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Characterization of insecticide resistance in Hemipteran crop pests with special reference to aphids and whiteflies

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Für meine Eltern,
meine Schwester
und Niklas

Abstract

Agriculture faces enormous challenges: In addition to the increasing demand for agricultural products with less available agricultural landscapes, there is a need to protect the environment and biodiversity and to minimize climatic emissions. In addition, agricultural food production is challenged by biotic factors as weed, pathogens and insect pests. The green peach aphid *Myzus persicae*, the pea aphid *Acyrthosiphon pisum* and the cabbage whitefly *Aleyrodes proletella* are among the most economically important insect pests worldwide. The one-sided usage of chemical insecticides is often causing insecticide resistance. In the present work, not yet documented insecticide resistance cases of the three pest species could be observed. By bioassays as well as molecular and biochemical diagnostic analyzes, resistance mechanisms were investigated to contribute to a successful integrated pest management.

In the green peach aphid, a 403-fold increase in tolerance to the 'transient receptor potential vanilloid' (TRPV) channel modulator pymetrozine was observed. In two comparative RNA sequencing approaches using Illumina and 'Oxford Nanopore Technology' (ONT) as well as amplicon sequencing using ONT, no mutations in the TRPV channel were detected. In contrast, overexpressed esterases FE4 and E4 as well as cytochrome P450s monooxygenases (CYP, P450s), were uncovered in the pymetrozine resistant strain. In other insects as the cotton whitefly *Bemisia tabaci*, overexpressed P450s have already led to pymetrozine resistance. The influence of overexpressed esterases and P450s on pymetrozine resistance in *M. persicae* needs to be elaborated in further studies.

In contrast, a P450 gene, *CYP6CY12*, could be linked to >180-fold pyrethroid resistance in pea aphids *A. pisum*. After detecting no relevant mutations in the sodium channel, the target-site of pyrethroids, an over 30-fold overexpression of *CYP6CY12* was reported by Illumina transcriptome sequencing. UPLC-MS/MS analysis and enzyme kinetic studies provided evidence that deltamethrin is degraded by the protein to its metabolite 4'OH-deltamethrin. In addition to CYP6CY12, three other CYP6CY P450s are already known which have led to insecticide resistance in aphids. For example, CYP6CY3 which caused resistance to nicotine and neonicotinoids in *M. persicae*.

In addition to the demonstrated metabolic resistance in pea aphids, target site resistance to spiromesifen and cross-resistance to spirotetramat were uncovered in *A. proletella*. The A2083V mutation which is located in the CT domain of Acetyl-CoA carboxylase (ACC) was identified by pyrosequencing in several strains from Europe. The mutation already resulted in ketoenol resistance in *B. tabaci*.

In the present work, important insecticide resistance cases were analyzed and uncovered. The elucidation of insecticide resistance mechanisms provides an important contribution to successful integrated pest management.

Zusammenfassung

Die Landwirtschaft steht aktuell vor großen Herausforderungen: Neben dem steigenden Bedarf an Agrarprodukten bei immer weniger zur Verfügung stehenden landwirtschaftlicher Nutzfläche, sind der Schutz der Umwelt und der Biodiversität sowie die Minimierung von klimaschädlichen Emissionen zu bewältigen. Darüber hinaus wird die Produktion von pflanzlichen Erzeugnissen durch zahlreiche biotische Schadfaktoren wie Unkraut, Pathogene und Schadinsekten gefährdet. Unter den tierischen Schaderregern zählen die Grüne Pfirsichblattlaus *Myzus persicae*, die Erbsenblattlaus *Acyrthosiphon pisum* sowie die Kohlmottenschildlaus *Aleyrodes proletella* zu den ökonomisch bedeutendsten Schadinsekten weltweit. Der Einsatz von Insektiziden führt häufig zu Insektizidresistenzen. In der vorliegenden Arbeit, konnten noch nicht dokumentierte Insektizidresistenzfälle der drei Schädlingsarten beobachten werden. Mittels Biotests sowie molekular- und biochemischen Diagnostikanalysen, wurde versucht die Resistenzmechanismen aufzudecken, um zu einem erfolgreichen integrierten Pflanzenschutz beizutragen.

Bei *M. persicae* wurde eine 403-fach erhöhte Verträglichkeit des 'transient receptor potential vanilloid' (TRPV) Kanal Modulators Pymetrozine beobachtet. In zwei vergleichenden RNA-Sequenzierungsansätzen mittels Illumina und 'Oxford Nanopore Technology' (ONT) sowie einer Amplicon-Sequenzierung mit ONT, konnten keine Mutationen im TRPV Kanal nachgewiesen werden. Hingegen wurden überexprimierte Esterasen FE4 und E4 sowie Cytochrom-P450s-Monooxygenasen (CYP, P450s), in dem Pymetrozine resistenten Stamm aufgedeckt. Auch in anderen Insekten, wie der Baumwollmottenschildlaus *Bemisia tabaci*, führten überexprimierte P450s zu einer Pymetrozineresistenz. Der Einfluss von überexprimierten Esterasen und P450s auf die Pymetrozineresistenz bei *M. persicae* muss in weiteren Studien erarbeitet werden.

Nachweislich wurde hingegen ein P450 Gen, *CYP6CY12*, mit einer >180-fachen Pyrethroid-Resistenz bei *A. pisum* in Verbindung gebracht. Nachdem keine relevanten Mutationen im Natriumkanal, dem Wirkungsort von Pyrethroiden, gefunden wurden, wurde eine >30-fache Überexpression von CYP6CY12 mittels Illumina Transkriptom-Sequenzierung analysiert. Eine UPLC-MS/MS Analyse sowie enzymkinetischen Studien erbrachten den Nachweis, dass Deltamethrin durch das Protein zu seinem Metaboliten 4'OH-Deltamethrin abgebaut wird. Neben CYP6CY12, sind bereits drei weitere CYP6CY P450s bekannt, die zu Insektizidresistenzen in Blattläusen geführt haben. Beispielsweise CYP6CY3, welches in *M. persicae* zu einer Resistenz gegenüber Nikotin und Neonicotinoide führte.

Neben der nachgewiesenen metabolischen Resistenz in *A. pisum*, konnte eine Wirkortresistenz (Target-Site) gegenüber Spiromesifen sowie die Kreuzresistenz zu Spirotetramat in der *A. proletella* gefunden werden. Die in der CT-Domäne der Acetyl-CoA

carboxylase (ACC) liegende A2083V Mutation wurde mittels Pyrosequenzierung in mehreren Stämmen aus Europa nachgewiesen. Die genannte Mutation führte bereits zu einer Ketoenolresistenz in *B. tabaci*.

In der vorliegenden Arbeit konnten bedeutsame Insektizidresistenzfälle analysiert und aufgedeckt werden. Die Aufklärung von Resistenzen liefert einen wichtigen Beitrag für einen erfolgreichen integrierten Pflanzenschutz.

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Table of contents

List of abbreviations

ABC	ATP-binding cassette
ACC	Acetyl-CoA carboxylase
ACh	Acetylcholine
AChE	Acetylcholinesterase
APRD	Arthropod Pesticide Resistance Database
ATP	Adenosine-triphosphate
BC	Biotin carboxylase
BCCP	Biotin carboxyl carrier protein
β1	Beta 1
BFC	7-benzyloxy-4-(trifluoromethyl)-coumarin
BLRV	Bean (pea) leaf roll virus
BMEL	Federal Ministry of Food and Agriculture
BOMFC	7-benzyloxymethoxy-4-trifluoromethylcoumarin
BOMR	7-benzyloxymethoxy resorufin
BOR	7-benzyloxyresorufin
bp	Base pairs
CABI	Centre for Agriculture and Bioscience International
cDNA	Complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
CEST	Carboxylesterase
CI	Confidence interval
CNS	Central nervous system
CO_2	Carbon dioxide
CPR	Cytochrome P450 reductase
CRISPR	Clustered regularly interspaced short palindromic repeats
CT	Carboxyltransferase
CYP	Cytochrome P450 monooxygenase
DDT	Dichlorodiphenyltrichloroethane
DEF	S,S,S-tributyl phosphorotrithioate

DEG	Differentially expressed genes
DEM	Diethylmaleate
DNA	Deoxyribonucleic acid
dPCR	Digital PCR
DTT	Dithiothreitol
EC	7-ethoxy coumarin
EDTA	Ethylenediamine tetra-acetic acid
EFC	7-ethoxy-4-trifluoromethyl coumarin
e.g.	Exempli gratia
EPPO	European and Mediterranean Plant Protection Organization
ER	Endoplasmic reticulum
ER	7-ethoxyresorufin
FAD	Flavin adenine dinucleotide
FAO	Food and Agriculture Organization
FC	Fold change
FMN	Flavin mononucleotide
FPKM	Fragments Per Kilobase Million
g	Gram
g	Relative centrifugal force / g-force
+G	Gamma-shape parameter
GABA	Gamma-aminobutyric acid
Gb	Gigabase
GO	Gene ontology
GSH	Glutathione
GST	Glutathione-S-transferase
h	Hour
ha	Hectare
HC	7-hydroy-4-(trifluoromethyl)-coumarin
HeR	Heterozygote resistant
HoR	Homozygote resistant
HoS	Homozygote susceptible

HPLC	High Performance Liquid Chromatography
HPLC-MS/MS	HPLC connected with mass spectrometry
Hz	Hertz
IAV	Inactive
i.e.	id est (that is)
IPPC	International Plant Protection Convention
IPM	Integrated Pest Management
IRAC	Insecticide Resistance Action Committee
IRM	Integrated Resistance Management
JTT	Jones-Taylor-Thornton
kb	Kilobase
kdr	Knockdown resistance
K_m	Michaelis-Menten constant
LC	Lethal concentration
M	Million
M	Molar
MACE	Modified acetylcholinesterase
MC	7-methoxy coumarin
MEAM	Middle Est-Asia Minor
MFC	7-methoxy-4-trifluoromethyl coumarin
mM	Millimolar
mm	Millimeter
µL	Microliter
µM	Micromolar
min	Minute
MoA	Mode of Action
MR	7-methoxyresorufin
mRNA	Messenger RNA
n	Number
nAChR	Nicotinic acetylcholine receptor
NADH	Nicotinamide adenine dinucleotide hydride

NADPH	Nicotinamide adenine dinucleotide phosphate
NAN	Nanchung
NGS	Next generation sequencing
nm	Nanometer
OD	Oil dispersion
OD	Optical density
ONT	Oxford Nanopore Technology
OOMR	Octyloxymethoxyresorufin
OP	Organophosphate
P450	Cytochrome P450 monooxygenase
PBO	Piperonyl butoxide
PC	Principal component
PC	7-n-pentoxy coumarin
PCA	PC analysis
PCR	Polymerase chain reaction
PEMV	Pea enation mosaic virus
PLRV	Potato leaf roll virus
ppm	Parts per million
PR	7-n-pentoxyresorufin
pyrosequencing	Pyrophosphate-based sequencing
Rdl gene	Resistance to dieldrin gene
RNA	Ribonucleic acid
RNAi	RNA interference
RNAseq	RNA-sequencing
RPL12	Ribosomal Protein L12
RR	Resistance ratio
rRNA	Ribosomal RNA
RT-qPCR	Real time quantitative PCR
Ry-R	Ryanodine receptor modulator
s	Second
SC	Suspension concentrate

SD	Standard deviation
SDG	Sustainable Development Goal
SE	Standard error
skdr	Super-*kdr*
SLC protein	Solute carrier protein
SNP	Single nucleotide polymorphism
TRP	Transient receptor potential
TRPV	Transient receptor potential vanilloid
TYLCV	Tomato yellow leaf curl virus
UDP	Uridine diphosphate
UGT	UDP-glycosyltransferase
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry
US	United States
VGSC	Voltage-gated sodium channel
V_{max}	Maximal enzyme velocity at substrate saturation

Chapter 1

Introduction

As the “bedrock of our nutrition” described the Federal Ministry of Food and Agriculture (BMEL) of Germany arable farming (BMEL, 2019). In recent times, agricultural production is more important than ever. In 1979, Knipling stated, that modern agriculture must deal with increased numbers of humans that need to be fed (Handler, 1970; Knipling, 1979). With a 50 % increase of food requirement between 2012 and 2050, there is a demand for greater production with less resources (Alexandratos & Bruinsma, 2012; FAO, 2017). Therefore, food security needs to go along with environmental sustainability. However, increased production of food should not necessitate agricultural expansion. On the contrary, crop yields should be increased on existing farmland, including landscapes that are not performing (Foley et al., 2011). These challenges require new agricultural approaches, such as precision agriculture or the improvement of market infrastructure. However, there are many opportunities, for example the right fertilizer management and nutrient recycling, contributing to an increased balance between agricultural production and environmental sustainability (Foley et al., 2011). In 2015, the United Nations summit defined the “Transforming our world: the 2030 Agenda for Sustainable Development” (United Nations, 2015). The agenda was defined for stakeholders and countries all over the world for sustainability and resilience till 2030. Due to that, 17 Sustainable Development Goals (SDG) were defined. These address the economic, environmental, and social criteria, defined as being the dimensions of sustainable development. Next to gender equality and education for all, the goals aim ending hunger and poverty (United Nations, 2015). In respect to the Covid-19 pandemic, up to 132 million more people were found to suffer from hunger in 2020, meaning an increase of 2 % compared to 2019. Additionally, food loss on farms after harvest, in their transport and storage as well as processes, counted 13.9 %, equivalent to US $ 400 billion per year, worldwide (FAO, 2021). Included are actions to reduce biodiversity losses, land degradation and desertification (United Nations, 2015).

1.1 Crop pests

Climate change is a well-known problem worldwide, making agricultural production more and more difficult. The reduction of food quantity goes hand-in-hand with smaller yields, nutrient values and higher crop plant pest problems (IPPC Secretariat, 2021). Because of increased light due to warmer and sunny days as well as enhanced water amounts caused by meteorological disturbance, it is expected that insects, weeds and pathogens will be harder to control in the future. Climate change affects not only the development and dispersal of

agricultural crop pests, but also the effectiveness of pesticides. This includes their persistence influenced by rainfall as well as temperature and light. Additionally, crops are more stressed due to climate changes, and through that they are more affected by pests (Iglesias & Rosenzweig, 2007). Warmer temperatures influence a faster development of insects. Despite that, insects are cold-blooded and sensitive to climate conditions. This can also influence their longevity which is reduced with higher temperatures. However, more insects are surviving the warmer temperatures in winter times with enhanced development rates (Iglesias & Rosenzweig, 2007). Despite that, drought influences the physiology of plants that can have an impact on the species feeding on them. That, on the other hand, can reduce natural enemies, such as birds. In addition, wet and cool climates are known to facilitate the infestation of plant pathogens and insects (Iglesias & Rosenzweig, 2007).

Crop losses due to arthropod infestations were estimated with 18-20 %, accounting US $ 470 billion, in 2017 worldwide (Sharma et al., 2017). Crop losses are caused by abiotic and biotic factors. Water and nutrient availability, as well as temperatures and irradiation, are environmental circumstances named as abiotic stressors. Examples for biotic factors are crop losses by weeds, pathogens or animal pests. Both, abiotic and biotic factors, are leading to an extreme crop loss. Due to that, pest control with physical, biological or chemical management strategies is of high importance (Oerke, 2006). Oerke et al., (2006), modeled possible scenarios of crop yield with and without crop protection usage. The authors concluded that without crop protection, there would be loss potential of e.g., approximately 75 % losses of potato production. In tandem with this is the prospect of no pesticide usage which would result in decreased yield rates (Oerke, 2006). In addition to plant pathogens, such as viruses, bacteria and fungi, and competitive plants, such as weeds, are animal pest species including mites and insects, challenging agricultural production worldwide (Oerke, 2007; Savary et al., 2019).

1.2 Hemipteran crop pests

Many insect species of the order Hemiptera are known as crop pests and characterized by sucking and piercing mouthparts, as well as hardened forewings. During their development to adults, the insects pass through nymphal stages, being similar to adults in their morphology as well as in their behavior. The order Hemiptera, the true bugs, is split into the Heteroptera and Homoptera suborders (Alford, 2007). The former are two winged predacious, sometimes phytophagous insects. The latter are normally small insects with and without membranous or hardened wings. The phytophagous insects are divided into the Auchenorrhyncha and Sternorrhyncha (= Phytophthires). Their differences lay in the length of the insects' antenna. Species of Auchenorrhyncha have very short ones, whereas the latter are characterized by

their long and small antenna (Alford, 2007). In addition, the name Sternorrhyncha describes the location of the insects' mouthparts. The Sternorrhyncha group comprise 16,000 species of which most members belong to four major groups: Psylloidea and Coccoidea, as well as Aleyrodoidea (superfamily of Aleyrodidae) and Aphidoidea (superfamily of Aphidomorpha) (Gullan & Martin, 2009). Latter are for example aphid species as the green peach aphid *Myzus persicae* or the pea aphid *Acyrthosiphon pisum* (Dixon, 1998). Aleyrodidae are whitefly species as the cotton whitefly *Bemisia tabaci*, the greenhouse whitefly *Trialeurodes vaporariorum* and the cabbage whitefly *Aleyrodes proletella* (Alford, 2007; Brødsgaard & Albajes, 1999).

1.2.1 Aphids (Homoptera: Aphididae)

Aphids are 1-10 mm long (Dixon, 1998) and are known for their variable way of life (Coaker, 1992). The species can develop multiple phenotypes and characteristics – a phenomenon called polyphenism (Brisson et al., 2016). "Polyphenism is a special case of phenotypic plasticity; it refers to the ability of animals with the same genotype to develop two or more distinctly different alternative phenotypes without intermediates. The phenotype that is expressed depends on the environmental conditions that the organism encounters during its development" (Nijhout, 1999). Moreover, there are aphid species that are affecting different host plants during summer and winter life cycle, highlighting the insects' phenotypic flexibility (Brisson et al., 2016). For example, the green peach aphid *M. persicae* Sulzer, with sexual and viviparous reproduction (Fig. 1A and B), can survive as a wingless (apterae) aphid in mild winters (Coaker, 1992). This species can live autoecious or heteroecious as well as holocyclic but also anholocyclic (Leather, 1993). Holocyclic aphid species stay on one host plant for the whole year, as the pea aphid *A. pisum*, the grain aphid *Sitobion avenae* or the cabbage aphid *Brevicoryne brassicae* L. (Coaker, 1992; Leather, 1993). Moreover, the dispersal and reproductive behavior of aphids are an additional characteristic of the insects' polyphenism. Changing environmental conditions can influence the embryonic development of nymphs inside the stem mothers' abdomen. The different phenotypes of the offspring are more adapted to the new environmental conditions (Brisson et al., 2016). For example, nymphs are developing wings for a possible dispersal to other host plants when plants are becoming crowded. Also, the reproduction strategy of the insects can change within the photoperiod length. The species are reproducing in cyclic parthenogenesis, e.g., asexual viviparity of females in summer cycle (Fig. 1A) (Brisson et al., 2016). A parthenogenetic female aphid can reproduce up to 90 embryos in its life. As the pre-oocytes are not stepping in meiosis in the early oogenesis, the pre-oocytes stay diploid and the produced nymphs are genetically identical to its mother (Brisson et al., 2016).

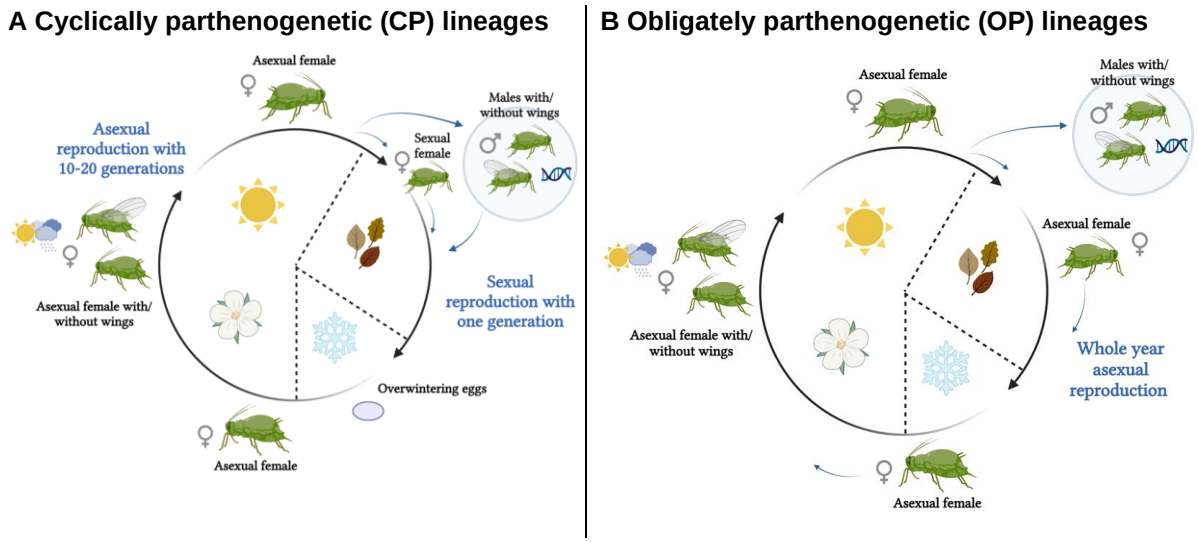

Fig. 1. Pea aphid life cycle. **A:** Pictured is the asexual reproduction in spring and summer cycle, followed by sexual production in autumn and winter cycle. Winged and wingless females can also occur in spring and summer cycle (Created with BioRender.com; modified after Brisson et al., 2016). **B:** The viviparous females produce clones of themselves throughout the year. Only in autumn are males produced, which can be winged or wingless (Created with BioRender.com; modified after Brisson et al., 2016).

These economically important aphid species feed on various plants worldwide (Coaker, 1992; Dixon, 1998). Due to feeding of plant phloem sap, which is rich of plant growth promoters as well as essential food materials, leaves of their hosts are curled, stunted and fall (Alford, 2007; Brødsgaard & Albajes, 1999; Coaker, 1992). Some species are also feeding on flowers, their buds and roots (Brødsgaard & Albajes, 1999). The damage caused by the insects includes not only the direct feeding on the plant, but also the transmission of plant diseases and the production of honeydew (Coaker, 1992; Brødsgaard & Albajes, 1999). While sucking plant sap, aphids often transmit viruses to their hosts. The transfer of permanent or non-permanent viruses can happen rapidly after the infected aphid feeds on the plant (Coaker, 1992). In addition, feeding areas are entry points for plant pathogens (Brødsgaard & Albajes, 1999). Aphids' honeydew contains several sugars, such as glucose, fructose and sucrose, as well as amino acids, especially non-essential amino acids such as glutamine and serine, and secondary plant compounds (Douglas, 1993; Mittler, 1958; van Helden, et al., 1994). The honeydew liquid can stick leaves and sooty molds can easily grow. This reduces not only photosynthesis, but also the optical value of ornamentals and fruits (Brødsgaard & Albajes, 1999).

1.2.1.1 Green peach aphid, *Myzus persicae*

The green peach aphid *M. persicae* is an economically important pest species which causes worldwide crop losses, e.g., from approximately 31 to 100 % (Fig. 2) (Alford, 2007; CABI, 2022d; Sharma et al., 2022). The polyphagous pest infests plants of over 40 families, e.g., peach, nectarine and almonds (Alford, 2007; Blackman & Eastop, 2017). The species is characterized by its green, red or sometimes black color. The insect is known as being genetically variable, pictured for example in their color ranges or its mechanisms to overcome insecticides' toxicity (Blackman & Eastop, 2017). The species' life cycle includes wingless aphids hatched out of eggs laid on Prunus trees such as peach and plum, between February and March. The winged aphids develop in May or June and relocate to its summer hosts. In fall, the green peach aphid migrates back to its primary host, Prunus trees, for sexual reproduction (Alford, 2007; Blackman & Eastop, 2017; Shands et al., 1969). However, the species is also overwintering viviparous, parthenogenetically on winter crops, weed species, brassica and other secondary hosts, e.g., potatoes (Alford, 2007; Margaritopoulos et al., 2002). As a virus vector, green peach aphids are of economic importance. The insect was described in being able to carry and transmit > 100 plant viruses (Blackman & Eastop, 2017). For example, the 'Potato leaf roll virus' (PLRV), which *M. persicae* transmits, can reduce yield of 448.2 g tuber/hill to 175.7 g tuber/hill in 100 % infected tuber (Jeger et al., 1998; Rahman & Akanda, 2010).

