and Molecular Biology. 144: 103761.

Njiru, C., Xue, W., De Rouck, S., Alba, J.M., Kant, M.R., Chruszcz, M., Vanholme, B., Dermauw, W., Wybouw, N., Van Leeuwen, T., 2022. Intradiol ring cleavage dioxygenases from herbivorous spider mites as a new detoxification enzyme family in animals. BMC Biology. 20: 1–23.

Xue, W., Snoeck, S., <u>Njiru, C.</u>, Inak, E., Dermauw, W., Van Leeuwen, T., 2020. Geographical distribution and molecular insights into abamectin and milbemectin cross-resistance in European field populations of *Tetranychus urticae*. Pest Management Science. 76: 2569–2581.

Christine, W.N., Huxley, M.M., John, M.K., Edwin, O.M., Charles, M.M., 2018. Bio-prospecting of macro-algae for potential industrial dyes. African Journal of Biotechnology. 17: 804–810.

Oral* and poster presentations

2023 74th International Symposium on Crop protection (Ghent, Belgium)

2021* 1st international Biotechnology Webinar series (Visayas State University, Philippines)

Awards

2016-2018 VLIR-UOS Scholarship to study Inter-university master program of Molecular Biology in Belgium.
2014-2016 Government of Kenya Research Endowment fund through the National commission for Science Technology and Innovation (NACOSTI). Grant No. NACOSTI/RCD/ST&I 5th call INNOV/068.

Other academic services

2019-2021 Undergraduate mentoring (Ghent University). Students mentored: Jeff Van Dingenen, Jacob Van De Weghe and Joppe De Vriese