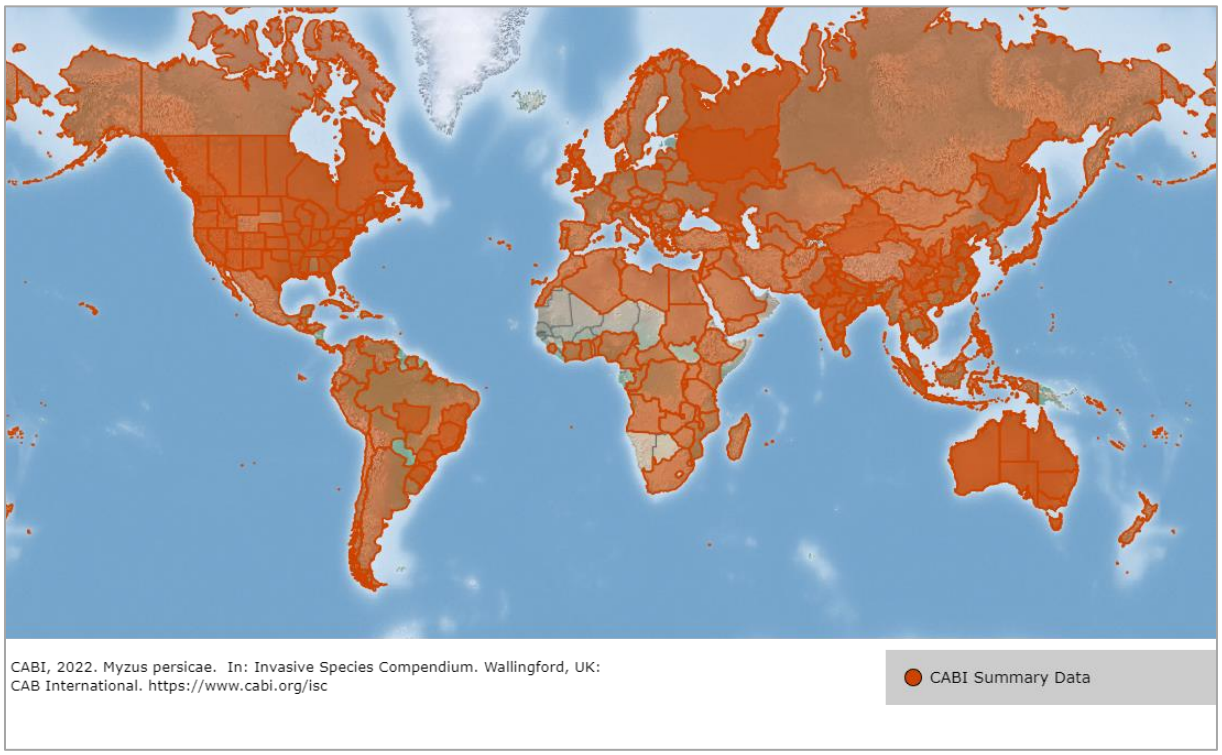

Fig. 2. Worldwide distribution of *Myzus persicae* pictured in orange color (CABI, 2022d).

1.2.1.2 Pea aphid, *Acyrthosiphon pisum*

The worldwide distributed pea aphid, *A. pisum* (Fig. 3) infests leguminous plants such as the economically important bean species *Phaseolus* as well as Faboideae Hedysareae *Hippocrepis* or other species such as Loteae *Lotus* spp. (Blackman & Eastop, 2017; CABI, 2022a). The insect is green or pink colored and one of the larger aphid species (Blackman & Eastop, 2017). The damage of the pest includes the sucking of plant sap and the excretion of honeydew (Kunjwal & Srivastava, 2018). In addition, the insect is known to transmit over 30 persistent and non-persistent viruses to their host plants (Blackman & Eastop, 2017) e.g., the persistent 'Bean (pea) leaf roll virus' (BLRV) is known in causing yield losses of around 80 % in bean cultivations (Blackman & Eastop, 2017; Heathcote & Gibbs, 1962). Another virus, the 'Pea enation mosaic virus' (PEMV), can lower yield by up to 50 % (Heathcote & Gibbs, 1962). The pea aphid life cycle is comparable to other aphid species. After sexual reproduction in fall, the eggs are laid on alfalfa or clover. In spring, the pea aphid population is relocating to pea plants (Kunjwal & Srivastava, 2018). In parthenogenesis, the female aphids produce up to 150 nymphs. From nymph to adult stage, it takes around 12 days. Due to the high reproduction rate, there can be up to 20 generations per year (Kunjwal & Srivastava, 2018).

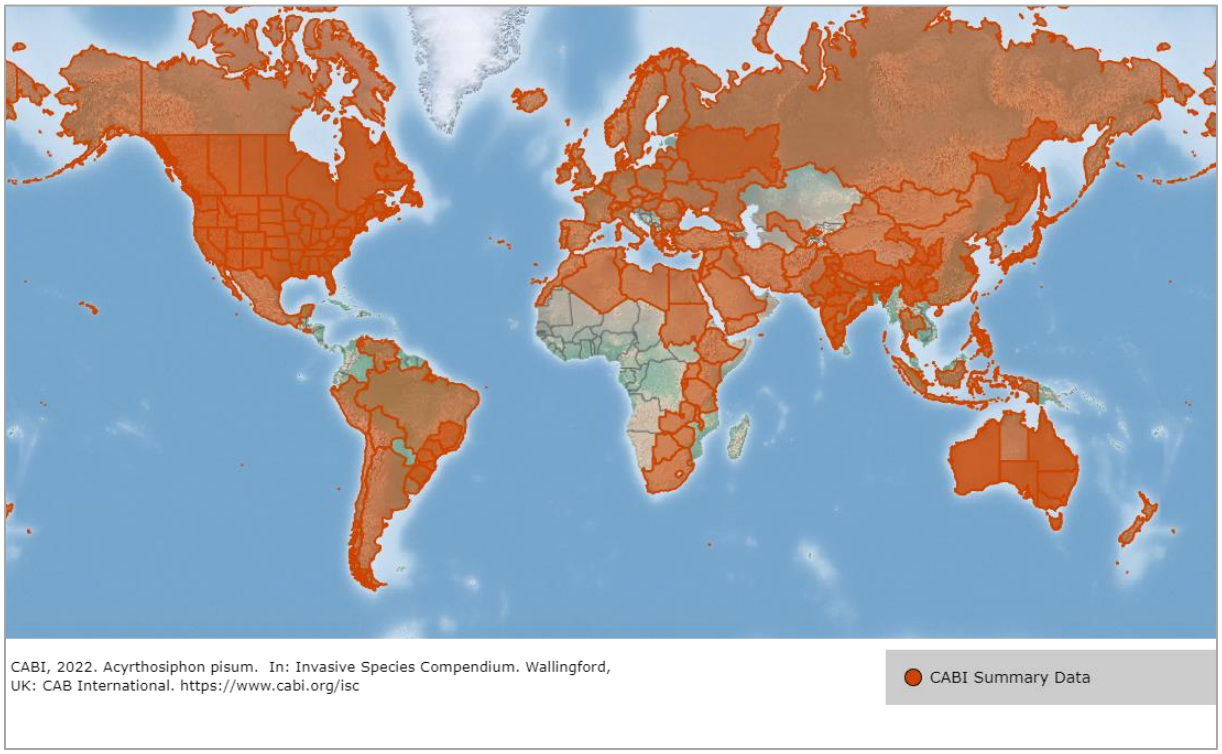

Fig. 3. Worldwide distribution of *Acyrthosiphon pisum* pictured in orange color (CABI, 2022a).

1.2.2 Whitefly species (Hemiptera: Aleyrodidae)

Whitefly species are belonging to the Aleyrodidae Sternorrhyncha and infest agricultural crops, such as beans, tomatoes and cotton (Borror et al., 1989; Thompson, 2011). Around 1,560 whitefly species are described worldwide (Gullan & Martin, 2009) and approximately 85 % of them are oligophagous (Brødsgaard & Albajes, 1999). However, most whitefly pests are polyphagous, as the greenhouse whitefly *T. vaporariorum*, with around 300 host plants (Brødsgaard & Albajes, 1999). Whiteflies undergo five instars, from egg to adult, in their development (Borror et al., 1989, Thompson, 2011). Female insects are diploid and arise out of fertilized eggs, whereas males are haploid and develop from unfertilized eggs (Ghanim & Czosnek, 2016). Adults and nymphs are phloem feeders. Heavily infested plants typically show reduced growth which goes along with leaf fall. In addition, comparable to aphids, excreted honeydew of whitefly adults and nymphs, favors fungal growth such as sooty mold (Brødsgaard & Albajes, 1999). Whiteflies are virus vectors which can cause enormous economic losses, such as the transmission of the 'Tomato yellow leaf curl virus' (TYLCV) (Brødsgaard & Albajes, 1999; Moriones & Verdin, 2020).

1.2.2.1 Greenhouse whitefly, *Trialeurodes vaporariorum*

The greenhouse whitefly *T. vaporariorum* is, like *B. tabaci*, a major polyphagous pest species and is distributed worldwide (Fig. 4) (CABI, 2022e; Capinera, 2001; Gullan & Martin, 2009). It is supposed that the species derived from the southwest of the USA or Mexico. The polyphagous *T. vaporariorum* feeds on over 300 plant species, including bean, cucumber, tomato, eggplant, squash and lettuce. Cabbage, pepper, potato, sweet potato as well as ornamental species are also sometimes infested (Capinera, 2001). Damage of their host can result in field losses (Johnson, et al., 1992). While using their sucking mouthparts, direct damage of plants results in a hosts' leaf loss, yellowing and spotting as well as growth reduction (Capinera, 2001). The excreted honeydew is affecting fruit quality (Capinera, 2001) and is a growth medium for fungi diseases as sooty mold in tomato cultivations (Johnson et al., 1992). The fungus causes damage by its black spots on reducing photosynthesis as well as gas exchange (Capinera, 2001).

The *T. vaporariorum* life cycle takes around one month at a temperature of 18 °C (Fig. 5). With higher temperatures, the development time decreases. For a complete development, a temperature between 15 to 32 °C is required. At 25 °C the eggs need around five days to develop to the first instar (Gamarra et al., 2020). Female whiteflies were reported to lay up to 581 eggs. On average, one female produces 5.7 eggs per day (de Vis & van Lenteren, 2002). After hatching, first instar nymphs, called crawler, bear legs as well as antennae (Gill, 1990

and references cited therein). Due to that, the nymphs are moving till they settle down for feeding (van Roermund & van Lenteren, 1992; Weber, 1931). In the following, second and third development stages, the nymphs are immobile. The last nymph stage, the fourth, is often classified as “pupa”, although the development cannot be compared with holometabolous insects because the resting, non-feeding stage is missing (Gullan & Martin, 2009).

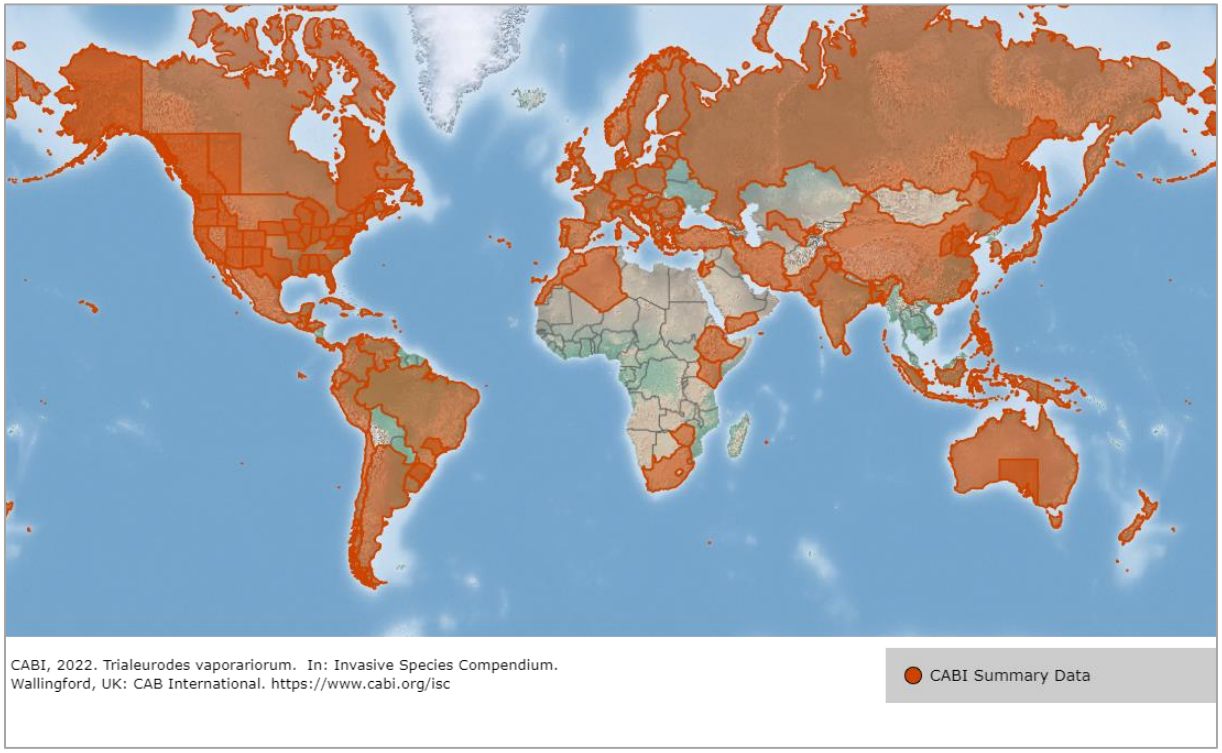

Fig. 4. Worldwide distribution of *Trialeurodes vaporariorum* pictured in orange color (CABI, 2022e).

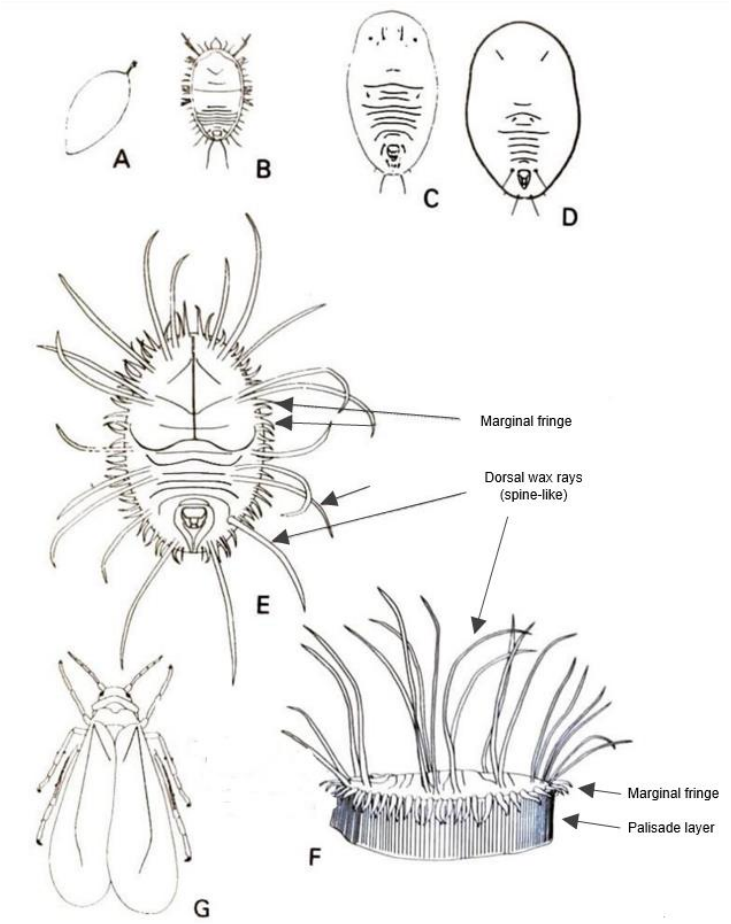

Fig. 5. The life stages of the greenhouse whitefly *Trialeurodes vaporariorum*. Pictured are the egg (A), first instar, also named crawler (B), second instar (C) and third instar (D. The fourth instar and the formed pupae in dorsal view (E) and lateral view (F) as well as the adult stage (G) (modified after: Gill, 1990; Morrill, 1905).

1.2.2.2 Cotton whitefly, *Bemisia tabaci*

The cotton whitefly *B. tabaci* is distributed throughout the world especially in tropical and subtropical climates (Fig. 6) (CABI, 2022c; McKinlay et al., 1992). Due to its ability to adapt quickly to a broad range of regions, the insects have also become a pest problem in the northern hemisphere. The favorite hosts of this species are sweet potato, cotton and tobacco, however, there is a series of additional plants, wild and cultivated, being affected by *B. tabaci* (McKinlay et al., 1992). *B. tabaci* develops within four weeks from egg to adult stage. Females can lay up to 400 eggs (Fig. 7). First instar nymphs are called crawler and are mobile. After settling, the nymph pass through three additional stages, before they pupate in the last, fourth nymphal stage. Adults develop out of the puparium after seven days and live approximately five weeks (Ghanim & Czosnek, 2016).

There are some slight differences reported for all development stages of *T. vaporariorum* and *B. tabaci*. For example, the dark, nearly black eggs of *T. vaporariorum* are laid in circles around their feeding position, whereas the golden-brown eggs of *B. tabaci* are clustered on the leaf surface (Hill, 1969). However, the main differences between both species can be seen in fourth instar nymphs: the “pupa” of *T. vaporariorum* is yellowish-white, non-transparent and covered with white wax as well as waxy filaments. In contrast, the “pupa” of *B. tabaci* is not surrounded by wax and lack waxy filaments. Additionally, differences can be observed in the insects’ adult stages: while both being yellow colored and having four wings covered with white wax, *B. tabaci* adults are darker yellow and smaller as *T. vaporariorum* (Hill, 1969). While resting, *B. tabaci* is holding its wings at an angle, whereas *T. vaporariorum* hold their wings horizontally while resting (Capinera, 2001).

There are many different biotypes of *B. tabaci*. 11 biotypes are known to have different biological characteristics, as for example the host range or the transmission of viruses (Bedford et al., 1994; De Barro et al., 2011). Today it is known that these 11 biotypes are originally from Asia, Mediterranean, New World, Middle East-Asia Minor (MEAM), as well as Sub-Saharan Africa (Boykin et al., 2007; De Barro et al., 2011; Dinsdale et al., 2010). The called biotype MEAM-1, formerly biotype B, includes insects known as silverleaf whiteflies *Bemisia argentifolii* (De Barro et al., 2005; Perring et al., 1993). Today, there are numerous additional biotypes known, causing pest problems all over the world (Perring, 2001). Furthermore, like the other sucking pests, *B. tabaci* are known to transmit viruses to their host plants. The damage is visible in curled, chlorosis affected, leaves. Also, the infested plants show reduced growth as well as stunting (de Sá et al., 2008). For example, one hundred percent of virus losses were reported in the Dominican Republic, resulting in US $ 10 million (Gilbertson et al., 2007).

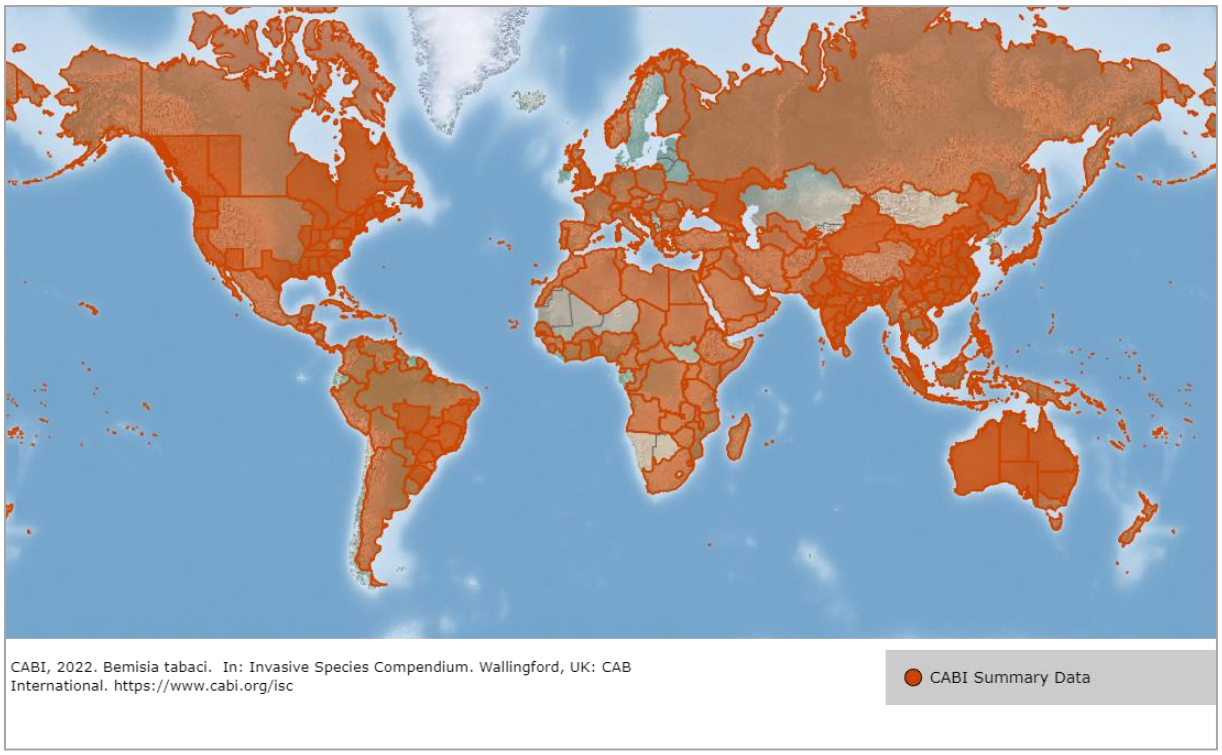

Fig. 6. Worldwide distribution of *Bemisia tabaci* pictured in orange color (CABI, 2022c).

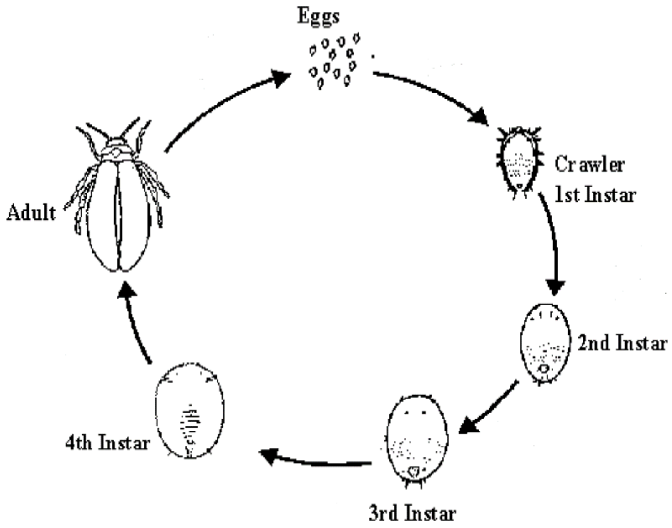

Fig. 7. The life cycle of *Bemisia tabaci*. Modified after Palumbo et al., 2001.

1.2.2.3 Cabbage whitefly, *Aleyrodes proletella*

The cabbage whitefly *A. proletella* is a pest of vegetable brassicas in Europe (Fig. 8) (Alford, 2007; CABI, 2022b; CABI Data Mining, n.d.; CABI, n.d.; Jansen & Ivanova, 2018; Malumphy et al., 2009; Malumphy & Ostrauskas, 2013). The species is also described in Egypt, Turkey, on the Bermuda Islands and in the United States of America as well as in China (CABI, 2022b;

CABI Data Mining, n.d.; Koca & Kütük, 2020; Seebens et al., 2017). There is also a report about its presence in New Zealand and Australia (Dale et al., 1976; De Barro & Carver, 1997; Finch & Thompson, 1992). Hosts of this pest are brassica crops such as late and early cauliflower, broccoli as well as red cabbage (Nebreda et al., 2005). The life cycle of *A. proletella* is similar to the cotton and greenhouse whiteflies. The adults and the laid eggs overwinter on the underside of leaves. As soon as temperature reaches 15 °C, the adults start reproducing and lay their eggs in circles. Nymphal stages develop similar as previously described for *T. vaporariorum* (1.2.2.1). Due to the feeding of plant sap and the excretion of honeydew, the affected leaves begin to build white to yellow patches which reduces the quality of marketable plant material (Finch & Thompson, 1992). Cabbage whiteflies are not considered as virus vectors (Ramsey & Ellis, 1994; Tomlinson et al., 1972).

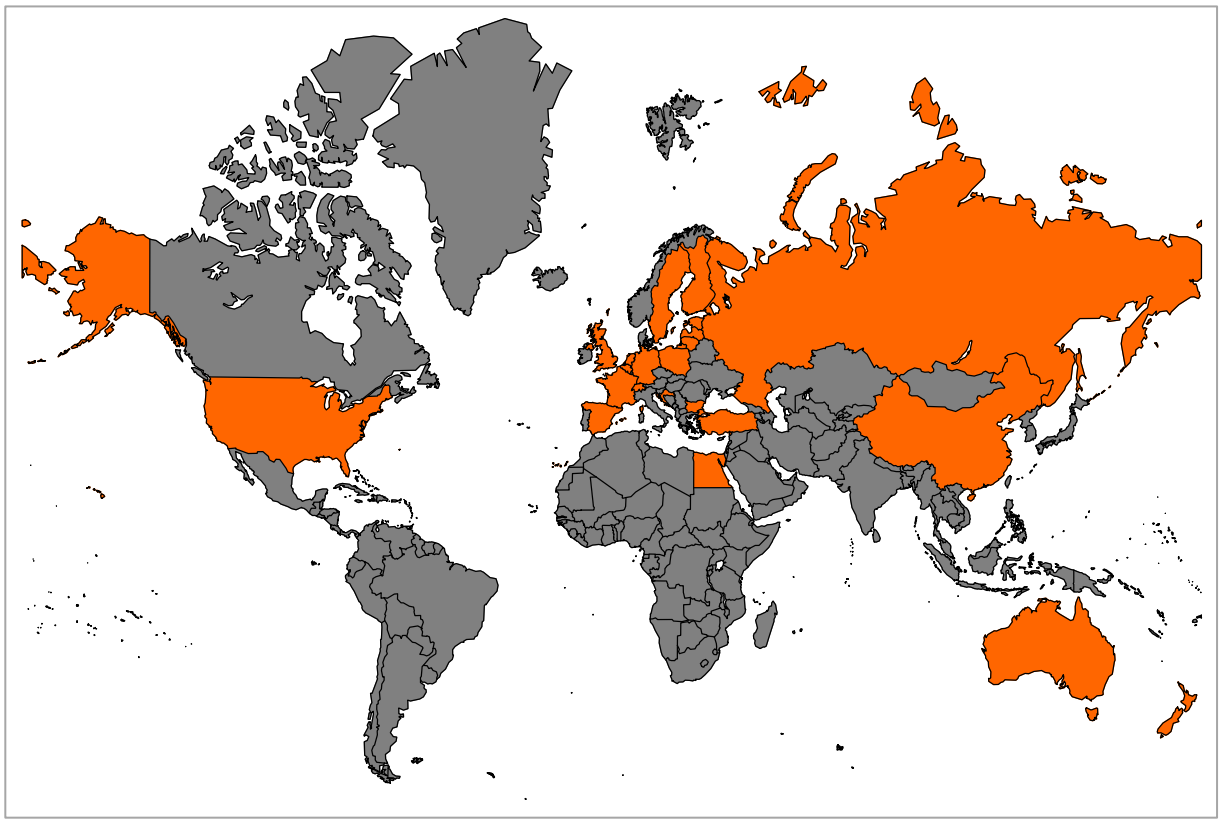

Fig. 8. World distribution of *Aleyrodes proletella* pictured in orange color. The species is distributed in the African continent in Egypt, in Asia in Turkey and China, Australia, New Zealand as well as in North America on the Bermudas and in the United States (CABI, 2022b; CABI Data Mining, n.d.; Dale et al., 1976; De Barro & Carver, 1997; Finch & Thompson, 1992; Koca & Kütük, 2020; Seebens et al., 2017). In European countries, *A. proletella* is present in Germany, Finland, Bulgaria, Sweden, the UK, Estonia, Latvia, Lithuania and the Union of Soviet Socialist Republics (CABI, 2022b; CABI Data Mining, n.d.; CABI, n.d.; Jansen & Ivanova, 2018; Malumphy et al., 2009; Malumphy & Ostrauskas, 2013). Within this study, the species was also found in the Netherlands, Poland, Switzerland, Spain, Belgium, France and Croatia (chapter 4).

1.3 Control mechanisms of Hemipteran crop pests

In general, sucking pest species such as aphids and whiteflies can be controlled by biological and chemical measures, or both strategies are combined in integrated pest management recommendations. The effectiveness of a control method depends on several factors including pest abundance, but also agricultural practices (Knipling, 1979). However, as this thesis is centered around chemical control of devastating sucking pest species and mechanisms of insecticide resistance, an introduction to biological and other control methods is considered out of scope and not described.

1.3.1 Chemical control methods using insecticides

The control of aphids and whiteflies as destructive pests in many settings largely relies on the application of chemical insecticides (Fig. 9A and B) (Cantrell et al., 2012). Most of the active ingredients registered from 1997 to 2010 were of synthetic origin, followed by synthetic natural derivates. Biologicals or natural products were of minor importance (Fig. 9A) (Cantrell et al., 2012). For the control of insects and mites, comparable allocation was found in the market (Fig. 9B) (Cantrell et al., 2012). Conventional insecticides used for plant protection are classified in the ‘Mode of Action’ (MoA) classification scheme of the ‘Insecticide Resistance Action Committee’ (IRAC). The classification by MoA is useful for integrated pest management (IPM) strategies. Also, it provides agricultural costumers, professional pest control specialists, as well as scientists, information about available insecticide and acaricide compounds. The chemical active ingredients are categorized regarding their mode of action and target region (IRAC, 2021a). In 2001, plant protection compounds accounted for US $ 7.56 billion sales. Thirty percent were sold for control of sucking insects (Beckmann & Haack, 2003; and references cited therein). The chemical compounds that are recommended for controlling aphids and whiteflies are listed in table 1 (IRAC, 2018a; IRAC, 2018b; IRAC, 2019a). The “first generation” insecticides were often of natural origin. Compounds of this generation were based on toxicants, which comprise fluorine or arsenic, or of botanicals, such as pyrethrins or nicotine. Since 1940, insecticides of the “second generation” are performing with increased efficacy and reduced costs (Casida, 1980). Today, the main classes of insecticides include the neonicotinoids as well as pyrethroids (Cantrell et al., 2012; Nauen & Bretschneider, 2002). The former is comprising US $ 4,752 million of the insecticidal market, whereas pyrethroids and pyrethrins account US $ 2,978 million (Fig. 10) (Sparks et al., 2020).

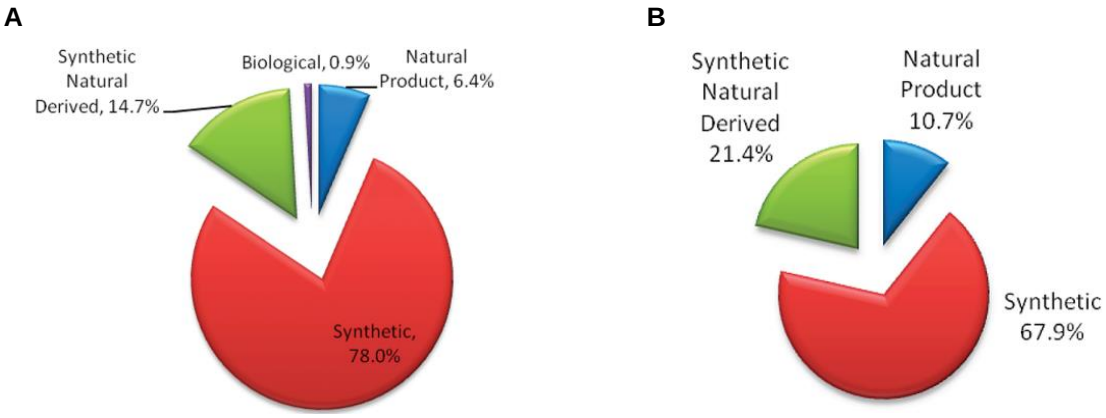

Fig. 9. A: Active ingredients of conventional pesticides registered between 1997 to 2010 (Cantrell et al., 2012). **B:** Active ingredients for control of insects and mites registered between 1997 to 2010 (Cantrell et al., 2012).

Table 1. Chemical classes and actives for a control of aphids and whiteflies (modified after (IRAC, 2018a; IRAC, 2018b; IRAC, 2019a). Abbreviations are named: GABA = y-aminobutyric acid, nAChR = nicotinic acetylcholine receptors, TRPV = transient receptor potential vanilloid, ATP = adenosine triphosphate, Acetyl-CoA = acetyl coenzyme A, CHS1 = chitin synthase 1.

Sucking pest family	IRAC main group	Mode of action	Sub-group	Chemical class or exemplifying active
Aphidomorpha & Aleyrodidae	1	Acetylcholinesterase inhibitors	A	Carbamates
			B	Organophosphates
	2	GABA-gated chloride channel antagonists	A	Endosulfan
	3	Sodium channel modulators	A	Pyrethroids
	4	nAChR competitive modulators	A	Neonicotinoids
			C	Sulfoxaflor
			D	Flupyradifurone
	9	Chordotonal organ TRPV channel modulators	B	Pyridine azomethine derivates
			D	Afidopyropen
	12	Inhibitors of mitochondrial ATP synthase	A	Diafenthiuron
	23	Inhibitors of acetyl-CoA carboxylase	-	Spirotetramat
			-	Spiropidion
	28	Ryanodine receptor modulators	-	Diamides

Sucking pest family	IRAC main group	Mode of action	Sub-group	Chemical class or exemplifying active
Aphidomorpha & Aleyrodidae	29	Chordotonal organ modulators – undefined target-site	-	Flonicamid
Aleyrodidae	7	Juvenile hormone mimics	A	Juvenile hormone analogues
			C	Pyriproxyfen
	15	Inhibitors of chitin biosynthesis affecting CHS1	-	Benzoylureas
	16	Inhibitors of chitin biosynthesis, type 1	-	Buprofezin
	21	Mitochondrial complex I electron transport inhibitors	A	METI acaricides and insecticides
	23	Inhibitors of acetyl-CoA carboxylase	-	Spiromesifen
	UN	Unknown or uncertain mode of action	-	Azadirachtin

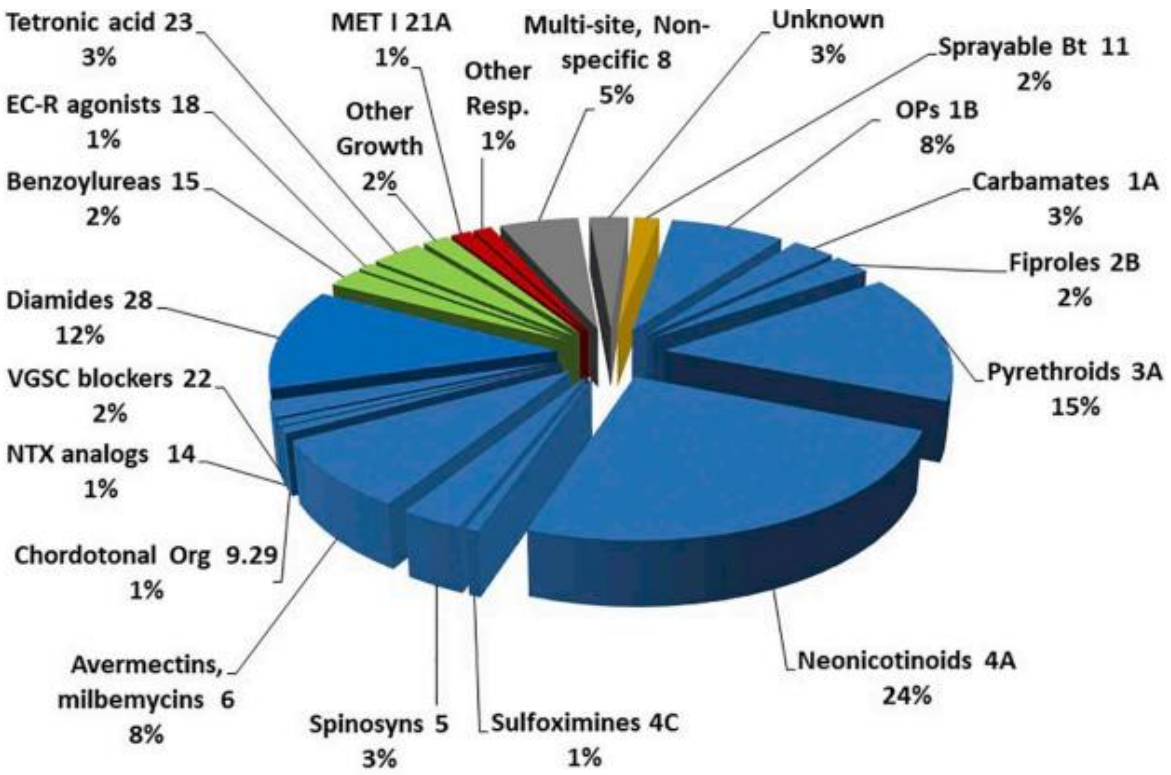

Fig. 10. Sales allocation of insecticides grouped in IRAC Mode of Action (MoA) classes. The listed chemical classes are colored in their different targets: blue = nerve and muscle, green = growth and development, red = respiration, yellow = midgut and gray = non-specific or unknown insect functions. The total end user sales comprised US $ 19.8 billion (Sparks et al., 2020).

1.3.1.1 Sodium channel modulators

Sodium channel modulators target the nerve and muscle function of insects. The compounds are targeting the voltage-gated sodium channel (VGSC) connected to nerve axons (Fig. 11 and 12) (Davies et al., 2007; Urkude et al., 2019). The knockdown is effectuating muscular paralysis which ends in death of the treated insects (Urkude et al., 2019). Sodium channel modulators are classified in group 3 of the IRAC MoA classification. Next to pyrethroids/pyrethrins (sub-group 3A), the class is split into dichlorodiphenyltrichloroethane (DDT) and methoxychlor (sub-group 3B) (Fig. 13) (IRAC, 2021b). Pyrethroids are toxic for sucking and biting insects (e.g., cypermethrin and deltamethrin) as well as mites (e.g., acrinathrin) (Beckmann & Haack, 2003; and references cited therein). The natural pyrethrin is the origin of modern, synthetic pyrethroids and was first extracted from *Chrysanthemum cinerariaefolium*, later called pyrethrum flowers (McLaughlin, 1973). The synthesis of pyrethroids out of the natural pyrethrin led to insecticides with high insecticidal activity, lower costs and enhanced photostability. Furthermore, the synthetic compounds are more selective and less toxic against mammals (Katsuda, 1999). The compounds are widely used not only in agriculture but also for controlling household and public health pests such as mosquitoes (Beckmann & Haack, 2003; Katsuda, 1999; and references cited therein). Pyrethroids are lipophilic and mainly act by contact or feeding (Beckmann & Haack, 2003; and references cited therein). In the 1970's and 80's the "third generation" of pyrethroid insecticides was established. Permethrin, deltamethrin, cypermethrin and fenvalerate are more stable and active as their precursors, such as allethrin or bioallethrin (Dubey & Patyal, 2007). Today, there is a series of pyrethroids or pyrethrins established as well as DDT and Methoxychlor, all included in group 3 of the IRAC MoA classification (Fig. 13) (IRAC, 2021b). DDT is no longer used in agriculture, but there are still cases of its usage in the public health sector, e.g., for the control of malaria-transmitting mosquitoes (Rehwagen, 2006; Yu, 2008b). Pyrethroids are grouped into type I and type II chemical compounds (Yu, 2008b). The formers are lacking an alpha-cyano group, whereas type II pyrethroids contain an alpha-cyano substituent which alters the symptomology of poisoning (Aznar-Alemany & Eljarrat, 2020). In 2019, synthetic pyrethroids accounted for 15 % of the worldwide insecticidal market (Fig. 10) (Sparks et al., 2020).

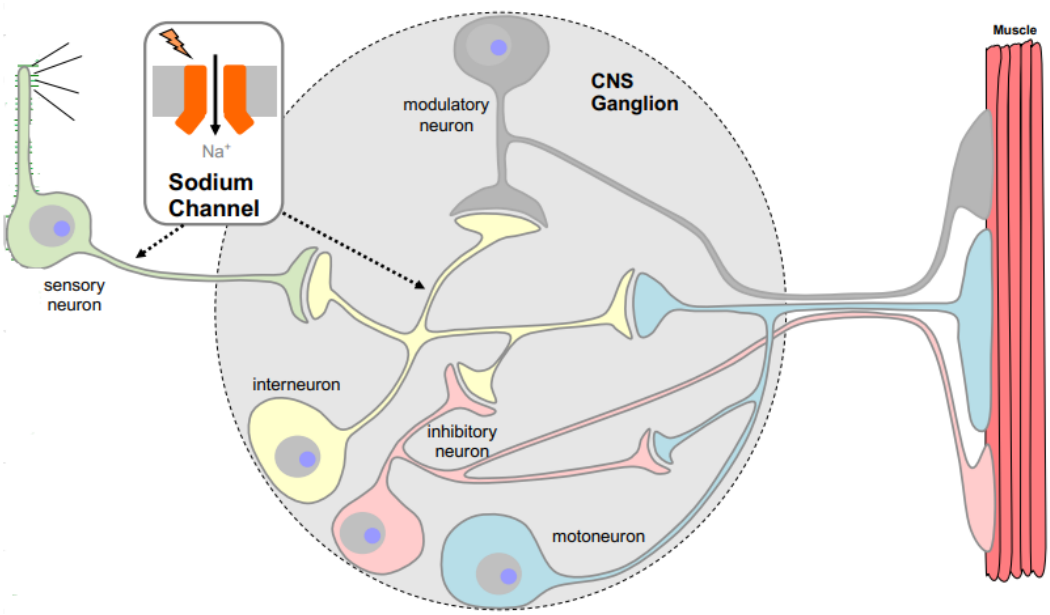

Fig. 11. Localization of voltage-gated sodium channels (VGSC) along the axons of connecting neuronal cells in the insect central nervous system (IRAC, 2019b).

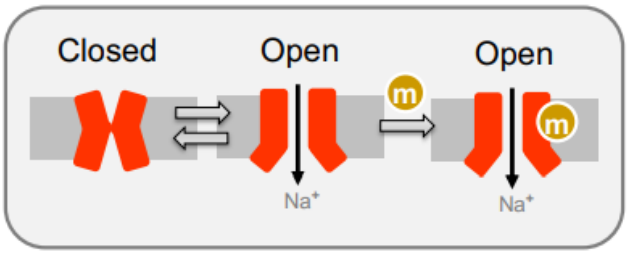

Fig. 12. Mode of action (MoA) of sodium channel modulators, pictured in the orange. Upon binding these compounds keep the sodium channels in an open state which led to a permanent flow of sodium ions into the neuron (IRAC, 2019b).

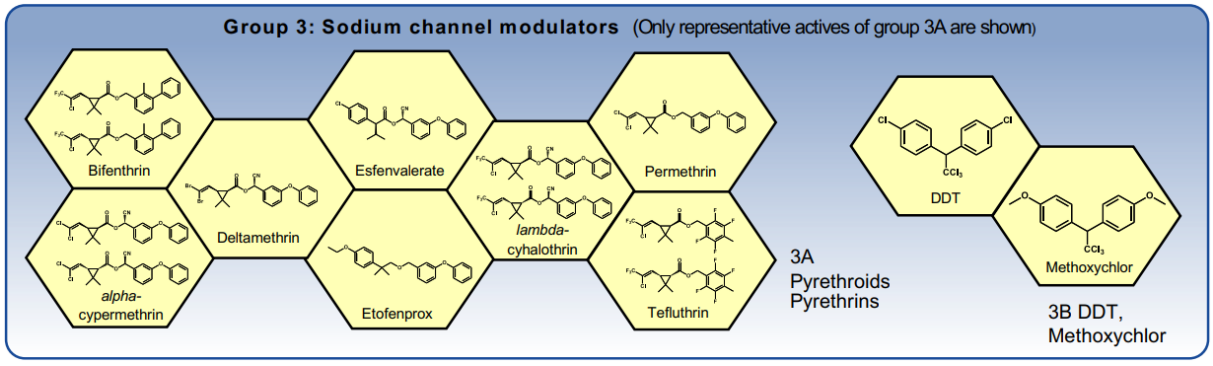

Fig. 13. Classification of sodium channel modulators in group 3 of the IRAC Mode of Action (MoA) classification**.** All sodium channel modulators target nerve and muscle function of pest insects (IRAC, 2021b).

1.3.1.2 Nicotinic acetylcholine receptor competitive modulators

Another important class of insecticides are neonicotinoids, classified as nicotinic acetylcholine receptor (nAChR) competitive modulators (Cantrell et al., 2012; IRAC, 2021b). The insect nAChR is a pentameric ligand-gated ion channel located in the central nervous system of insects and activated by the endogenous excitatory neurotransmitter acetylcholine (ACh) (Fig. 14) (Ihara, 2022; Tomizawa & Casida, 2003; and references cited therein). The neurotransmission is happening when the presynaptic membrane is releasing ACh which binds to the orthosteric site of nAChRs located in the extracellular domain (Fig. 15) (Dubey & Patyal, 2007; IRAC, 2019b; Yu, 2008b). Afterwards, the receptor is undergoing a conformational change, that stimulates the opening of the ion channels. The enzyme acetylcholinesterase hydrolyzes released ACh rapidly to prevent neuronal overstimulation. nAChR competitive modulators are acting as ACh mimics not hydrolyzed by acetylcholinesterase, therefore resulting in continuous nAChR activation (Fig. 15). This leads to overstimulation of the cholinergic synapses which induces excitatory symptoms, paralysis and finally death of targeted pest insects (Dubey & Patyal, 2007; Yu, 2008b).

Neonicotinoids are sub-grouped in the IRAC MoA class 4 along with other, chemically different nAChR modulators, including nicotine, sulfoximines, butenolides, mesoionics and pyridylidenes (Fig. 16) (IRAC, 2021b). Neonicotinoids are by far the most important insecticides globally and bind to the orthosteric site of insect nAChRs (Elbert et al., 2008; Matsuda, 2021; Taly et al., 2009). In contrast to nicotine, there is almost no interaction of neonicotinoids with vertebrate nAChRs (Tomizawa & Casida, 1999; Tomizawa et al., 2000; Tomizawa et al., 2001; Tomizawa & Casida, 2009; Yamamoto & Casida, 1999), thus explaining their benign mammalian toxicological profile compared to nicotine (Yu, 2008b). The first neonicotinoid launched to the market was imidacloprid in 1991 (Jeschke & Nauen, 2008). The insecticide originates from nithiazine, an insecticidal nitromethylene chemical (Dubey & Patyal, 2007). The compound is active against sucking insects, such as aphids and whiteflies (Yu, 2008b). Imidacloprid can be used on a wide range of plants, for example vegetables, cotton, fruits or cereals (Jeschke & Nauen, 2008). Several other neonicotinoid insecticides were derived from imidacloprid, such as the systemic compound acetamiprid, which is used against aphids and whiteflies as well (Dubey & Patyal, 2007; Yu, 2008b). Also, useful against sucking insects are the chemicals thiacloprid, nitenpyram, dinotefuran, thiamethoxam and clothianidin (Yu, 2008b). One advantage of neonicotinoids is the wide application range due to their physicochemical characteristics. They can be applied within a seed treatment, foliar, on stem, or as soil drench (Jeschke et al., 2013).

In 2014, an additional nAChR competitive modulator, the butenolide flupyradifurone, was first registered in Guatemala and Honduras (Central America). The compounds' toxicology safety

profile to mammals and ecosystem, as non-target species, are favorable: e.g., a dosage rate of up to 205 g/ha has not shown any negative effect on honeybees in oilseed rape. Flupyradifurone can be applied as drench, seed treatment, or foliar and is especially active against sucking pest species (Nauen et al., 2015a).

In 2006, neonicotinoid insecticides accounted for 17 % of the insecticide market worldwide, comprising US $ 1.56 billion (Jeschke & Nauen, 2008) Today, its usage has increased to 24 %, meaning the largest market share in 2019 (Fig. 10) (Sparks et al., 2020).

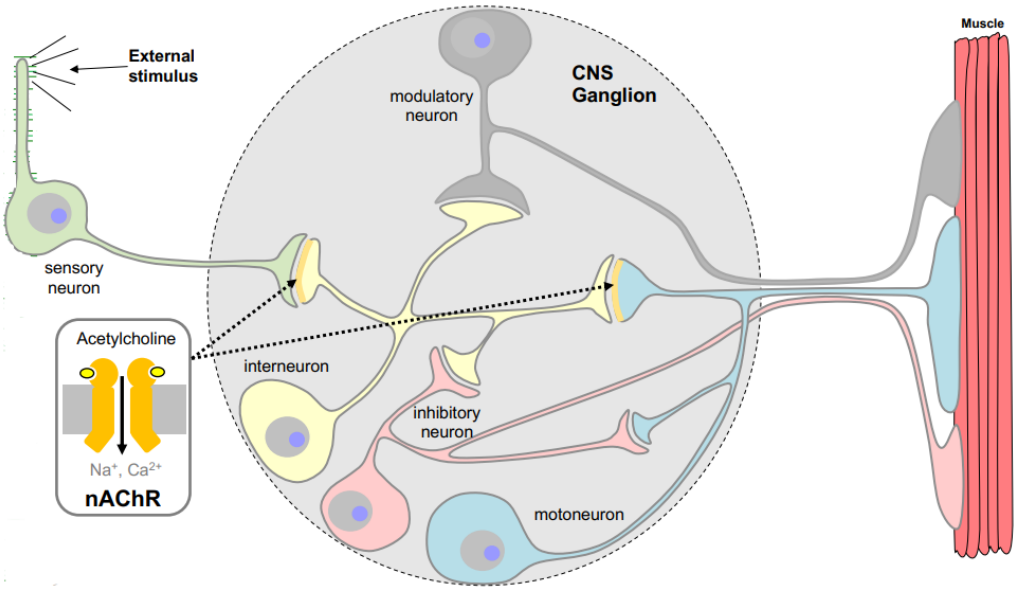

Fig. 14. Presynaptic localization of nicotinic acetylcholine receptors (nAChR) at synaptic junctions in the insect central nervous system (CNS). nAChR are the target-site of neonicotinoids, nicotine, sulfoximines, butenolides, mesoionics and pyridylidenes (IRAC, 2019b).

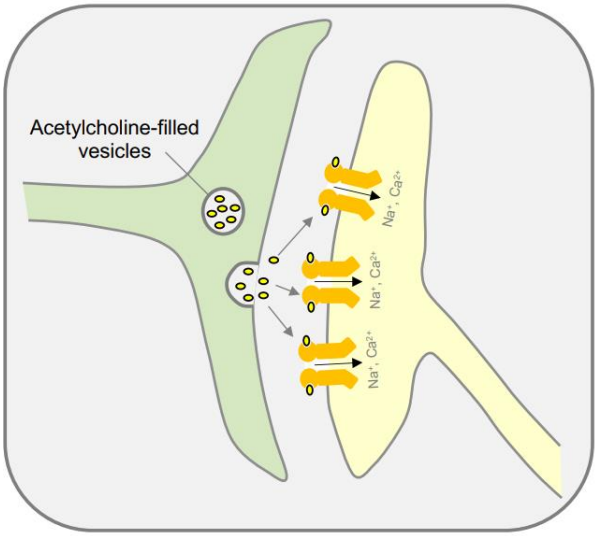

Fig. 15. Mode of action of nicotinic acetylcholine receptors (nAChR) competitive modulators. The compounds bind to the orthosteric site of presynaptic nAChRs and act as full agonists, partial agonists, or even antagonists (mesoionics) (Ihara et al., 2017; IRAC, 2019b; Wonnacott, 2020; Yu, 2008b).

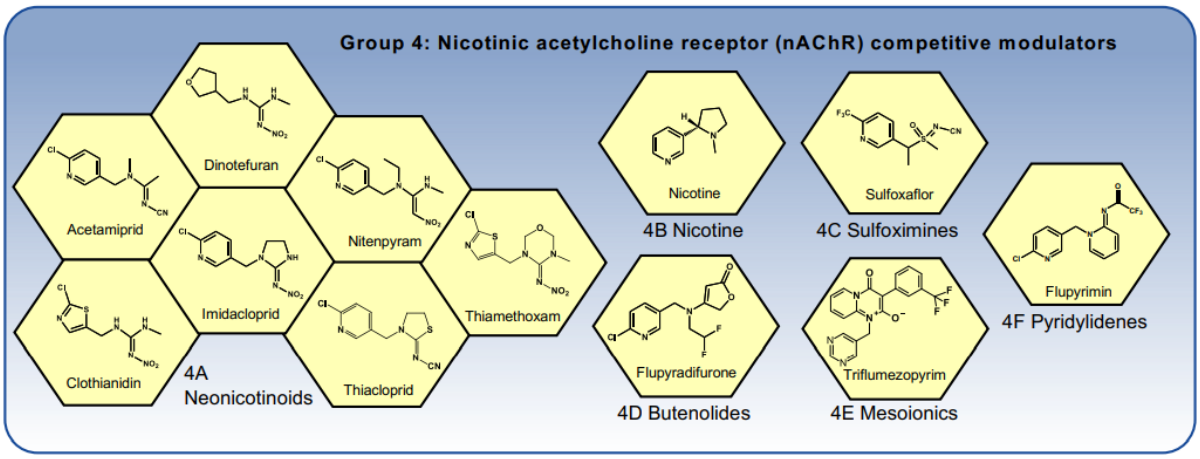

Fig. 16. Classification of nicotinic acetylcholine receptor (nAChR) competitive modulators in group 4 of the Insecticide Resistance Action Committee (IRAC) Mode of Action classification. Neonicotinoids are grouped in 4A. All nAChR competitive modulators target nerve and muscle function of insects (IRAC, 2021b).

1.3.1.3 Acetyl-CoA carboxylase (ACC) inhibitors

On top of nAChR and sodium channel modulators, tetronic and tetramic acid derivates, ketoenols, are used for sucking pest control (Fig. 17A and B) (Yu, 2008b). The insecticide class shares US $ 652 million of the insecticidal market (Sparks et al., 2020). They are classified as group 23 of the IRAC MoA classification and target the growth and development

of pests (Fig. 18A) (IRAC, 2021b). The tetramic and tetronic acid derivates act as lipid biosynthesis inhibitors (Nauen et al., 2002; Nauen et al., 2003; Nauen et al., 2005; Nauen et al., 2006; Wachendorff et al., 2000; Wachendorff et al., 2002). However, its active enol form is binding to acetyl-CoA carboxylase (ACC) of insects and mites (Bretschneider et al., 2012; Lümmen et al., 2014). ACC catalyzes the rate-limiting step in fatty acid biosynthesis by converting acetyl-CoA to malonyl-CoA (Wakil et al., 1983). In the first reaction step, biotin-carboxylase (BC) catalyzes the biotin cofactors N1' atom. This reaction is magnesium- and adenosine-triphosphate (ATP)-dependent and bicarbonate is used as carbon dioxide (CO_2) donor. Biotin is carried by the biotin carboxyl carrier protein (BCCP). In the next steps, the CO_2 transfer is catalyzed from carboxybiotin to the carboxyl acceptor group. This reaction is executed by the carboxyltransferase (CT) domain of ACC (Tong, 2013). Due to the inhibition of ACC in the first committed step of fatty acid synthesis, ketoenol insecticides prohibit the formation of malonyl-CoA (Fig. 18B) (IRAC, 2019b; Lümmen et al., 2014).

Tetronic acid derivates include the compounds spiromesifen and spirodiclofen (Nauen et al., 2003; Nauen et al., 2005; Wachendorff et al., 2000; Yu, 2008b). Spiromesifen is active against whiteflies and mites colonizing cotton, fruits, vegetables, or ornamentals (Nauen et al., 2003; Yu, 2008b). Although it's a non-systemic compound, it was studied having some translaminar activity (Nauen et al., 2003). Spirodiclofen is a non-systemic acaricide being toxic for phytophagous spider mites but also rust mites in pome fruits, citrus, nuts and grapes (Nauen et al., 2003; Wachendorff et al., 2000). Tetramic acid derivates include spirotetramat as well as spiropidion (Muehlebach et al., 2021; Yu, 2008b). The latter is systemically controlling sucking pests, such as aphids and whiteflies, affecting field crops, vegetables, or fruits (Muehlebach et al., 2021). Spirotetramat is acting systemically in stone-, pome-, or tropical fruits as well as grapes, nuts, tea, cotton, vegetables, citrus, almonds and hops. Due to its systemic action, it is passing through the entire vascular system, meaning distribution through xylem and phloem. Spirotetramat is controlling sucking insects such as aphids and whiteflies, but also scales and mealy bugs (Nauen et al., 2008). In general, ketoenol insecticides (prodrugs) need to be hydrolyzed to their active enol form, after application to and taken up by the plant. This allows the transport in xylem and phloem (Brück et al., 2009; Lueke et al., 2020).

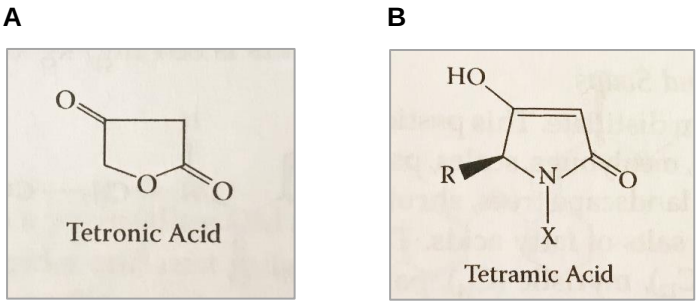

Fig. 17. Chemical structure of tetronic **(A)** and tetramic **(B)** acids (Yu, 2008b).

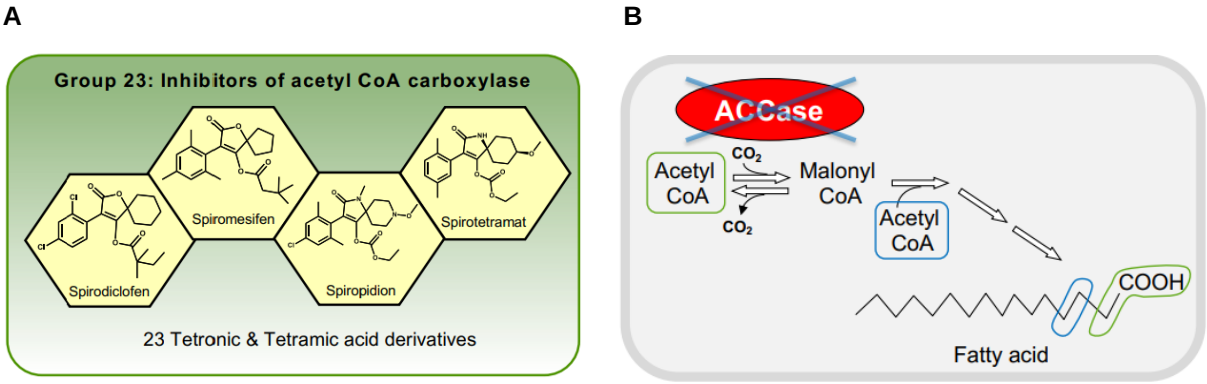

Fig. 18. A: Acetyl-CoA carboxylase (ACC) inhibitors are members of group 23 of the IRAC Mode of Action (MoA) classification. All insecticides listed in this group are tetronic or tetramic acid derivates and target growth and development of insect pests (IRAC, 2021b). **B:** MoA of ACC inhibitors (IRAC, 2019b).

1.3.1.4 Chordotonal organ modulators

The chordotonal organ transient receptor potential vanilloid (TRPV), channel modulators pyridine azomethine derivates (9B), as well as pyropenes (9D) are found in group 9 of the IRAC MoA classification (Fig. 19A) (IRAC, 2021b). The chemical class 9B shares US $ 70 million of the insecticidal market (Sparks et al., 2020). Flonicamid, also a chordotonal organ modulator, which target-site is yet unclear, is listed in MoA group 29 in the classification system (Fig. 19B) (IRAC, 2021b). Chordotonal organs are only present in insects and crustacean, but not in other arthropod classes. The mechanoreceptors are present in most exoskeletal joints, also between body segments and limbs. In a chordotonal organ sensilla are clustered, which are linked to the tracheal system or to moving parts of the skeletal cuticle. Sometimes, the organs are also included in connective tissue strands (Field & Matheson, 1998).

Pyridine azomethine derivates (pymetrozine and pyrifluquinazon) as well as pyropenes (afidopyropen) act as modulators of the chordotonal organ TRPV channel (Fig. 19A and 20). The Transient Receptor Potential (TRP) are cation channels which are responsible for sensory

signaling (Salgado, 2017). The TRPV cation channel includes a loop, which separates two transmembrane segments. However, it contains six transmembrane segments in total. In the fruit fly *Drosophila melanogaster* two TRPV genes are found. In humans, five genes of the superfamily are present (Montell et al., 2002). The insect superfamily consists of two channel subunits, Nanchung (NAN) and Inactive (IAV), being present in the chordotonal organs (Gong et al., 2004; Kim et al., 2003). In further studies using *D. melanogaster* flies as well as the brown planthopper *Nilaparvata lugens*, pymetrozine and pyrifluquinazon were shown to activate NAN and IAV heterodimers directly (Nesterov et al., 2015; Wang et al., 2019). This was also confirmed for afidopyropen: The compound was shown to stimulate TRPV channels of the fruit fly and the pea aphid *A. pisum*. However, the mammalian TRPV4 channel could not be activated by this insecticide. Analysis of the action on the subunits revealed NAN as main binding interface, whereas the binding affinity highly increased after IAV activation (Kandasamy et al., 2017). Next to it, the insecticide flonicamid could not be shown in activating the insect TRPV channel, but it is supposed that it also modulates chordotonal organs (IRAC, 2021b; Kandasamy et al., 2017; Sparks & Nauen, 2015; Taylor-Wells et al., 2018). Even though the compound was first expected in targeting potassium channels, the exact MoA remains elusive (Hayashi et al., 2005; Taylor-Wells et al., 2018). All chordotonal organ modulators are active against sucking insects such as aphids and/or whiteflies (Maienfisch, 2019).

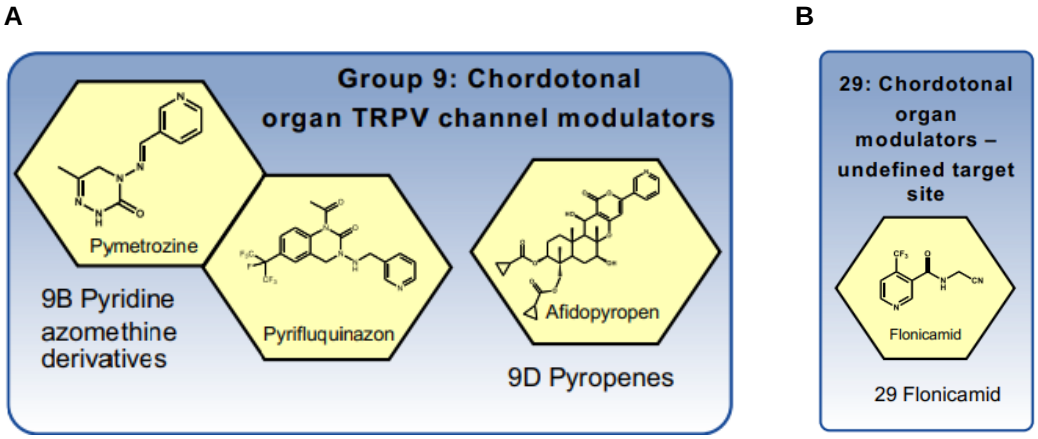

Fig. 19. A: Classification of chordotonal organ transient receptor potential vanilloid (TRPV) channel modulators are listed in group 9 of the Insecticide Resistance Action Committee (IRAC) Mode of Action (MoA) classification targeting the nerve and muscle function of insects (IRAC, 2021b). **B:** The chordotonal organ modulator flonicamid is classified in group 29 due to its yet undefined target-site (IRAC, 2021b).

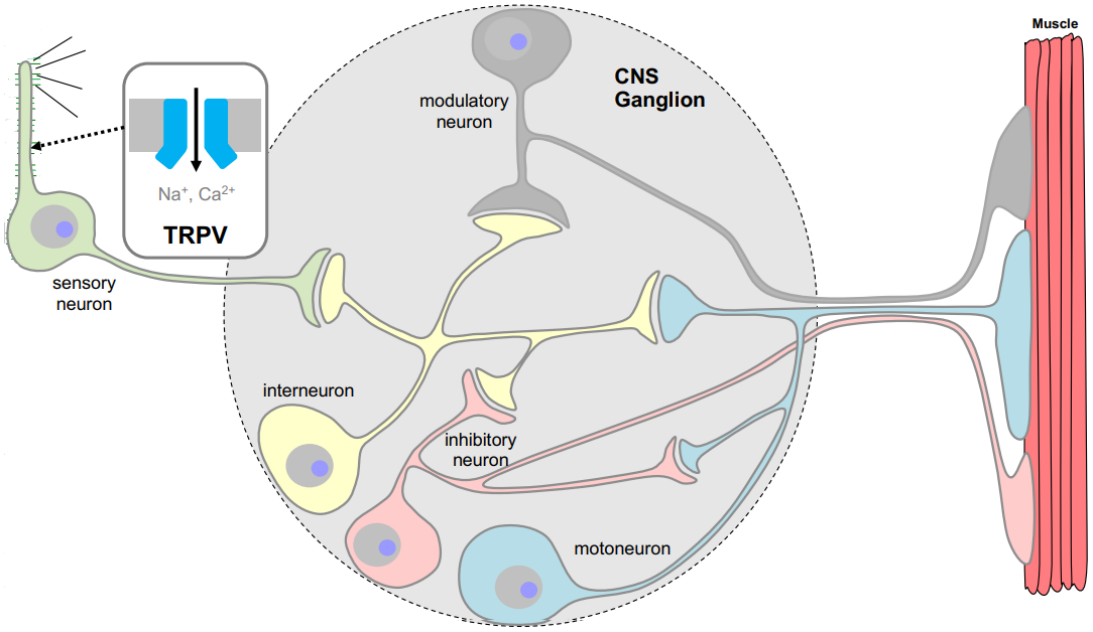

Fig. 20. Localization of the transient receptor potential vanilloid (TRPV) channels in specialized sensory neurons. TRPV channels are the target-sites of the chordotonal organ modulators pyridine azomethine derivates and pyropenes (IRAC, 2019b).

1.3.1.5 Other modes of action for Hemipteran pest control

Other classes used for hemipteran pest control are acetylcholinesterase (AChE) inhibitors, classified in group 1 of the IRAC Mode of Action Classification (Fig. 21A), as well as ryanodine receptor modulators (Ry-R), group 28 (Fig. 21B), (IRAC, 2021b). The former hydrolyzes the neurotransmitter acetylcholine in cholinergic synapses in the insect central nervous system (IRAC, 2019b; Schwenk & Burr, 2021). “In this catalytic process, the choline moiety binds to the “anionic site” in the catalytic center of the enzyme and the acetyl part of the molecule to the “ester site.” Subsequently, acetylcholine gets cleaved. Choline is released and the acetate-enzyme bond gets hydrolyzed” (Schwenk & Burr, 2021). The organophosphates (OP) (IRAC group 1B) reaction with AChE undergoes the same action as of AChE and acetylcholine. However, the final reaction step of OP and cholinesterase differs. The deacetylation happens faster than the dephosphorylation (Dubey & Patyal, 2007). This irreversible AChE inhibition is not only caused by organophosphate, but also by carbamate insecticides (Aldridge, 1950; Boublik et al., 2002; Chaize & Fournier, 2004). Today, OP shares US $ 1,467 million and carbamates US $ 550 million of the insecticide market, meaning it is the fourth most important MoA (Sparks et al., 2020).

The Ry-R diamides are widely used, accounting for US $ 2,336 million of the insecticidal market. The insecticides are the third most used class (Sparks et al., 2020) and target the Ry-

R, a large tetrameric calcium channel without voltage effect. While regulating the intracellular release of calcium stores, the receptor coordinates muscle contraction (Lahm et al., 2009). Despite that, flubendiamide and chlorantraniliprole are phthalic and anthranilic acid diamides respectively that mimic the function of Ry-R. The mammalian toxicity is lower and the action of the compounds is similar as of ryanodine insecticides, although they are not analogs (Bloomquist, 2021).

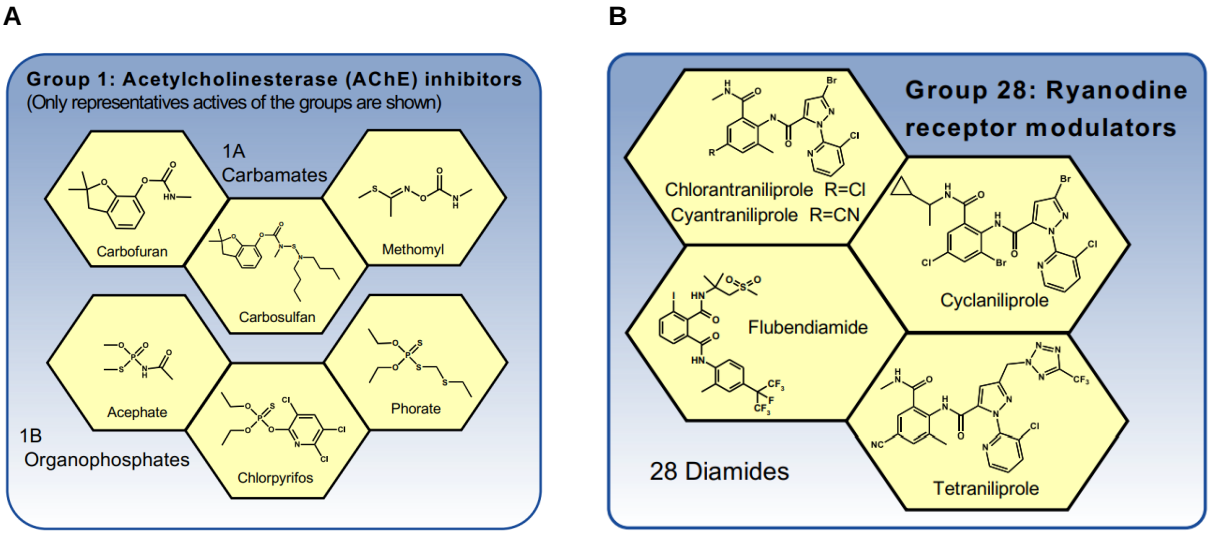

Fig. 21. A: Acetylcholinesterase (AChE) inhibitors are listed in group 1 of the IRAC Mode of Action (MoA) classification. Carbamates are grouped in 1A and organophosphates in 1B (IRAC, 2021b). **B:** The ryanodine receptor modulators (Ry-R) diamides are classified in group 28. All listed insecticides target nerve and muscle function of insect pests (IRAC, 2021b).

1.4 Insecticide resistance

Due to the intensive usage of insecticides, pest species have developed resistance to overcome the insecticides' toxicity (Sparks et al., 2021). Resistance can be described as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" (IRAC, 2021a). There are different types of insecticide resistance. The most common types of insecticide resistance are target-site resistance and metabolic resistance (IRAC International, 2021). Besides, also a modified cuticle can lead to a slower uptake of the insecticide (IRAC International, 2021; Balabanidou et al., 2018). Often this resistance mechanism is present along with other resistance forms, as described for neonicotinoid resistant *M. persicae* clones (IRAC International, 2021; Puinean et al., 2010). Another type of resistance is the so-called behavioral resistance. The resistant insects

recognize the insecticide, stop feeding, move to other plants, to the non-spayed part of the plants or deeply inside the crop canopy. This resistance mechanism is described for several insecticide classes, including pyrethroids, organophosphates and carbamates as well as organochlorines (IRAC International, 2021). Behavioral resistance has been observed in neonicotinoid resistant *M. persicae* adults (Fray et al., 2014). This thesis only describes target-site and metabolic resistance cases.

Insects can develop cross-resistance to structurally related insecticides and/or multiple resistance affecting structurally unrelated chemicals from different MoA groups (Cochrane, 2007; Metcalf, 1955). The first resistance case described dates back to 1914 (Fig. 22A) and was discovered in the homopteran Diaspididae species San Jose scale *Quadraspidiotus perniciosus* against lime sulphur (Melander, 1914). The development and intensive usage of synthetic organic insecticides came along with a rapid increase of insecticide resistance cases (Fig. 22A and B) (Sparks et al., 2021 and references cited therein). With increased resistance cases described, the IRAC was founded in 1984, a consortium of crop protection companies technically addressing insecticide resistance issues. IRAC gathered experts out of companies as well as universities to develop strategies to overcome increasing issues of insecticide resistance (Sparks et al., 2021). IRAC also developed the insecticide MoA classification scheme to provide orientation and clarity on the sites of action of insecticides and acaricides (IRAC, 2021a). Today, companies working for the IRAC are developing compounds that are comprising around 81 % of the world insecticide market (Phillips, 2020; Sparks et al., 2021). The current mission of IRAC is to educate and communicate sustainable strategies to overcome insecticide and insecticidal trait resistance (Sparks et al., 2021).

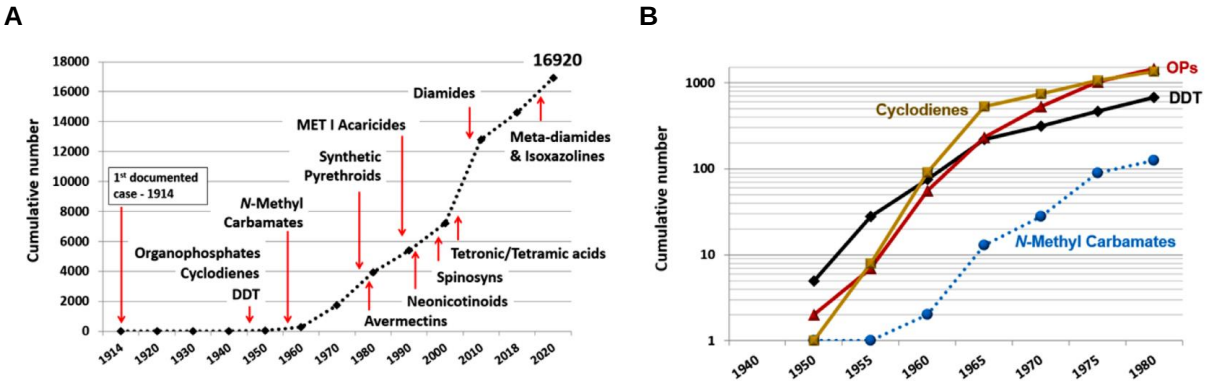

Fig. 22. A: Insecticide resistance drastically increased in the last 50 years. Red arrows represent the year of introduction of the major insecticide classes (Sparks et al., 2021). **B:** Resistance cases to major classes of insecticides from 1940 to 2010 (Sparks et al., 2021 and references cited therein).

To document resistance cases worldwide, IRAC is one of the founders of the 'Arthropod Pesticide Resistance Database' (APRD, http://www.pesticideresistance.org). This database lists globally described resistance cases in arthropods by species and MoA. The database chronicles 3,609 resistance cases in the order Homoptera. With 697 instances, the cotton whitefly *B. tabaci* is on top of the list, followed by the green peach aphid *M. persicae* (Table 2). The greenhouse whitefly *T. vaporariorum* is listed with 112 resistance events and the cabbage whitefly *A. proletella* with 6 cases. To date, the pea aphid *A. pisum* is not listed with any resistance case (Mota-Sanchez & Wise, 2022).

Table 2. Top five insect species of the order Homoptera that comprises the most insecticide resistance cases described (Mota-Sanchez & Wise, 2022).

Genus Species	Taxonomy (family of the order Homoptera)	Common Name(s)	# Cases
Bemisia tabaci	Aleyrodidae	Sweetpotato whitefly	697
Myzus persicae	Aphididae	Green peach aphid	487
Nilaparvata lugens	Delphacidae	Brown planthopper	453
Aphis gossypii	Aphididae	Melon and cotton aphid	302
Sogatella furcifera	Delphacidae	White-backed planthopper	222

1.4.1 Target-site resistance

Target-site resistance usually confers cross-resistance to an entire chemical class, for example by a mutation of an amino acid residue due to a single nucleotide polymorphism (SNP) in the gene expressing the insecticide target (IRAC International, 2021).

1.4.1.1 Voltage-gated sodium channel target-site resistance

In 1951, resistance to DDT was first detected in the house fly *Musca domestica* and described as *kdr* (Busvine, 1951; Milani, 1954). Later, the resistance was explained by the decreased neuronal sensitivity to DDT and was also described for pyrethroids (Sawicki, 1985). Insects carrying the *kdr* were shown to have an altered binding of DDT and pyrethroid compounds to VGSC (Chang & Plapp, 1983; Pauron et al., 1989). Sequencing of the *para* gene, encoding the VGSC in house flies revealed 24 mutations. One mutation comprises a leucine at position 1014 which is substituted by a phenylalanine and designated as L1014F mutation. Position

1014 is in the transmembrane region of segment 6 of the second domain of the VGSC (Williamson et al., 1996). The *kdr* allele was also described in several other insects, e.g., in mosquito species such as *Anopheles gambiae*, *Culex pipiens* or *Anopheles coluzzii* (Bkhache et al., 2016; Ibrahim et al., 2014; Martinez-Torres et al., 1998). Furthermore, the mutation was detected in numerous aphid species, including *S. avenae* and *M. persicae* (Foster et al., 2014; Martinez-Torres et al., 1999). Another mutation, M918T is located in the intracellular linker of S4 and S5 in the second domain of the VGSC (Williamson et al., 1996). The mutation known as super-*kdr* (*skdr*) was first detected in house flies *M. domestica* (Williamson et al., 1996). However, in *T. vaporariorum*, a M918L mutation was described and shown to confer pyrethroid resistance, too (Karatolos et al., 2012a). Additionally, the *skdr* mutation M918V as well as the polymorphism L925I were detected in *B. tabaci* resistant to a mixture of pyrethroid and OP, whereas only L925I could be associated with resistance (Morin et al., 2002). Another mutation described in pyrethroid-resistant *B. tabaci* is T929V (Roditakis, et al., 2006), but T929I was shown earlier to be acting like a *skdr* resistance (Vais et al., 2001). Aphids were also shown to have *skdr* mutations, such as the cotton aphid *Aphis gossypii* and *M. persicae*: Cotton aphids from China were found carrying the M918V and the R81T (1.4.1.3.) mutation (Munkhbayar et al., 2020). The latter one is present in loop D of the β1-subunit of nAChR in resistant aphids (Bass et al., 2011b). The simultaneous presence of L1014F and M918T was associated with high levels of pyrethroid resistance (RR up to 455) in green peach aphids, much higher than in aphids carrying the *kdr* mutation only (Eleftherianos et al., 2008). Furthermore, the M918L substitution was also found in *M. persicae* collected from oilseed rape and was also shown to carry the L932F mutation next to others (Fontaine et al., 2011). The L932F as well as the T929I mutations were also associated with permethrin resistance in human head lice *Pediculus capitis* (Yoon et al., 2003).

1.4.1.2 Acetylcholinesterase target-site resistance

AChE insensitivity has been linked to carbamate insecticide sensitivity. The target of carbamates and OP is the *AChE1*, also named *ace1*, enzyme (Benting & Nauen, 2004). In insects, the *ace1* is the primary loci that carry polymorphisms, that are responsible for insecticide resistance (Lee et al., 2015). The amino acid substitution S431F confers resistance in *M. persicae* strains against the carbamate pirimicarb (Andrews et al., 2002; Nabeshima et al., 2003). The mutation in green peach aphids was found in being homologous to the wild type of the amino acid F331 present in analyzed *Torpedo californica* (Andrews et al., 2004). Next to the S431F mutation, the A201S and its homologous were shown in causing OP resistance in several insect species, such as the cotton aphid *A. gossypii* (Andrews et al., 2004; Toda et al., 2004), the fall armyworm *Spodoptera frugiperda* (Carvalho et al., 2013), the rice stem borer

Chilo suppressalis (Jiang et al., 2009), the spider mite *Tetranychus urticae* (Khajehali et al., 2010) or the tomato leaf miner *Tuta absoluta* (Haddi et al., 2017). This polymorphism is present in the active site of AChE (Toda et al., 2004). In addition, OP resistance in *B. tabaci* was linked to overexpressed carboxylesterases (CEST) but also to the point mutation F392W in *ace1* (Alon et al., 2008). The mutation was found in being homologous to the F331W, that was clustered at active site motif in AChE resistant *T. californica* (Alon et al., 2008; Oakeshott et al., 2005). Besides, the mutation was also found in *T. urticae* strains which showed resistance towards OPs (Anazawa et al., 2003; Khajehali et al., 2010).

In *A. gossypii*, insensitivity towards OPs and carbamates are associated with the A302S mutation (Andrews et al., 2004). In other aphid species, such as *S. avenae* or *R. padi*, additional mutation sites were detected (Chen et al., 2007a; Chen et al., 2007b). In the former one, two mutations, the L436S (L336S in *T. californica*) in *ace1* and the W516R (W435R in *T. californica*) in *AChE2*, also named *ace2*, evolved and are shown to confer 161.8-fold resistance against pirimicarb (Chen et al., 2007b). In the OP and carbamate resistant *R. padi* aphids, three mutations, one in *ace1* and two in *ace2* were found (Chen et al., 2007a). The F368L (F290L in *T. californica*) mutation sits in the acetyl-pocket of *ace2* (Chen et al., 2007a). Published data shows that polymorphisms at this location are responsible for insecticide resistance in *D. melanogaster* (Mutero et al., 1994; Villatte et al., 2000) and *M. domestica* (Walsh et al., 2001). The other polymorphisms present in *ace2*, the V435A (V356A in *T. californica*) and the S329P (S228P in *T. californica*) in *ace1* are influencing the structure of the enzyme (Chen et al., 2007a).

1.4.1.3 Nicotinic acetylcholine receptor target-site resistance

The first target-site resistance responsible for neonicotinoid resistance in aphids, was observed in an imidacloprid resistant *M. persicae* strain. Radioligand binding studies using tritiated imidacloprid showed that the [3H]-imidacloprid binding site was almost lost in the resistant aphids. The low binding affinity of the insecticide to the nAChR target was later found due to the target-site mutation R81T (Bass et al., 2011b). nAChR ß1-subunit is split into the loops D, E and F, whereas the corresponding alpha subunit is split into loops A, B and C (Grutter & Changeux, 2001). The R81T mutation was also found in *A. gossypii*, selected for 60 generations on imidacloprid-treated plants in the laboratory (Shi et al., 2012). In field populations of *A. gossypii* sampled in South-Korea also the R81T polymorphism was detected (Koo et al., 2014). On top of the R81T polymorphism, amino acid exchanges of valine to isoleucine (V62I) and lysine to glutamic acid (K264E) were reported in imidacloprid resistant *A. gossypii*. Both mutations were speculated to be involved in imidacloprid resistance in cotton aphids (Chen et al., 2017). However, the R81T mutation in imidacloprid resistant *M. persicae*

strains, seems to have less effect on binding affinity to sulfoxaflor (Wang et al., 2016), a sulfoximine insecticide also known to bind to the orthosteric site (Ihara et al., 2017).

In an imidacloprid resistant *N. lugens* laboratory strain a single point mutation Y151S, present in a conserved region of two subunits Nlα1 and Nlα3 in nAChR, was shown to confer imidacloprid resistance, based on reduced binding (Liu et al., 2005). However, it was found that the mutation has only minor effects on the toxicity of neonicotinoid agonists (Liu et al., 2006; Liu et al., 2009; Yixi et al., 2009). Furthermore, neonicotinoid resistant *B. tabaci* collected in China, showed SNPs corresponding to the β1-subunit of nAChR in *M. persicae*: Interestingly in one of the analyzed strains, a 45 bp fragment was missing, encoding 15 amino acids in nAChR ß1. Included was the arginine R79, corresponding to the R81T mutation in *M. persicae* (Wang et al., 2017).

1.4.1.4 Other target-site mutations conferring insecticide resistance

Apart from mutations found in the VGSC, AChE and nAChR, a target-site mutation in ACC was found conferring ketoenol resistance in insects, first detected in *T. vaporariorum* (Karatolos et al., 2012c). In the spiromesifen resistant insects a glutamic acid to lysine substitution at position 645 (E645K) was associated with resistance. The mutation is located in between the enzyme's domains BC and BCC (Karatolos et al., 2012c). However, it was later also found in populations from Greece but could not be clearly linked to spiromesifen resistance in these insects (Kapantaidaki et al. 2018). Ketoenol insecticides have been shown to bind to the CT domain of ACC (Lümmen et al., 2014). Therefore, the E645K mutation is unlikely to confer target-site resistance as it lays outside the CT domain (Karatolos et al., 2012c; Lueke et al., 2020). In the spider mite *T. urticae* resistant to spirodiclofen, an amino acid substitution A1079T was found in 2010, but was not associated with a resistant phenotype (Khajehali, 2010). Also, it is not present in the conserved CT region of ACC, and genome edited *D. melanogaster* lines bearing the A1079T mutation were not found to be resistant against spirodiclofen (Khajehali, 2010; Lueke et al., 2020).

Recently two mutations, A2083V and A2151V, have been detected in spiromesifen resistant *B. tabaci*. It was shown that genome edited *D. melanogaster* lines carrying the A2083V mutation in ACC were resistant against spiromesifen, spirodiclofen and spirotetramat, whereas the A2151V mutation has no impact on ketoenol binding as it is located in a less conserved region of the ACC (Lueke et al., 2020). The mutation site A2226V, corresponding to A2083V in *B. tabaci*, was detected in spirotetramat resistant green peach aphids *M. persicae* recently (Singh et al., 2021; Umina et al., 2022). Furthermore, molecular analysis of spirotetramat resistant *A. gossypii*, revealed several amino acid substitutions in the BC and CT domain. In

the CT domain, one mutation, P2170S, was found to be present in all analyzed resistant cotton aphids. It was speculated that the mutations found in the CT domain can be associated with spirotetramat resistance in this aphid species though no functional evidence was provided (Pan et al., 2017).

1.4.2 Metabolic resistance

Metabolic resistance is based on an enhanced metabolic degradation of insecticides due to elevated levels of detoxification enzymes. By increased expression, resistant insects can detoxify insecticides faster than susceptible pests (IRAC International, 2021). Cytochrome P450 monooxygenases (CYP, P450), esterases or glutathione-S-transferases (GST), are examples for enzyme families that detoxify insecticidal compounds. Xenobiotic metabolism (including insecticides) can basically be separated in three phases (Fig. 23) (Amezian et al., 2021; Yu, 2008a). In phase I, the compound is oxidized, hydrolyzed and reduced. This step usually decreases the biological activity of the compound as it becomes more hydrophilic and is functionalized for further detoxification steps (Yu, 2008a). The metabolites formed in phase I are typically less toxic than the primary xenobiotic, however, sometimes phase I processing of a compound results in more toxic products (Kennedy & Tierney, 2013). Normally, the formed metabolites are further converted in the following phase II (conjugation), but sometimes, due to their higher hydrophilicity, they can be excreted directly via phase III transporters such as ATP-binding cassette (ABC) efflux transporters (Amezian et al., 2021; Kennedy & Tierney, 2013; Yu, 2008a). In phase II, the metabolites are bound to endogenous compounds, such as sulfates, sugars, amino acids, phosphates or glutathione (Yu, 2008a). In this phase, water-soluble products are formed and can be excreted as mentioned above (Kennedy & Tierney, 2013). Insects of diverse species express different amounts of detoxification genes, as indicated in table 3.

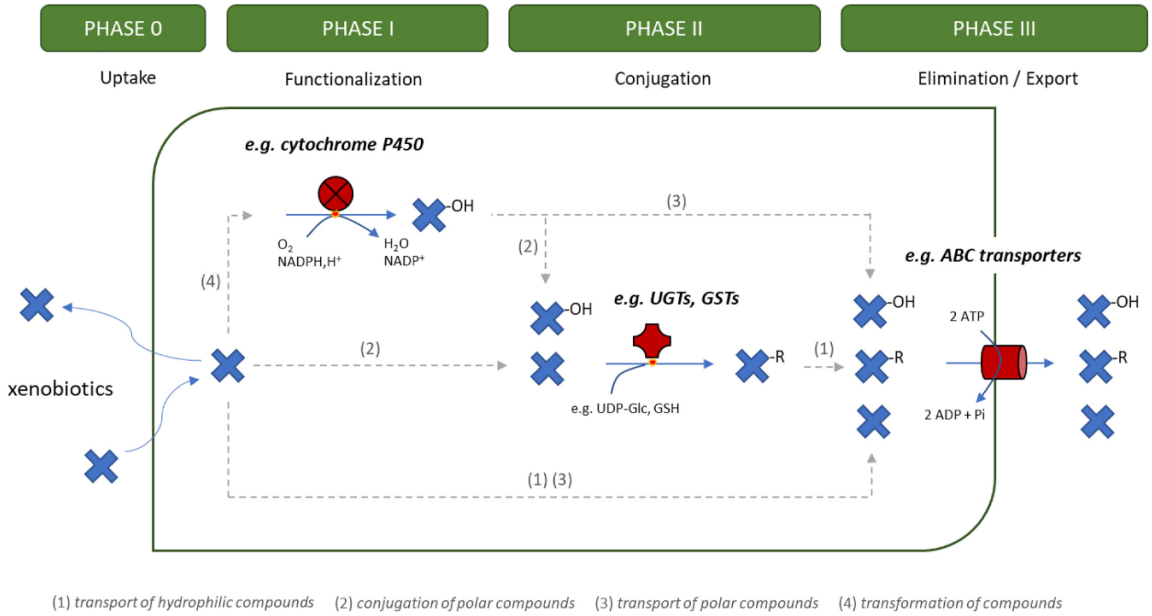

Fig. 23. Detoxification pathways of xenobiotics. In phase I and II enzymes as cytochrome P450 monooxygenases (P450s) and Glutathione-S-transferases (GSTs) or uridine diphosphate (UDP)-glycosyl transferase (UGTs) are eliminating by functionalization and conjugating. The xenobiotic is exported in Phase III with, for example, ABC transporters out of cells. Depending on the present xenobiotic, the pathways can occur simultaneously, or, as in most cases, step-by-step (Amezian, et al., 2021).

Table 3. Numbers of detoxification genes identified in different insect species (modified after Chen et al., 2016, Claudianos et al., 2006, Ramsey et al., 2010, Xia et al., 2019 and Xie et al., 2012). Listed are quantities of the cytochrome P450s monooxygenases (P450s), UDP-glucuronosyltransferases (UGTs), Glutathione-S-transferases (GSTs), Carboxylesterases (CESTs) as well as ABC transporters (ABCs). The analyzed species are abbreviated with their dedicated 'European and Mediterranean Plant Protection Organization' (EPPO) codes (https://gd.eppo.int) with *Bemisia tabaci* (BEMITA), *Acyrthosiphon pisum* (ACYRON), *Nilaparvata lugens* (NILALU), *Pediculus humanus* (PEDIHO), *Anopheles gambiae* (ANPHGB), *Apis mellifera* (APISME), *Bombyx mori* (BOMBMO), *Drosophila melanogaster* (DROSME), *Tribolium castaneum* (TRIBCA) and *Tetranychus urticae* (TETRUR).

	BEMITA	***ACYRON***	***NILALU***	***PEDIHO***	***ANPHGB***	***APISME***	***BOMBMO***	***DROSME***	***TRIBCA***	***TETRUR***
P450s	131	83	76	39	106	46	82	85	128	123
CESTs	42	29	57	22	51	24	76	35	60	85
GSTs	25	20	9	12	31	10	23	38	35	31
ABCs	50	126	92	38	52	41	55	56	73	103
UGTs	81	72	20	4	24	11	38	35	27	81

1.4.2.1 Cytochrome P450s monooxygenases

Oxidation and reduction, in the first phase of xenobiotic metabolism, is catalyzed by membrane bound CYPs (Kennedy & Tierney, 2013; Yu, 2008a). The smooth endoplasmic reticulum (ER) is the location where P450s are acting (Gems & McElwee, 2005). The enzymes can be found in insects, higher plants, bacteria, yeast, fish, crustaceans, mollusks, reptiles, birds as well as mammals (Yu, 2008a). CYPs are coupled to their redox partner, the oxidated nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase (CPR), also located in the ER. This system is necessary for oxidative, reductive as well as peroxidative metabolism of xenobiotics (Kennedy & Tierney, 2013). The microsomal membranes require electrons and protons, twice each, for the insertion of oxygen into the substrate (De Mot & Parret, 2002). Electrons from NADPH are transmitted by CPR, which contains flavin mononucleotide (FMN) as well as flavin adenine dinucleotide (FAD) (De Mot & Parret, 2002; Yu, 2008a). The reaction is described as follows:

$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O \text{ (Yu, 2008a).}$$

RH is the substrate and ROH is the released hydroxylated product (De Mot & Parret, 2002).

Besides insecticides, CYPs also metabolize endogenous compounds, for example bile acids, steroids or fatty acids as well as xenobiotics, such as drugs, natural plant products, environmental pollutants or alcohol (Nebert et al., 1991). CYPs are an enzyme superfamily which is one of the largest and oldest (Feyereisen, 1999). Insect P450s genes can be grouped into four clans: Clan 2, 3 and 4 as well as the mitochondrial clan (Feyereisen, 2006; Nelson, 1998). Studies revealed that particularly P450s of the CYP3 clan are involved in secondary plant metabolite (e.g., alkaloids) and chemical insecticide metabolism (Feyereisen, 1999; Mao et al., 2006; Ramsey et al., 2010; Scott, 1999; Snyder & Glendinning, 1996). Additionally, members of the CYP4 clan were found in detoxifying pheromones and xenobiotics (Feyereisen, 2005; Feyereisen, 2006; Feyereisen, 2012; Maïbèche-Coisne et al., 2004; Ramsey et al., 2010) as well as CYP2 clan being responsible for hormone detoxification, next to others (Feyereisen, 1999; Feyereisen, 2006; Maïbèche-Coisne et al., 2004; Ramsey et al., 2010). Numbers of cytochrome P450 genes and their classifications can be reviewed in table 3 and 4. Of the species analyzed, exhibited *B. tabaci* the most P450s genes, followed by *M. persicae* (Table 4). Furthermore, the spider mite *T. urticae* was also found having high numbers of CYP genes expressed (Table 3) (Chen et al., 2016).

Enhanced CYP activity is also related to several described insecticide resistance cases. The first gene that was detected being responsible for neonicotinoid imidacloprid resistance was CYP6CM1 in *B. tabaci* B- and Q-type (Karunker et al., 2008). Studies revealed that the CYP6CM1 gene depletes imidacloprid to its 5-hydroxy form (Karunker et al., 2009).

Additionally, three single-nucleotide polymorphisms were present in the CYP6CM1 intron region. Resistant insects were carrying only the resistant alleles but not the susceptible one (Karunker et al., 2008). Additional studies revealed cross-resistance to pymetrozine as well as pyriproxyfen, by metabolism studies with functionally expressed CYP6CM1 (Nauen et al., 2013; Nauen et al., 2015b). Besides, imidacloprid resistance is also associated with the overexpressed CYP6ER1 gene, which was found in resistant *N. lugens* (Bass et al., 2011a). With subsequent transgenic expressions in *D. melanogaster*, ribonucleic acid (RNA) interference (RNAi) as well as simulation and molecular docking studies, it was confirmed that the CYP6ER1 gene is responsible for the neonicotinoid resistance in *N. lugens* (Pang et al., 2016). In addition, in the green peach aphid *M. persicae*, imidacloprid resistance was also associated with enhanced oxidase activity (Philippou et al., 2010). An overexpressed P450 gene, CYP6CY3, was associated with resistance against neonicotinoids in green peach aphids (Puinean et al., 2010). In addition, a German greenhouse whitefly population was resistant against pyriproxyfen, with the mode of action of juvenile hormone mimics in group 7 of the IRAC MoA classification system (IRAC, 2021b; Karatolos et al., 2012b). Microarray analysis followed by 'Real time quantitative polymerase chain reaction' (PCR) (RT-qPCR) revealed the CYP4G61 P450 gene being 81.7-fold overexpressed in the resistant insects (Karatolos, et al., 2012b).

Table 4. Cytochrome P450 genes in insect species. Listed are predicted P450 genes (Total P450s), the clans CYP2, CYP3, CYP4, mitochondrial (MITO) as well as the source of publication (Source).

Species	Total P450s	CYP2	CYP3	CYP4	MITO	Source
Myzus persicae	115	3	63	48	1	Ramsey et al. (2010)
Acyrthosiphon pisum	83	10	33	32	8	Ramsey et al. (2010)
Trialeurodes vaporariorum	57	3	34	13	7	Karatolos et al. (2011)
Bemisia tabaci	131	6	84	26	15	Xie et al. (2012)
Apis mellifera	46	8	28	4	6	Claudianos et al. (2006); Ramsey et al. (2010)
Drosophila melanogaster	85	6	36	32	11	Ramsey et al. (2010)
Anopheles gambiae	106	10	42	45	9	Claudianos et al. 2006

1.4.2.2 Carboxylesterases

Besides P450s, esterases are an important multigene family involved in insects' xenobiotic defense. As described for P450s, resistant insects can overexpress ESTs, making them resistant against crop protection compounds such as OPs. Additionally, these enzymes can metabolize pheromones and specific hormones (Oakeshott et al., 2010). As hydrolases, esterases are splitting ester compounds while adding water, resulting in an alcohol and an acid moiety. Insecticides such as pyrethroids, OPs and carbamates contain an ester linkage making them susceptible for hydrolysis. CESTs as well as phosphatases/phosphorotriester hydrolases are known to metabolize the previously described insecticide classes. OPs are also detoxified by phosphatases. The difference to CESTs lays in the fact that phosphatases are not influenced by OP inhibition (Yu, 2008a). CESTs can be classified into eight subfamilies: α- and β-esterases, juvenile hormone esterases, gliotactins, neuroligins, neurotactins, acetylcholinesterases and glutactin type (Ranson et al., 2002). Ramsey et al. (2010) identified seven CESTs clades present in *M. persicae* and *A. pisum* (Table 5). In total, approximately 30 CEST genes were found in pea aphids (Table 3 and 5) (Chen et al., 2016; Ramsey et al., 2010). Besides, in *T. vaporariorum* approximately 30 genes were identified as possible "real" esterase genes (Table 5) and in the cotton whitefly approximately 40 genes (Table 3 and 5) (Chen et al., 2016; Karatolos et al., 2011; Xia et al., 2019). Several other insect species and their classification of CESTs genes are listed in table 5.

Overexpressed CESTs such as E4 and FE4 were identified for the first time in OP resistant *M. persicae*. The resistant insects exhibited higher levels of both genes making them resistant against organophosphate, carbamate as well as pyrethroid insecticides (Devonshire & Moores, 1982; Devonshire et al., 1983; Needham & Sawicki, 1971). Overexpressed CESTs linked to insecticide resistance were also identified in cotton whiteflies. An OP resistant *B. tabaci* population has been described to carry the point mutation F392W in *ace1* of the AChE and a 4-fold overexpressed CEST gene, named *coe1*. Probably, this gene modifies transcriptional control, while lacking higher gene amplifications (Alon et al., 2008).

Table 5. Carboxylesterase genes in insect species.

Species	Dietary			Pheromone and hormone processing				Neuro and developmental						Total	Source
	A	B	C	D	E	F	G	H	I	J	K	L	M		
Myzus persicae	5	0	0	0	12	0	0	0	1	3	1	0	0	22	Ramsey et al. (2010)
Acyrthosiphon pisum	5	0	0	0	18	0	0	0	1	2	1	3	0	29	Ramsey et al. (2010)
Trialeurodes vaporariorum	11	0	1	0	6	0	0	1	1	2	1	3	1	27	Karatolos et al. (2011)
Bemisia tabaci		6			1	1	0	3	15	4	10	1	1	42	Xia et al. (2019)*
Drosophila melanogaster	0	2	11	3	3	2	0	4	2	1	1	4	2	35	Claudianos et al. (2006)
Apis mellifera	8	0	0	1	3	0	1	0	2	2	1	5	1	24	Claudianos et al. (2006)
Anopheles gambiae	0	14	2	0	4	4	4	9	2	2	1	5	2	51	Claudianos et al. (2006)

* The carboxylesterase classes listed in Xia et al. (2019), were translated in subfamily abbreviations of carboxylesterase gene naming after Wu et al. (2018).

1.4.2.3 Glutathione S-transferases

In the second phase of xenobiotic defense, side groups are added to the unactive, often lipophilic compounds of phase I. This increases their solubility and its excretion out of the cells (Gems & McElwee, 2005). GSTs catalyze the conjugation of substrates, e.g., hydroxylated phase I metabolites, with glutathione (GSH). The mainly cytosolic enzymes are acting in xenobiotic resistance (Kennedy & Tierney, 2013). In the GSH conjugation, the tripeptide GSH is affecting the electrophilic carbon atoms of the compound. Due to that, the electrophilic compounds are not acting with other biomolecules, resulting in their detoxification. Consisting of glutamic acid, glycine and cysteine, the nucleophilic antioxidant is responsible for the protection of cells (Kennedy & Tierney, 2013). While GSH is only present in a reduced form, GSH reductase is found in high concentrations and in an active form. The enzyme is converting the oxidative form of GSH (Kennedy & Tierney, 2013). GST in insects consists of six known classes (Chelvanayagam et al., 2001; Ketterman et al., 2011). The epsilon, delta and omega classes are associated with insecticide resistance (Ranson et al., 2001; Vontas et al., 2002; Yamamoto et al., 2009). The different classes of GSTs and the distribution of genes different species are listed in table 6. In total, the *A. pisum* as well as *M. persicae* genomes comprises each approximately 20 GST genes (Table 3 and 6) (Chen et al., 2016; Ramsey et al., 2010).

Similar numbers were also described in *T. vaporariorum* (Karatolos et al., 2011) and *B. tabaci* (Chen et al., 2016).

Enhanced GST activity in insect pests is also responsible for several known insecticide resistance cases. In the two-spotted spider mite, *T. urticae*, enhanced GST activity leads to abamectin resistance of the insects (Stumpf & Nauen, 2002). Higher GST activity was also detected in a pyriproxyfen resistant population of the cotton whiteflies (Ma et al., 2010). In addition, GST triggered thiamethoxam (neonicotinoid) resistance in *B. tabaci* (Yang et al., 2016).

Table 6. Glutathione-S-transferase genes in insect species. Listed are the six classes known being present in insects, after Chelvanayagam et al. (2001) and Ketterman et al. (2011): Delta (D), Epsilon (E), Omega (O), Sigma (S), Theta (T) and Zeta (Z) - as well as Microsomal (M) gene, genes which could not ordered to one of the classes (Unk.) and the total number of glutathione-S-transferase genes detected (Tot.).

Species	D	E	O	S	T	Z	M	Unk.	Tot.	Source
Myzus persicae	8	0	0	8	2	0	2	-	21	Ramsey et al. (2010)
Acyrthosiphon pisum	10	0	0	6	2	0	2	-	20	Ramsey et al. (2010)
Trialeurodes vaporariorum	9	1	0	5	0	1	1	-	17	Karatolos et al. (2011)
Bemisia tabaci	14	0	1	6	0	2	2	-	25	Harari et al., (2020)
Drosophila melanogaster	11	14	5	1	4	2	1	0	38	Claudianos et al. (2006)
Apis mellifera	1	0	1	4	1	1	2	0	10	Claudianos et al. (2006)
Anopheles gambiae	12	8	1	1	2	1	3	3	31	Claudianos et al. (2006)

1.4.2.4 ATP-binding cassette transporters

In phase III the detoxified compounds are excreted out of the cells by solute carrier (SLC) proteins or ABCs (Kennedy & Tierney, 2013). These transporters are not only excreting detoxified xenobiotics or endogenous compounds out of the cells but are also responsible for defending the cells against the entrance of toxins (Epel et al., 2008; Kennedy & Tierney, 2013). ABC transporters are divided into eight subfamilies ranging from A to H (Dermauw et al., 2013; Dermauw & Van Leeuwen, 2014; Sun et al., 2017). While membrane-bound, the proteins are

using ATP for transporting the products out of the cells (Kennedy & Tierney, 2013). The total numbers of ABC genes detected in different insect species are listed in table 3. In *B. tabaci* 50 ABC transporters were found (Chen et al., 2016; Pym et al., 2019). In the greenhouse whitefly *T. vaporariorum* 46 ABC transporters were detected (Pym et al., 2019). Additionally, members of the ABCB family were also found in *T. vaporariorum.* The cotton whitefly *B. tabaci* exhibited high numbers of ABCG family members (Pym et al., 2019). The ABCG, ABCC as well as ABCB families were shown to confer transport and insecticide resistance (Dermauw & Van Leeuwen, 2014). Next to enhanced expressions of P450s, GSTs and CESTs, several overexpressed ABCGs were found in neonicotinoid-resistant *B. tabaci* and could be associated with insecticide resistance (Yang et al., 2013).

1.4.2.5 Others

Another class of phase II detoxification enzymes are uridine diphosphate (UDP)-glycosyltransferases (UGT), however their role in xenobiotic detoxification is not completely elucidated to date (Fig. 23) (Amezian et al., 2021; Li et al., 2018). UGTs are acting in the ER (Gems & McElwee, 2005). The glycosylation reaction is besides the glutathione conjugation another important step in the phase II detoxification of xenobiotics (Kennedy & Tierney, 2013). *B. tabaci* and *T. urticae* exhibited the highest numbers of UGT genes when compared to the other species analyzed (Table 3). Also, in the pea aphid slightly more UGT genes were found than in other insect species (Chen et al., 2016). The UGT50 family was described being present in a wide range of insect species, including *B. tabaci* but lacking in pea aphids and greenhouse whiteflies (Ahn et al., 2012; Pym et al., 2019). This family is homologous to the human UGT8 (Ahn et al., 2012). The main function of the family is the conjugation of endogenous lipophilic chemicals. Drug metabolism is relatively low. It is assumed that the family is responsible for the endobiotics' toxic accumulation and is monitoring the lipophilic signaling molecule levels (Meech et al., 2019).

Moreover, nicotine tolerance in *Myzus persicae nicotianae* was linked to overexpressed UGT330A3, UGT348A3, UGT349A3 and UGT344D5. After knockdown of the genes, the insects showed higher sensitivity against the plant secondary metabolite, suggesting an involvement in nicotine detoxification (Pan et al., 2019).

1.5 Integrated pest and resistance management

The Food and Agriculture Organization (FAO) defined IPM as "the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that

discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms" (FAO, 2022).

The integration of all possible ways of controlling a pest include the usage of resistant plants as an effective tool for controlling pest species. After planting, the genetic protection against pest attacks does not account additional costs and saves agricultural practices during the growing time. Additionally, the usage of resistant plants is safe for humans and the environment (Dedryver et al., 2010). The host plant *Prunus davidiana* was for example studied having a resistance allele for reducing aphids' phloem sap ingestions (Sauge et al., 2012). Moreover, resistant plant varieties were also studied for controlling whitefly species. For example, the watermelon specie *Citrullus colocynthis* was found being resistant against *B. tabaci* while reducing the size of populations (Simmons & Levi, 2002a; Simmons & Levi, 2002b; Simmons et al., 2010). Besides, crop rotation is well known having a positive impact in pest control management. Multi cropping is defined as the cultivation of two or more crops on same fields within one year (Andrews & Kassam, 1976). In a review by Lopes et al. (2016) the wheat-based crop rotation techniques for reducing pest populations successfully were used.

Cultural practices are also playing a major role in IPM. For example, the sanitation and pre-season cleanup reduces pest population infestations between crop cultivars. Furthermore, the right fertilizer usage is important e.g., nutrient is only applied when needed for optimal growth rates (Rathee et al., 2018). In addition, biological control is also proven to be effective against pest infestations. The predator *Chrysoperla carnea* was shown to reduce whitefly and aphid populations by around 80 % (Younes et al., 2013). However, chemical mechanism remains the most effective strategy for controlling pest species. The usage is economical, flexible to changing ecological or agronomic conditions, rapid in its action and highly effective. Chemical control is a reliable mechanism for effective pest management when the damaging populations converge or overcome the economic threshold (Rathee et al., 2018). However, in 'Integrated Resistance Management' (IRM) the attention of the economic threshold is necessary for knowing when to apply the insecticides. Also, a correct application of the chemicals as well as the regular evaluation of its efficacy is of importance. When an additional insecticide treatment is necessary for controlling the pest species, an insecticide with a different MoA should be used (Koch et al., 2018).

The combination of IPM and IRM is a suitable tool for minimizing upcoming insecticide resistance mechanism in diverse pest species, reducing environmental and human health impacts and decrease site effects to natural enemies.

1.6 Rationales and Objectives

The objective of this thesis was to investigate and uncover multiple resistance cases of Hemipteran crop pests against important chemical classes of insecticides. The global relevance of the green peach aphid *M. persicae* and the pea aphid *A. pisum* as well as the arising importance of the cabbage whitefly *A. proletella* across the world, underlines the importance of IPM strategies. The understanding of the mechanisms of resistances that the species evolved as well as possible cross-resistance cases, are important for future pest management methodologies.

The aim of the first study (chapter 2) was to investigate the mechanism of resistance the green peach aphid evolved against pymetrozine, a TRPV channel modulator. Two ribonucleic acid (RNA)-sequencing (RNAseq) approaches, Oxford Nanopore Technology (ONT) and the gold standard Illumina sequencing were compared. The study aims to find upregulated detoxification enzymes or polymorphism that could be of relevance for pymetrozine resistance in *M. persicae*. Moreover, cross-resistance patterns were evaluated, setting the pymetrozine resistance in concern with other insecticide resistance cases known to be present in green peach aphids.

The second study (chapter 3) aims to uncover pyrethroid resistance present in field populations of pea aphids. This study provides the globally very first evidence of an insecticide resistance case in pea aphids. The usage of a broad range of experiments, resulted in the confirmation of the presence of a resistance mechanism conferring pyrethroid resistance at high levels. Next to molecular investigations (pyrophosphate-based sequencing (pyrosequencing), transcriptome sequencing, real time quantitative polymerase chain reaction (PCR) (RT-qPCR), functional expression of upregulated detoxification genes and biochemical approaches (e.g., 'High Performance Liquid Chromatography' (HPLC) connected with mass spectrometry (HPLC-MS/MS), Michaelis-Menten kinetics), helped to understand the underlying mechanisms of resistance. Besides, the obtained knowledge is the basis for giving advice on pea aphid management strategies including alternative chemical compounds.

In addition, the third study (chapter 3) examined resistance against ketoenol insecticides evolved in the cabbage whitefly *A. proletella*. Because the European based pest is spreading across the globe, the employment of IPM is of high importance. Till now in this species only pyrethroid resistance was detected (Springate & Colvin, 2012), however ketoenol-resistance was found in other whitefly species (Karatolos et al., 2012c; Lueke et al., 2020). The aim of the study was to estimate the ketoenol resistance mechanism in cabbage whiteflies. As this pest was genetically relatively unknown, sequencing approaches were conducted, in order to provide transcriptomic resources for the species. Furthermore, a pyrosequencing protocol was established to increase the monitoring activities to detect resistant populations across Europe.

This helped to give recommendations for IPM strategies to farmers and advisors, as a reaction towards the rapid spread of the species across the globe.

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Chapter 2

Whole genome sequencing study of pymetrozine resistant green peach aphids *Myzus persicae* comparing Illumina Sequencing and Oxford Nanopore Technology

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Abstract

The green peach aphid *Myzus persicae* is a worldwide distributed polyphagous pest species that causes economically important crop losses. Although being controlled with insecticides different classes, several resistance cases were already detected. However, the transient receptor potential vanilloid (TRPV) channel modulator pymetrozine was still reported in being effective for controlling *M. persicae*. In this study the first pymetrozine resistance case with a resistance ration (RR) of 403, in a green peach aphid strain, named 11/18, from Spain was identified. Within dose-response bioassays, pymetrozine resistance as well as cross-resistance to important insecticide classes were tested in several *M. persicae* strains of different geographic origins. Strain 11/18 was found to be cross-resistant to the neonicotinoid acetamiprid (RR of 479) and the pyrethroid deltamethrin (RR of >606). Some individuals of the 11/18 strain were separated for cultivation under selection pressure on 40 ppm pymetrozine insecticide (the resulting strain was called PYR-R1). In addition to the pymetrozine resistant 11/18, PYM-R1 and the susceptible reference strain HS, two nicotinic acetylcholine receptor (nAChR) competitor modulator resistant strains, IMDR and 10/18, were identified in the bioassays conducted. Thereafter, a wide selection of *M. persicae* populations from different European origins was evaluated for the presence of target-site mutations, known causing insecticide resistance in aphids, by pyrophosphate-based sequencing (pyrosequencing) approach. Interestingly, the main differences between the IMDR and 10/18, resistant to nAChR competitor modulators, to the pymetrozine resistant 11/18 / PYM-R1 strain, was the missing knockdown resistance (*kdr*) (L1014F) mutation in the voltage-gated sodium channel (VGSC) in the latter populations as well as different allele binding in super-*kdr* (*skdr*) M918L/T. Moreover, the five strains, 11/18, PYM-R1, 10/18, IMDR and the susceptible HS, which were of most interest, were analyzed in a comparable ribonucleic acid (RNA)-sequencing (RNAseq) approach using Illumina and Oxford Nanopore Technology (ONT). In the PYM-R1 insects, upregulated esterase E4 and FE4-like genes as well as probable cytochrome P450 monooxygenase (P450) 6a13 and 6a14 genes were identified. However, due to low expressions of the Illumina sequenced TRPV channel, no target-site mutations could be evaluated. Besides, for checking sequencing preciseness, a conducted amplicon sequencing approach using ONT, screened the *M. persicae* strains for the presence of the R81T mutation in nAChR as well as the TRPV channel for relevant mutation sites. It could be confirmed that the 10/18, 11/18, PYM-R1 and IMDR strains are carrying the polymorphism R81T. However, although a series of polymorphisms were detected in the TRPV channel, low quality and coverages are reducing the possibility to check their relevance in pymetrozine resistance. In further studies, the involvement of the identified upregulated detoxification genes in green peach aphids' pymetrozine resistance needs to be analyzed. Also, when improving the

sequencing quality, the ONT could be an important tool for direct RNA-sequencing in areas with limited technical resources in the future.

1 Introduction

1.1 The green peach aphid *Myzus persicae*

The green peach aphid *Myzus persicae* is a polyphagous pest species which is worldwide distributed and is known in causing economically important crop losses (Alford, 2007; CABI, 2022; Sharma et al., 2022). Aphids damage plants not only by sucking plant sap but also by transferring more than 100 viruses (Blackman & Eastop, 2017; Coaker, 1992). Green peach aphids are known to feed on more than 40 plant families (Blackman & Eastop, 2017). Next to the heteroecious feeding behavior described for *M. persicae*, some aphid species are known to feed on just one host plant, i.e., living an autoecious life (Leather, 1993). For overcoming winter times, the species can live holocyclic with a sexual production of diapausing eggs in autumn (Blackman, 1975; Margaritopoulos et al., 2002). Besides, green peach aphids can also survive winter times as parthenogenetic morphs (Margaritopoulos et al., 2002). Because of their ability to adapt to environmental conditions, their different color varieties as well as the relationship to their host and life cycle, a good pest management strategy is of high importance (Blackman & Eastop, 2017; Margaritopoulos et al., 2002).

1.2 Insecticide resistance cases in *Myzus persicae*

The ‘Insecticide Resistance Action Committee’ (IRAC) provided management guidelines for *M. persicae* to control or delay insecticide resistance (Bass et al., 2014; IRAC, 2018). The intensive and one-sided usage of insecticides is the reason for numerous insecticide resistance cases described for *M. persicae* (Bass et al., 2014). First resistance cases were identified for carbamate, organophosphates (OP) as well as pyrethroids and caused by the overproduction of the carboxylesterases (CEST) E4 and FE4 (Devonshire & Moores, 1982; Devonshire et al., 1983; Needham & Sawicki, 1971). In the following years, there were globally numerous resistance cases reported. Next to the overexpressed E4 and FE4 genes, polymorphisms in other genes were identified in causing resistance to insecticides of different classes. For example, the amino acid substitution, S431F in acetylcholinesterase (AChE) confers insensitivity of AChE against the carbamate class of insecticides (Andrews et al., 2002; Nabeshima et al., 2003). Moreover, knockdown resistance (*kdr*), L1014F, and its allelic form super-*kdr* (*skdr*), M918L/T, in voltage-gated sodium channels (VGSC) were reported in causing pyrethroid resistance in green peach aphids (Eleftherianos et al., 2008; Fontaine et al., 2011; Martinez-Torres et al., 1997; Martinez-Torres et al., 1999; Panini et al., 2015). Furthermore, the intensive usage of cyclodiene insecticides (e.g., dieldrin), triggered the development of an A302S mutation in the gamma-aminobutyric acid (GABA)-gated chloride channels, encoded by the ‘resistance to dieldrin’ (rdl) gene (Anthony et al., 1998). All these mechanisms did not confer cross-resistance to a new class of insecticides introduced in the

early 1990s, the neonicotinoids (Nauen & Denholm, 2005). However, after several years of use, an overexpressed cytochrome P450 monooxygenase (P450), CYP6CY3, was shown to confer low resistance to neonicotinoid insecticides as well as nicotine, a secondary plant metabolite (Bass et al., 2013; Puinean et al., 2010). However, not only the CYP6CY3 gene was associated with neonicotinoid resistance, but also an upregulation of genes, which encode cuticular proteins involved in penetration resistance (Puinean et al., 2010). After almost 20 years of use, a target-site mutation was identified in a *M. persicae* clone from Southern France by sequencing of the nicotinic acetylcholine receptor (nAChR), the neonicotinoid target-site. A point mutation R81T in the region of loop D of the beta 1 (β1)-subunit was identified and shown to confer high levels of resistance (Bass et al., 2011). The nAChR β1-subunit is split into loop D, E and F. The former is, when combined with the loops A, B and C of subunit α, the binding site of acetylcholine as well as of specific agonists, such as neonicotinoids (Bass et al., 2014; Grutter & Changeux, 2001). Furthermore, the IRAC is recommending chordotonal organ transient receptor potential vanilloid (TRPV) channel modulators like pymetrozine for green peach aphid control (IRAC, 2018). The insect TRPV channel consists of two protomers, Nanchung (NAN) and Inactive (IAV), which are located in the chordotonal organs (Gong et al., 2004; Kim et al., 2003). The TRPV channel modulators are targeting nerve and muscle function in insects, resulting in a feeding stop, inability to walk and eventually death of the pest species (IRAC International, 2021a). There was no TRPV channel modulator resistance identified in green peach aphids yet (Mota-Sanchez & Wise, 2022).

1.3 History of RNA and DNA sequencing systems

For detection of the nucleotide order in ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) molecules, sequencing methods have developed rapidly in the last decade (Grada & Weinbrecht, 2013). First-generation, Sanger sequencing, was used since 1975 and became popular due to the Human Genome Project which was completed in 2003 (Grada & Weinbrecht, 2013; Sanger et al., 1977). Since that time, next generation sequencing (NGS) was developed, which allowed high throughput sequencing with lower costs (Grada & Weinbrecht, 2013). A main difference to first-generation sequencing, is the usage of cell-free NGS libraries, instead of the previous method where DNA fragments were bacterial cloned. Additionally, no electrophoresis is necessary to detect the sequencing output. It is directly visible through the cyclic and parallel performed base interrogation (Margulies et al., 2005; van Dijk et al., 2014). The pyrophosphate-based sequencing (pyrosequencing) was the first technology that was launched in the market as NGS method (Margulies et al., 2005; van Dijk et al., 2014). With the introduction of Illumina sequencing, a technology with lower costs per sequence, high-throughput and great coverage was developed. Next to high scale sequencing methods as HiSeq2000 of Illumina with over 50 Gb sequencing productions per day, was the

MiSeq platform developed for 1.5 Gb sizes per day, 150-bases paired-end reads for 5 M times (Caporaso et al., 2012). Furthermore, with Oxford Nanopore Technology (ONT) a third-generation sequencing technology was introduced into the market (Dumschott et al., 2020). This platform does not need a DNA fragment sharing. Also, no amplification step or the usage of fluorescent labeled nucleotides is necessary. Additionally, for the detection of fluorescent labels the system is not dependent on the installation of optical instrumentations (Masoudi-Nejad et al., 2013). The nanopore sequencing system consists of two proteins, whereas the upper one separates the double-stranded DNA in its single strands. The single-stranded DNA is threading through the proteins to the nanopore. The second protein sits above the membrane and forms the microscopic pore into it. Inside the protein is an adapter molecule which controls the speed of nucleotides that pass through the nanopore. Due to the flow of passing ions, a current is created. With the continuous flow of bases, the current is altered base dependent. The controlled flow of nucleotides through the adapter allows an exact identification of bases in the sequenced DNA strand (Masoudi-Nejad et al., 2013).

1.4 Aim of the study

In this study, pymetrozine resistance and cross-resistance pattern in *M. persicae* strains of different geographic origin, were analyzed. Utilizing of two direct RNAseq approaches with a subset of samples allowed a direct comparison of both sequencing systems, NGS technology Illumina and ONT. Both technologies were used for the evaluation of differential expressed genes (DEGs) with special reference to upregulated detoxification genes, as well as polymorphisms in TRPV, the target-site of pymetrozine, and other targets. Besides, an amplicon sequencing was conducted using ONT, for specifically analyzing mutations in TRPV channel. The sequencing of the nAChR β1-subunit and its relevant mutation site R81T, helped to evaluate the sequencing preciseness of ONT as well. This study deals with challenges and expectations of both sequencing systems and uncovers the suitability of using ONT in small scale applications in sample collection areas with limited technical resources, because the system is portable. Finally, a first resistance management advice is given, for dealing with pymetrozine resistance in *M. persicae*.

2 Material and methods

2.1 Insects

Green peach aphid strains of different origins were reared in the laboratory for studying their insecticide resistance mechanisms (Table 1). All strains were reared on untreated Chinese cabbage *Brassica pekinensis*. After the detection of imidacloprid resistance in the IMDR strain as well as pymetrozine resistance in the 11/18 strain, aphids of these strains were cultivated under insecticide selection pressure. The selected 11/18 strain, named PYM-R1, was reared under continuous treatments with 40 ppm pymetrozine, while strain IMDR was cultivated on Chinese cabbage plants treated with 100 ppm imidacloprid. All *M. persicae* populations were evaluated for their insecticide resistance in bioassay studies and sequenced for known mutation sites using pyrosequencing method. Of high interest for this study were especially the pymetrozine resistant 11/18 population, the selected PYM-R1, the nAChR competitor modulator resistant strains 10/18 and IMDR as well as the susceptible HS strain. These five strains were further evaluated in an amplicon sequencing with ONT as well as RNAseq studies using Illumina. The susceptible HS strain as well as PYM-R1 were also evaluated in an RNAseq approach with ONT. All aphid strains were maintained at the following conditions: 23 ± 1 °C, 50 % relative humidity and a photoperiod of L 16h:D 8h.

Table 1. Characteristics and origin of different *Myzus persicae* strains used in this study.

Name	Year	Country	Venue	Host
HS	1967	Germany	Bonn, North Rhine-Westphalia	-
3/14	2014	Australia	Canberra, New South Wales	-
13/15	2015	France	Poitou, Nouvelle-Aquitaine	Winter oilseed rape
20/15	2015	Netherlands	Grashoek, Limburg	-
7/16	2016	Greece	Meliki, Central Macedonia	Peach/Nectarine
IMDR	2017	UK	Harpenden, Hertfordshire	Cabbage; selected: 100 ppm imidacloprid
2/18	2018	Italy	Cerveteri, Rom	Peach
3/18	2018	Italy	Canosa di Puglia, Barletta-Andria Trani	Peach
7/18	2018	Italy	Spinazzino, Ferrara	Nectarine
9/18	2018	Italy	Bologna, Emilia Romagna	Peach

Name	Year	Country	Venue	Host
10/18	2018	Spain	Jumilla, Murcia	Flat peach
11/18	2018	Spain	Saragossa, Aragon	Nectarine
PYM-R1	2018	Spain	Saragossa, Aragon	11/18 selected: 40 ppm pymetrozine
12/18	2018	France	Vernaison, Auvergne-Rhône-Alpes	Peach
13/18	2018	France	Mornant, Rhône-Alpes	Peach

2.2 Chemicals

All chemicals used in this study were of analytical grade. Pymetrozine, deltamethrin, flonicamid, chloroform, Triton X-100 and agar were purchased from Sigma-Aldrich. Pyrifluquinazon, afidopyropen, sulfoxaflor, pirimicarb, flupyradifurone and imidacloprid were of analytical grade and provided internally. Acetamiprid was ordered from Thermo-Fisher Scientific and Trizol Reagent from Invitrogen, Thermo-Fisher Scientific (Waltham, MA, USA).

2.3 *Myzus persicae* chordotonal organ modulator toxicity bioassays

The bioassays were conducted according to the IRAC susceptibility test method 019 (IRAC International, 2021b). After preparation of a 1.5 % agar solution filled in pots, leaves of Chinese cabbage were dipped for three seconds in different insecticide concentrations. Full dose response assays with concentration between 0.003 to 1,000 ppm of the chordotonal organ TRPV channel modulators pymetrozine, pyrifluquinazon and afidopyropen, were executed. Flonicamid, a chordotonal organ modulator with undefined target-site, was tested in full dose response assays as well. Pymetrozine was also tested in a single diagnostic dose assay of 8 ppm against all *M. persicae* populations reared in the laboratory (Table 1). All dilutions were prepared with aqueous 0.02 % Triton-X100, which also served as control treatment. The dipped leaves were dried for around 20 min and placed onto the prepared agar. Thirty insects per concentration were tested in total, ten per replicate. All experiments were evaluated after 72 h and the number of alive, dead and symptomatic aphids was counted. The latter was estimated by uncontrolled movement of body parts, as legs and antenna. All results were corrected for control mortality using Abbott's formula (Abbott, 1925). Mean values ± SD (n=30) and significant differences between strains were calculated with Graph Pad Prism v8 (GraphPad Prism Inc., CA, USA) and Polo PC (LeOra Software, Berkeley, California) software.

2.4 *Myzus persicae* cross-resistance bioassays

Additionally, cross-resistance levels of the analyzed *M. persicae* strains to insecticides of different classes registered in the EU were examined. Full dose response studies were performed for testing the nAChR competitive modulators acetamiprid, flupyradifurone and sulfoxaflor, the pyrethroid deltamethrin as well as the AChE inhibitor pirimicarb. The studies were mainly executed with the most important *M. persicae* populations 11/18, PYM-R1, 10/18, IMDR and the susceptible reference strain HS. Additionally, all *M. persicae* populations reared in the laboratory (Table 1) were evaluated in their acetamiprid and sulfoxaflor resistance in a 8 ppm single discriminating dose assay. In addition, the neonicotinoid imidacloprid was tested in concentrations of 10 and 100 ppm against the HS, IMDR and 11/18 insects. The evaluation and analysis of the performed cross-resistance bioassays were done as described in 2.3.

2.5 Pyrosequencing of *Myzus persicae* strains

All strains were evaluated for the presence of common mutations described above, such as *kdr*, *skdr*, modified acetylcholinesterase (MACE) and R81T. Before sequencing, the genomic DNA of single green peach aphids (n=10) was isolated in 200 µL nuclease-free water and homogenized with 3 mm grinding balls (Qiagen) in a bead mill (Retsch MM300). After performing two cycles at 25 Hz for 30 s, homogenates were incubated at 65 °C for 15 min. When completed, an additional homogenization step using the bead mill at 20 Hz for 15 s was conducted. The DNA was transferred to a polymerase chain reaction (PCR)-plate and incubated for another 5 min at 98 °C using a C1000 Touch Thermal Cycler (Bio-Rad). The isolated genomic DNA was subjected to PCR using the 2 x JumpStartTM Taq Ready Mix (Sigma-Aldrich) as polymerase and performed with the C1000 Touch Thermal Cycler (Bio-Rad). The pyrosequencing approach was conducted using the PyroMark Q96 ID (Qiagen) as well as the PyroMark Gold Q96 Reagents (Qiagen) according to the manufacturers' instructions. The sequencing primers to detect the amino acid substitutions L1014F, M918L/T, R81T and S431F were included in the assays (Table S1). After completing the pyrosequencing approach, the PyroMark Q96 ID Software 2.5 (Qiagen) converted the resulting data into pyrograms which were evaluated for the presence of target-site mutations.

2.6 RNA-sequencing of *Myzus persicae* strains

Besides, an RNAseq approach using Illumina sequencing was performed for the most important green peach aphid strains HS, 11/18, PYM-R1, IMDR as well as 10/18. ONT was used for an additional transcriptome sequencing of the strains HS and PYM-R1. For isolating necessary RNA, pools of 10 insects per sample were flash frozen and homogenized with 3 mm stainless steel beads at 20 Hz for 2 x 10 s with a MM300 laboratory bead mill (Retsch).

The crushed insects were incubated for 5 min at room temperature, after 0.5 ml Trizol (Qiagen) was added. Afterwards, 100 µL chloroform were pipetted to the homogenized samples, inverted for 15 s and incubated for 3 min at room temperature. A centrifugation step with 10,000 *x g* for 15 min at 4 °C followed. For further RNA purification with the RNeasy Mini Kit (Qiagen), 200 µL of the aqueous phase were used. High quality RNA (OD 260/280 1.8-2.0 and OD 260/230 2.0-2.2) measured with the Infinity M200Pro plate reader using a Nano Quant plate (Tecan Trading AG), were utilized for RNA library preparation. A TruSeq stranded messenger RNA (mRNA) (poly (A) enriched) was performed externally and created according to manufacturer's instructions as well as sequenced using NextSeq (100 bp paired end, 16 M reads). Direct RNA sequencing was performed using the ONT GridION instrument. In total, a sequencing depth of 20 M paired end reads were anticipated in Illumina libraries whereas approximately 1 M single RNA molecule reads were sequenced using ONT. Long reads from ONT sequencing were aligned to the reference genome with minimap2.17 and quantified with NanoCount v0.1.a4 (Gleeson, et al., 2022). Short reads were aligned to the genome with STAR aligner and quantified via RSEM. Conversion of alignment files was performed with samtools v1.9 (Danecek, et al., 2021). Differential gene expression of ONT and Illumina derived gene counts was performed with DESeq2 v1.26.0 (Love, et al., 2014). Gene ontology (GO) enrichment was performed with goseq v1.38.0 (Young, et al., 2010). The results were evaluated for DEGs and their function as well as relevant target-site mutations in the TRPV channel of the insects.

2.7 Amplicon sequencing of *Myzus persicae* strains

In addition, an amplicon sequencing was performed for testing the sequencing precision of the ONT device, by screening the nAChR for the presence of the R81T mutation. Also, NAN and IAV of the TRPV channel were analyzed for relevant target-site mutations. Therefore, the DNA of *M. persicae* population IMDR, 10/18, 11/18, PYM-R1 and the susceptible HS were isolated. Pools of ten insects per strain were extracted using the DNeasy Blood and Tissue Kit (Qiagen). After concentration and quality measurement with the Nano Quant plate and the Infinity M200Pro plate reader (Tecan Trading AG), PCRs were conducted. Multiple amplicons of nAChR β1-subunit for analyzing R81T mutation as well as IAV and NAN for checking target-site mutations in TRPV channel, were prepared. As polymerase, the Platinum SuperFi PCR Master Mix (Invitrogen, Thermo-Fisher Scientific) was used. Due to their length of > 40 kb, NAN and IAV were amplified in overlapped pieces. IAV was split into three and NAN into seven PCR reactions, each with sequence length of four to seven kilo bases (Table S2). The duplicated fragments were purified using the AM-Pure PCR purification kit (Beckman Coulter). The final concentration was measured with the Qubit 3.0 Fluorometer (Invitrogen, Thermo-

Fisher Scientific). The amplicons were pooled per strain and sequenced using ONT MinION. Sequences were aligned to the reference genome of *M. persicae* (GenBank ID: GCA_001856785.1) using minimap v2.17 and post processed using samtools v1.9 (Danecek, et al., 2021). Variants were detected using deepvariant v1.1.0 (Poplin, et al., 2018) and further analyzed with Geneious Bioinformatic Software v2019.1.3.

3 Results

3.1 *Myzus persicae* chordotonal organ modulator toxicity bioassays

In dose-response toxicity bioassays, the 11/18 strain exhibited pymetrozine resistance ratios (RR) of >400 when compared to the susceptible reference strain HS (Table 2). In contrast, the 10/18 and IMDR strains showed lower RR. Cross-resistance to pyrifluquinazon was not observed in any of the tested strains. Whereas afidopyropen resistance was found in strains 11/18 and IMDR, resulting in RRs of 16 and 36, respectively. The 8 ppm pymetrozine single-dose toxicity assay with all *M. persicae* strains (Table 1), identified two strains (3/14 and 2/18) with approximately 40 % mortality after 72 h insecticide exposure (Table S3). All other strains tested showed 60 to 100 % mortality after single-dose pymetrozine exposure. Flonicamid resistance was rather low, particularly in strain 11/18, excluding cross-resistance issues upon pymetrozine selection (Table 2).

Table 2. Log-dose probit-mortality data for chordotonal organ modulator insecticides against *Myzus persicae* adults (n=30) of different strains. Evaluation of affected green peach aphids was done 72 h after leaf-dip bioassay application. Abbreviations: IRAC, Insecticide Resistance Action Committee; TRPV, Transient receptor potential vanilloid.

IRAC insecticide class	Insecticide	Strain	LC_{50} [mg L^{-1}]	95% CI[a]	Slope ± SE	RR[b]
Chordotonal organ TRPV channel modulators	pymetrozine	HS	0.14	0.06-0.3	0.9 ± 0.06	
		IMDR	0.78	0.47-1.33	0.84 ± 0.05	5.57
		10/18	0.39	0.08-1.69	0.49 ± 0.04	2.79
		11/18	56.4	25.5-129	1.11 ± 0.08	403
	pyrifluquinazon	HS	0.49	0.42-0.58	3.12 ± 0.31	
		IMDR	0.99	0.23-5.49	0.98 ± 0.06	2.02

IRAC insecticide class	Insecticide	Strain	LC_{50} [mg L^{-1}]	95% CI[a]	Slope ± SE	RR[b]
Chordotonal organ TRPV channel modulators	pyrifluquinazon	11/18	2.51	1.84-3.43	1.53 ± 0.11	5.12
	afidopyropen	HS	0.01	0.01-0.02	23.3 ± 2.65	
		IMDR	0.36	0.3-0.45	1.81 ± 0.13	36
		11/18	0.16	0.1-0.25	1.58 ± 0.1	16
Chordotonal organ modulators – undefined target-site	flonicamid	HS	0.41	0.19-0.94	1.5 ± 0.1	
		IMDR	6.53	1.82-23	1.2 ± 0.08	15.9
		11/18	3.19	1.27-8.62	1.18 ± 0.07	7.78

[a] 95% confidence interval (95% CI); [b] Resistance ratio (RR) = LC_{50} of each strain divided by LC_{50} susceptible HS.

3.2 *Myzus persicae* cross-resistance bioassays

Due to resource restrictions, not all *M. persicae* strains could be tested in full dose response, however, all dose response data are compiled in table 3. Strain 11/18 showed cross-resistance to different insecticides such as acetamiprid and deltamethrin when compared to the HS strain. The cross-resistance to flupyradifurone, sulfoxaflor and pirimicarb in strain 11/18 was less pronounced. Strain PYM-R1 was tested in full dose response assays with flupyradifurone, deltamethrin and pirimicarb. Comparable RR as strain 11/18 were received when compared to HS, except for flupyradifurone exposure whereof it was less affected. The IMDR and 10/18 strains showed resistance against the neonicotinoid acetamiprid (RR of approximately 90 and 260) and were also tested under sulfoxaflor exposure. Both populations showed cross-resistance levels (Table 3).

Moreover, all *M. persicae* populations were analyzed for cross-resistance levels in single-dose assays with 8 ppm acetamiprid and sulfoxaflor. Individuals of strains 3/14 and 2/18 survived after exposure to 8 ppm acetamiprid in leaf-dip bioassays (Table S3). Nearly all insects of the 7/18 were still alive after the treatment as well. Strains 12/18 and 13/18 showed mortality rates of around 30 %, whereas all other strains tested, died nearly completely. The same was observed for 8 ppm sulfoxaflor treatments, except for strain 2/18. Single-dose leaf-dip bioassays using 10 and 100 ppm imidacloprid were conducted with strains HS, IMDR and 11/18 (Table S4). In contrast to the susceptible reference strain, approximately 20 % of the

aphids of strain 11/18 treated with 100 ppm imidacloprid died. All aphids of this strain were still alive after the 10 ppm imidacloprid application. The IMDR strain showed full resistance to both imidacloprid concentrations.

Table 3. Log-dose probit mortality data of insecticides with different Mode of Actions (MoA) against *Myzus persicae* adults (n=30) of different strains. Evaluation of affected green peach aphids was done 72 h after leaf-dip bioassay application. Abbreviations: AChE, Acetylcholinesterase; IRAC, Insecticide Resistance Action Committee; nAChR, Nicotinic acetylcholine receptor.

IRAC insecticide class	Insecticide	Strain	LC_{50} [mg L^{-1}]	95% CI[a]	Slope ± SE	RR[b]
nAChR competitive modulators - Neonicotinoid	acetamiprid	HS	0.14	0.09-0.22	1.14 ± 0.07	
		IMDR	13.1	2.95-168	0.39 ± 0.03	93.6
		10/18	36	8.97-624	1.11 ± 0.09	257
		11/18	67	19.3-414	0.58 ± 0.04	479
- Butenolides	flupyradifurone	HS	2.04	1.74-2.38	3.3 ± 0.36	
		11/18	10.9	2.58-67.6	1.34 ± 0.09	5.34
		PYM-R1	130	47.2-825	0.77 ± 0.07	63.7
nAChR competitive modulators - Sulfoximines	sulfoxaflor	HS	0.02	0.02-0.03	3.07 ± 0.27	
		IMDR	3.67	1.21-14.1	0.7 ± 0.04	184
		10/18	0.95	0.26-3.93	0.88 ± 0.05	47.5
		11/18	0.34	0.12-0.9	0.71 ± 0.04	17
Sodium channel modulators	deltamethrin	HS	0.33	0.18-0.63	1.29 ± 0.08	
		11/18	>200	-	-	>606
		PYM-R1	>200	-	-	>606
AChE inhibitors	pirimicarb	HS	1.25	0.87-1.82	1.92 ± 0.15	
		11/18	13.8	8.73-22.4	1.35 ± 0.1	11
		PYM-R1	6.87	3.76-12.8	1.23 ± 0.08	5.5

[a] 95% confidence interval (95% CI); [b] Resistance ratio (RR) = LC_{50} of each strain divided by LC_{50} susceptible HS.

3.3 Pyrosequencing of *Myzus persicae* strains

After bioassay-based toxicity studies, molecular diagnostic of four known polymorphisms being present in *M. persicae* was performed using pyrosequencing method (Table 4). Strain IMDR was homozygote resistant (HoR) for L1014F, M918T and R81T mutation, but homozygote susceptible (HoS) for S431F polymorphism. Strain 10/18 was mostly HoS and contained 12 % heterozygote resistant (HeR) aphids for the L1014F mutation. The aphids were also tested for the presence of the *skdr* mutation. It could be shown that most of the tested 10/18 aphids were HoR carrying both M918T/L, while approximately 40 % of the aphids were HoR (M918L). For the presence of the R81T mutation, HeR and HoR 10/18 insects were measured. None of the 10/18 individuals tested carried the MACE mutation S431F. Furthermore, all insects of the 11/18 strain were analyzed having the *skdr* M918L and R81T polymorphism but being wildtype for *kdr* and MACE. The susceptible reference strain HS was wildtype for all evaluated polymorphisms.

Table 4. Pyrosequencing of individual *Myzus persicae* adults (n=5-10) of different populations for detection of knockdown resistance (*kdr*) L1014F and super-*kdr* (*skdr*) M918L/T as well as the nicotinic acetylcholine receptor (nAChR) substitution R81T and the modified acetylcholinesterase (MACE) polymorphism S431F. Data shows the number of aphids [%] being homozygote susceptible (HoS), heterozygote resistant (HeR) or homozygote resistant (HoR) for the relevant mutation site.

	L1014F (*kdr*)			**M918L/T (*skdr*)**						**R81T (nAChR)**			**S431F (MACE)**		
Strain	HoS	HeR	HoR	HoS	HeR(T)	HeR(L)	HoR(T)	HoR(L)	HoR(T/L)	HoS	HeR	HoR	HoS	HeR	HoR
HS	100	0	0	100	0	0	0	0	0	100	0	0	100	0	0
3/14	100	0	0	0	0	100	0	0	0	100	0	0	0	100	0
13/15	100	0	0	0	0	100	0	0	0	100	0	0	0	100	0
20/15	100	0	0	0	0	100	0	0	0	100	0	0	0	100	0
7/16	100	0	0	0	0	100	0	0	0	100	0	0	0	100	0
IMDR	0	0	100	0	0	0	100	0	0	0	0	100	100	0	0
2/18	25	75	0	0	50	0	0	12	38	0	12	88	100	0	0
3/18	12	63	25	0	13	0	25	12	50	50	50	0	63	37	0
7/18	0	75	25	0	25	0	25	0	50	50	50	0	50	38	12
9/18	0	100	0	0	0	0	0	0	100	100	0	0	74	13	13
10/18	88	12	0	0	0	0	0	37	63	0	37	63	100	0	0
11/18	100	0	0	0	0	0	0	100	0	0	0	100	100	0	0

	L1014F (*kdr*)			M918L/T (*skdr*)						R81T (nAChR)			S431F (MACE)		
Strain	HoS	HeR	HoR	HoS	HeR(T)	HeR(L)	HoR(T)	HoR(L)	HoR(T/L)	HoS	HeR	HoR	HoS	HeR	HoR
12/18	0	100	0	0	63	0	0	0	38	0	100	0	100	0	0
13/18	0	63	37	0	50	0	38	0	12	12	88	0	88	12	0

3.4 RNAseq using Illumina sequencing technology

3.4.1 *Myzus persicae* gene expression profiles

Gene expression profiles of *M. persicae* strains were compared across the susceptible strain and some resistant populations employing Illumina sequencing. Both, the principal component analysis (PCA) and heat map revealed distinct expression patterns for individual strains (Fig. 1 A and B, Fig. S1). The variance in the principal component (PC) PC1 was 37.2 % and showed that the susceptible HS is well separated from the insecticide resistant strains (Fig. 1A). The PC2 is explaining 20.8 % of variance and split the analyzed samples into treated and non-treated. Here, the PYM-R1 and IMDR strains, both maintained under insecticide selection pressure, were separated from the other strains. This could illustrate a xenobiotic response under the continuous insecticide pressure in both strains. The second PCA consists of the PC1 and PC3. The latter is explaining 13 % of variance and is separating the PYM-R1 strain to all other strains analyzed. Only the samples of the 11/18 strain were distributed in all clusters. The third PCA consists of the comparison between PC2 and PC3. Here, the PYM-R1 and IMDR separation to all other strains is obvious again. The susceptible HS is clustered in the same area as the non-treated 11/18 and 10/18 samples. Furthermore, the illustrated heat-map (Fig. 1B) is showing the DEG of the analyzed green peach aphids when compared to the susceptible reference strain HS. The cluster of genes pictures a separation of the susceptible HS and all other analyzed *M. persicae* strains. The same observation was made in a Poisson distance evaluation (Fig. S1).

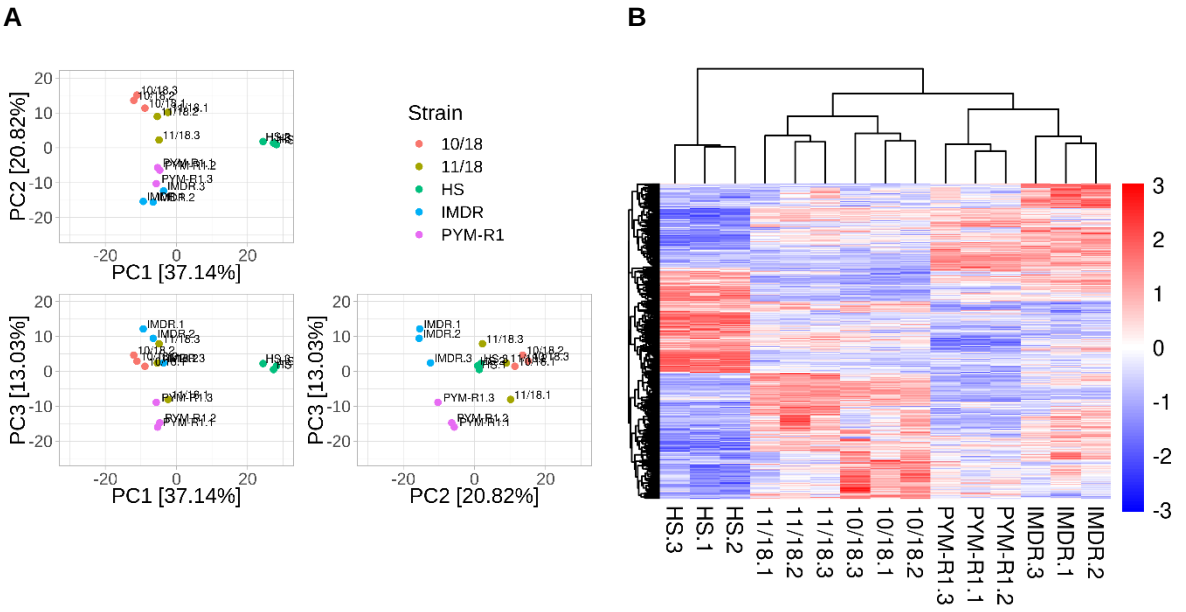

Fig. 1. A) Principal component analysis (PCA) of ribonucleic acid (RNA)-sequencing (RNAseq) data obtained by Illumina sequencing and clustering principal component (PC) 1 and PC2, PC1 and PC3, and PC2 and PC3 together. The PC1 is separating the susceptible reference strain HS from the other sequenced *Myzus persicae* populations 10/18, 11/18, PYM-R1 and IMDR. PC2 is considering the separation of insecticide treated and non-treated populations. PC3 is separating the pymetrozine resistant PYM-R1 to all other strains. **B)** Heat map of expression levels for several up- and down-regulated genes in strain HS compared to all other strains analyzed.

3.4.2 Differentially expressed genes in *Myzus persicae* populations

When considered individually, each insecticide resistant *M. persicae* population analyzed comprised approximately 900 to 1,200 DEGs in comparison to the susceptible reference strain HS. In sum this resulted in 1,910 DEGs found within this study and being present individually or in multiple populations evaluated (Fig. 2A). Of these DEGs, 368 were found to be regulated in all insecticide resistant strains 10/18, PYM-R1, IMDR and 11/18. In PYM-R1, 138 DEGs were detected which were not found to be regulated in any of the other strains (Fig. 2B). This represents 7.2 % of all 1,910 DEGs found within this study (Fig. 2A). Most of the DEGs, 114 in total, being only regulated in PYM-R1, were repressed (Fig. S2), considering a correlation to the continuous selection pressure of 40 ppm pymetrozine. When comparing the results to the DEGs found in the non-selected 11/18 strain, more numbers of induced genes were observed (Fig. S2). The same allocation of individual genes was measured in the IMDR and 10/18 strains. Most of the individual DEGs were induced (Fig. S2).

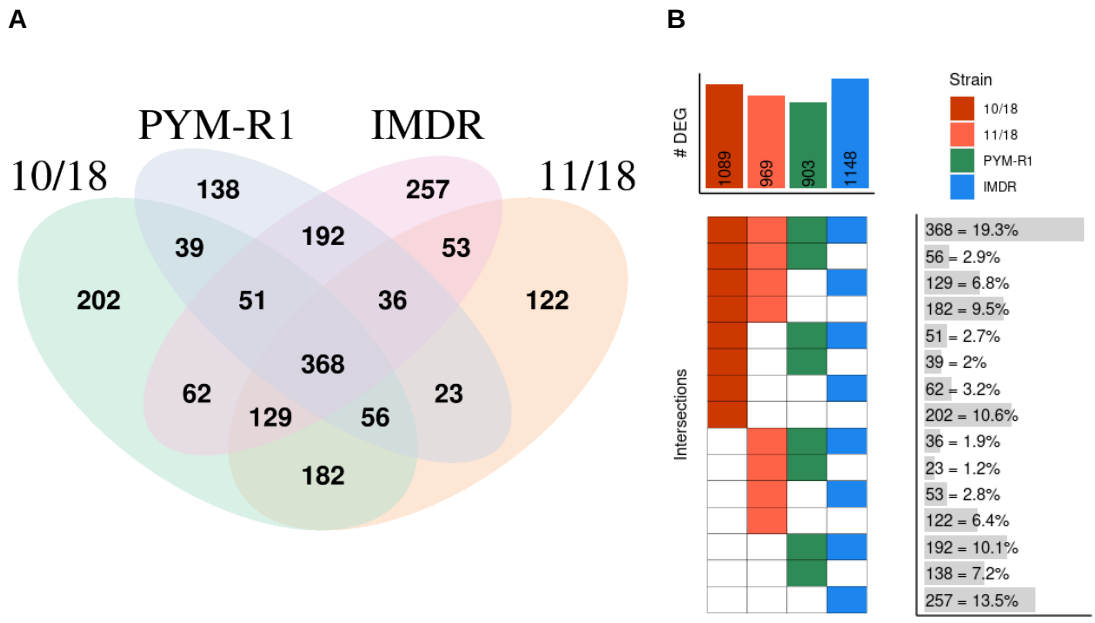

Fig. 2. A) Intersection of differentially expressed genes (DEG) (log_2FC) in *Myzus persicae* strains 10/18, 11/18, PYM-R1 and IMDR when compared to the susceptible reference strain HS, analyzed by RNAseq Illumina sequencing. In sum, 1,910 DEGs were found individually in one or in multiple green peach aphid strains analyzed. **B)** Numbers of DEGs (log_2FC) in *M. persicae* populations when compared to the susceptible reference strain HS. Data in percentage depict the number of genes in comparison to all clustered 1,910 DEGs that were detected in strains 10/18, 11/18, PYM-R1 and IMDR (Fig. 2A).

Furthermore, the detected DEGs of each *M. persicae* strain were screened for their function by GO clustering. The comparison between the pymetrozine resistant PYM-R1 and the susceptible HS revealed significant more DEGs e.g., related to oxidoreductases and proteolysis in the resistant strain (Fig. S3). The same was observed for strain 11/18 (Fig. S4). When comparing both pymetrozine resistant strains, the selected PYM-R1 insects regulated more genes related to DNA binding and structural constituents of cuticle (Fig. S5). The 11/18 aphids were analyzed having significant more DEGs with activities in e.g., zinc ion binding, DNA integration, responses to oxidative stress, peroxidase, nuclease and carbohydrate metabolic process. Among others, the 10/18 as well as IMDR strains exhibit high numbers of detoxification genes when compared to strain HS (Fig. S6, S7). Most of the upregulated genes belong to the GO terms oxidation-reduction process and proteolysis.

3.4.3 Mutations located in the TRPV channel of *Myzus persicae* populations

The expression levels of the subunits NAN and IAV were too low in the analyzed *M. persicae* populations to detect any mutations by Illumina sequencing technology.

3.5 Comparative analysis of Oxford Nanopore and Illumina sequencing RNAseq approaches

In a following study, the PYM-R1 sequencing results observed with Illumina sequencing (3.4.2) were compared to the PYM-R1 sequencing data obtained by direct RNAseq study with ONT. The measured data of PYM-R1 was mapped to the reference genome of the susceptible strain HS. Genes of the resistant strain that were found with ONT positively correlated with the ones identified by Illumina (Fig. S8A and B). Generally, genes with a high sequencing expression (base mean) in Illumina, were also detected with ONT. Ninety-two induced and 30 repressed DEGs were identified by both sequencing technologies (Fig. 3). This means that 20 % of the total 458 induced DEGs and 6.7 % of the total 445 repressed DEGs detected in PYM-R1 by Illumina sequencing were also found with ONT (Fig. S2). Most of the highly overexpressed genes in PYM-R1 which were verified by both sequencing methods were subunits of the respiratory chain and ribosomal proteins. Furthermore, several relevant detoxification genes could be identified in strain PYM-R1 within the RNAseq approaches of both technologies (Table 5). Genes with a high sequencing expression (base mean) in both technologies included esterase genes FE4 and FE4-like as well as E4 and probable cytochrome P450 6a13 and two P450 6a14. Several genes which were detected by Illumina but not by ONT (base mean of approximately zero) can be reviewed in Table S5.

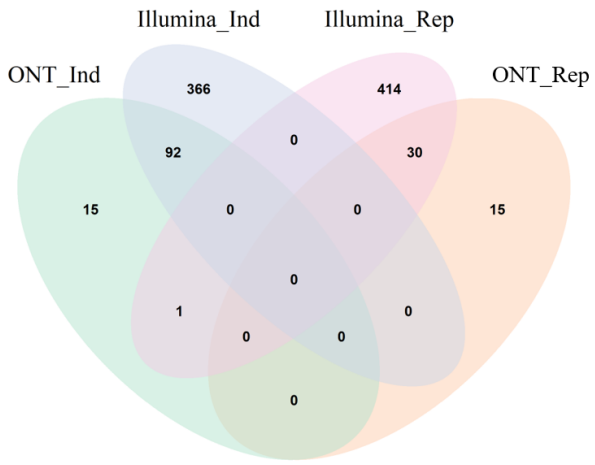

Fig. 3. Intersection of induced and repressed differentially expressed genes (DEG) (log_2FC) in *Myzus persicae* strain PYM-R1 when compared to the susceptible reference strain HS, analyzed by RNAseq Illumina sequencing and Oxford Nanopore Technology (ONT). Abbreviations: ONT_Ind, induced genes found by ONT; Illumina_Ind, induced genes found by Illumina; Illumina_Rep, repressed genes found by Illumina; ONT_Rep, repressed genes found by ONT.

Table 5. Upregulated detoxification genes detected by direct RNAseq approaches with Illumina sequencing and ONT in strain PYM-R1 compared to strain HS.

Transcript ID	Product	Illumina sequencing		ONT	
		Base mean	log_2FC	Base mean	log_2FC
XM_022314770.1	esterase FE4-like	2,810	7.04	0.58	2.56
XM_022316142.1	esterase E4	10,776	6.53	4.1	5.36
XM_022305797.1	esterase FE4-like	5,152	5.67	940	5.98
XM_022305796.1	esterase FE4	4,382	4.56	583	5.88
XM_022320240.1	uncharacterized LOC111037582	2,009	8.16	20.1	5.82
XM_022306862.1	probable cytochrome P450 6a14	5,772	4.55	183	4.63
XM_022306355.1	probable cytochrome P450 6a13	11,550	3.77	563	3.89
XM_022309346.1	probable cytochrome P450 6a13, transcript variant X2	17.4	3.62	0.3	1.57
XM_022314345.1	probable cytochrome P450 6a13	846	3.05	33.1	3.88
XM_022314335.1	probable cytochrome P450 6a14	1,204	2.23	22.5	1.6

3.6 Amplicon sequencing performed with Oxford Nanopore Technology

3.6.1 Sequencing of the nAChR β1-subunit for the presence of the R81T mutation

Amplicon sequencing by ONT of 10/18, 11/18, PYM-R1, IMDR and HS *Myzus persicae* samples revealed the presence of the R81T mutation. These results confirm the data obtained by pyrosequencing (3.3, Table 4). Besides, several additional mutations were found, but in non-coding (intron) regions of nAChR β1-subunit gene. No polymorphism was found by ONT in samples prepared from the susceptible reference strain HS.

3.6.2 Mutations located in the TRPV channel of *Myzus persicae* populations

The amplicon sequencing with ONT was also used for sequencing the TRPV channel for relevant mutation sites in the resistant *M. persicae* strains when mapped to the reference genome of HS. Despite the fact, that there were several mutations detected in either NAN or IAV, no polymorphism was found that could be of relevance for insecticide resistance in the

analyzed green peach aphids. The amplicon sequencing of the TRPV channel was limited through homopolymer problems and low sequencing qualities by ONT.

4 Discussion

4.1 *Myzus persicae* resistance to chordotonal organ modulator insecticides

With the introduction of TRPV channel modulators into the market, an increased usage and resistance risk comes along in pest species. In this study, the first resistance case of *M. persicae* against the TRPV channel modulator pymetrozine, is described. *M. persicae* clones with various insecticide resistance mechanisms were examined for their cross-resistance to pymetrozine. However, till now, pymetrozine provided efficacy against a range of green peach aphid populations collected across different geographies (Foster et al., 2002). In contrast, as the bioassays of this study showed, a high pymetrozine resistance was present in strain 11/18 (3.1, Table 2). The same as in previous studies, good efficacies of pyrifluquinazon and afidopyropen against all analyzed *M. persicae* strains were detected (Kang et al., 2012; Koch et al., 2020; Vafaie & Grove, 2018). There is no documented resistance case of any insect pest for both insecticidal compounds described yet (Mota-Sanchez & Wise, 2022). Additionally, good efficacy against green peach aphids resistant to pymetrozine or imidacloprid was also provided by applications with flonicamid in this study. When comparing the RR of <16 in the IMDR insects (Table 2) to documented flonicamid cross-resistance levels observed in a neonicotinoid resistant *Aphis gossypii* strain in previous studies, the detected RR-levels in this study are of minor relevance and rather low (Koo et al., 2014). Also, no relevant cross-resistance (RR of <8) was detected in the pymetrozine resistant strain 11/18 (Table 2). There is also no documented cross-resistance case between pymetrozine and flonicamid yet described in any aphid species (Mota-Sanchez & Wise, 2022).

4.2 Cross-resistance to nicotinic acetylcholine receptor (nAChR) insecticides

Besides the recommendation for using imidacloprid and pymetrozine insecticides in a mixture for increased mortalities of *A. gossypii* populations (Somar et al., 2019), resistance to pymetrozine was already detected in the Hemipteran crop pest *Bemisia tabaci* (Elbert & Nauen, 2000; Gorman et al., 2010). Later it was found that the lack of efficacy is based on an overexpressed P450 gene, CYP6CM1, which was detected in being also responsible for imidacloprid resistance in *B. tabaci* (Karunker et al., 2008; Nauen et al., 2013; Qiong et al., 2012). A correlation between pymetrozine and neonicotinoid resistance was also described in *Trialeurodes vaporariorum* strains (Karatolos et al., 2010). The same observation was made in the present study. Cross-resistance to nAChR competitive modulators was detected in the pymetrozine resistant 11/18 strain. First identified with high cross-resistance ratios of 479

against the neonicotinoid acetamiprid (Table 3, Table S3), was the 11/18 strain also analyzed with imidacloprid resistance (Table S4), confirming observations mentioned above. Also, the additional set of 8 ppm single-dose toxicity bioassays revealed cross-resistance between pymetrozine and nAChR competitive modulators in several *M. persicae* populations. In contrast, the highly imidacloprid resistant IMDR strain (Table S4) was not measured with cross-resistance to pymetrozine, although being resistant to acetamiprid and sulfoxaflor (Table 3).

The non-toxicity of neonicotinoids measured in that study can be explained by the presence of the R81T mutation in the β1-subunit of the nAChR. The polymorphism was reported in causing reduced sensitivity to neonicotinoids such as imidacloprid (Bass et al., 2011; Bass et al., 2014). The 11/18, IMDR and 10/18 insects were completely or predominantly HoR for R81T. In addition, other studies have shown that also other nAChR competitive modulator insecticides, such as sulfoxaflor and flupyradifurone, are having binding issues to insects' nAChR due to the presence of the R81T polymorphism (Bass et al., 2014; Cutler et al., 2013). The question of correlation between the mutation site and sulfoxaflor effectivity can also not be answered with this study. While the insecticide resistant *M. persicae* strains 11/18, 10/18 and IMDR were all carrying the R81T mutation, sulfoxaflor was still useful for an effective pymetrozine resistant insect control, but not for an enduring neonicotinoid resistant aphid control. *A. gossypii* field populations were also already measured with resistance levels up to 464 against sulfoxaflor, however, other resistance research studies investigated that there is low to no cross-resistance between neonicotinoids and sulfoxaflor (Koo et al., 2014; Perry et al., 2012). It is likely that the latter compound is less influenced in targeting the nAChR affected by R81T mutation, as it is described for neonicotinoids (Mezei et al., 2022; Wang et al., 2016). The insecticide is also recommended for replacing neonicotinoid insecticides and for an effective aphid control strategy to be applied in rotation with compounds of other chemical classes, such as pymetrozine or flonicamid (Sparks et al., 2013).

Moreover, due to limited resources, the neonicotinoid resistant strains 10/18 and IMDR could not be tested for flupyradifurone cross-resistance, which will be important in future studies. Besides, flupyradifurone caused a low RR of 5.34 in the pymetrozine resistant 11/18, which is of minor relevance. However, there seems to be a correlation of continuous pymetrozine selection pressure and flupyradifurone exposure, as seen in higher resistance levels of 63.7 in PYM-R1. There is no documented pymetrozine and flupyradifurone cross-resistance case described yet, although a first case of flupyradifurone resistance in green peach aphids from Greece was detected recently (Mota-Sanchez & Wise, 2022; Papadimitriou, et al., 2022).

4.3 Cross resistance to the pyrethroid deltamethrin

In contrast, cross-resistance levels to pymetrozine were already reported in deltamethrin and organophosphate resistant whiteflies *B. tabaci* (Houndété et al., 2010). Also in this study, high cross-resistance levels to deltamethrin were measured in strain 11/18 and PYM-R1 with RR of over 600-fold (Table 3). Generally, pyrethroid resistance in insects is often related to *kdr* (L1014F) and *skdr* (M918L/T). The *kdr* and *skdr* are known mutation sites that cause pyrethroid resistance not only in green peach aphids (Eleftherianos et al., 2008; Fontaine et al., 2011; Martinez-Torres et al., 1997; Martinez-Torres et al., 1999; Panini et al., 2015), but also in several other insect pests, such as mosquitos (Bkhache et al., 2016; Ibrahim et al., 2014; Martinez-Torres et al., 1998) or greenhouse whiteflies *T. vaporariorum* (Karatolos et al., 2012).

In this study, the high cross-resistance levels to deltamethrin observed in the pymetrozine resistant strains 11/18 and PYM-R1, are not related to *kdr* but to *skdr*. Aphids of strain 11/18 were completely HoS for *kdr* but HoR for *skdr* M918L (Table 4). Unfortunately, limited resources have not allowed the testing of deltamethrin cross-resistance in the neonicotinoid resistant strain IMDR. However, the molecular diagnostic with pyrosequencing revealed the presence of the *kdr* and *skdr* mutations in the *M. persicae* population. All insects of strain IMDR carried the L1014F and M918T polymorphism.

The difference between the pymetrozine resistant strain 11/18 and the nAChR competitive modulator resistant IMDR insects, was the missing *kdr* mutation in the former ones as well as the different allele binding in *skdr*. While the 11/18 insects were measured with the amino acid substitution of M918L, showed the IMDR strain the allele binding to Threonine instead of Methionine.

4.4 Cross-resistance to the AChE inhibitor pirimicarb

Moreover, because an application program of pirimicarb and pymetrozine is recommended for effective control of *M. persicae* (Foster et al., 2002), the susceptibility of the pymetrozine resistant 11/18 and PYM-R1 strains to the AChE inhibitor was tested as well (3.2). However, no relevant resistance ratios, that would indicate cross-resistance, were measured (Table 3). This result was also confirmed by the absence of the S431F mutation in AChE in all evaluated insects of strain 11/18 (Table 4). The polymorphism has caused high levels of green peach aphid resistance to the carbamate insecticide pirimicarb previously (Andrews et al., 2002; Nabeshima et al., 2003). However, current results made in this study suggest that pirimicarb seems to be still an effective insecticide to include in integrated resistance management (IRM) strategies for controlling pymetrozine resistant *M. persicae*.

4.5 Direct RNAseq approaches using Illumina sequencing and Oxford Nanopore Technology

Besides the fact, that the expression levels of IAV and NAN were too low for detecting any mutations with Illumina sequencing, several genes, that could be relevant in causing pymetrozine resistance were identified by the sequencing approaches (3.4.2, Fig. 2B). Genes which could be of interest for pymetrozine resistance, could be differentially regulated and only present in the 11/18 and PYM-R1 aphids. The comparable sequencing of PYM-R1 with ONT and Illumina uncovered several genes, that could be involved in pymetrozine resistance. Most relevant could be the detected esterase and P450s genes in PYM-R1 when compared to the susceptible HS, that were found with both sequencing methods (Table 5). Overexpressed E4 and FE4 esterase genes were previously described for causing organophosphate, carbamate and pyrethroid insecticide resistance in *M. persicae* (Devonshire & Moores, 1982; Devonshire et al., 1983; Needham & Sawicki, 1971). One candidate P450 is CYP6CY3 which has been previously shown to confer neonicotinoid insecticide and nicotine resistance (Bass et al., 2013; Puinean et al., 2010). P450s are generally interesting candidates, because it was already shown that pymetrozine resistance in *Nilaparvata lugens* (Hemiptera: Delphacidae) is associated with an overexpression of CYP6CS1 (Wang et al., 2021). The relevance of the upregulated P450 6a13, P450 6a14, esterase E4 and FE4 genes in pymetrozine resistance in green peach aphids needs to be analyzed in future studies. Besides P450s and esterase genes, detected both sequencing devices ribosomal proteins and subunits of enzymes of the respiratory chain in PYM-R1 when compared to the susceptible HS (3.5), although their contribution to the observed resistance is rather unlikely.

4.6 Amplicon sequencing using Oxford Nanopore Technology

With the comparison of the sequencing methods of NGS Illumina and ONT, it could be confirmed that ONT principally provides the same information as Illumina sequencing. First evaluated by pyrosequencing method, the relevant target-site mutation R81T causing nAChR insensitivity to IRAC Mode of Action (MoA) group 4 insecticides in green peach aphids (Bass et al., 2011), was also found in the ONT approach (3.6.1). The technology confirmed that the polymorphism is present in strains 10/18, 11/18, PYM-R1 and IMDR. The results are consistent with previous studies were ONT and Illumina sequencing data correlated (Quick et al., 2016).

4.7 Limitations and Learnings of the study

Generally, the RNAseq study with ONT device in the present work had to deal with two main limitations: a high error and mismatch of sequencing bases as well as a limited yield: A 10-fold higher error rate in comparison to Illumina sequencing could be observed (data not shown).

However, it was demonstrated that error rates decrease within using the ONT: The 10 % error rates detected in this study, were low, when compared to 32 % to 14 % decreases as described in other studies (Norris et al., 2016). However, the limitations of a high error rate and mismatch of sequencing bases became also visible with the low coverages and qualities of the detected polymorphisms in the TRPV channel of *M. persicae* strains (3.6.2). Due to that, no trustable polymorphisms were found that could be of relevance in causing pymetrozine resistance in green peach aphids. Another limiting factor of analyzing and comparing sequencing results in this study, was the software that was used for data evaluation. Many of the software packages favorably used in RNAseq methods are still not optimized for a direct RNAseq approach with ONT. It is expected that mismatches of direct RNA reads will decrease due to the usage of an optimal alignment tool (Garalde et al., 2018).

An advantage of using nanopore sequencer is the possibility of direct sequencing of long RNA sequences (Garalde et al., 2018). RNAseq with other sequencing devices requires a reverse transcription of RNA into complementary DNA (cDNA). Misamplifications or biases are possible strengthens of these additional steps (Kono & Arakawa, 2019). Other studies showed that ONT was winsome for long sequencing reads. Additionally, its portability and affordability will be important features for sequencing projects in field-stations with limited resources (Compton et al., 2020). Despite its high error rate, the MinION instrument was already effective in field studies for identification of the Ebola virus disease in Guinea (Quick et al., 2016). By using the instrument, the sequencing of Ebola virus samples took just 15-60 min and the detection of the virus was possible within 24 h. Additionally, the results were compared to Illumina sequencing and shown to correlate. All in all, in the research of Ebola virus disease identification in Guinea the bioinformatic approach worked quite well and no false positive samples were detected (Quick et al., 2016).

4.8 Summary and outlook

The aim of the study was to uncover candidate genes to be responsible for pymetrozine resistance observed in *M. persicae* strains. By employing an RNAseq approach comparing Illumina with ONT as well as an amplicon sequencing with ONT, the latter revealed high error and mismatching rates causing bad quality of polymorphisms identified in the amplicon sequencing within this study. However, it is expected that mismatches can be decreased when an optimal alignment tool for ONT is developed (Garalde et al., 2018). Nonetheless, the evidence that the ONT device is delivering relevant information, is demonstrated by the fact that the sequencing system successfully identified the R81T mutation in several neonicotinoid resistant *M. persicae* strains. With increased sequencing qualities of the device, the technology

can play an important future role in insecticide target-site resistance diagnostics by direct long-read sequencing, particularly in areas with limited resources.

In the RNAseq approaches of the current study, Illumina and ONT have identified DEGs which revealed overexpressed esterase and P450 genes in the pymetrozine resistant strain PYM-R1. The relevance of the genes in insecticide resistance could not be clarified finally. Due to that, a follow-up evaluation of the genes in RT-qPCR approaches is recommended. Upregulated genes which can be confirmed within this analysis, could also be recombinant expressed in insect cells, for evaluating their capacity to detoxify pymetrozine within pharmacogenetic studies.

Considering the cross-resistance studies conducted, it can be recommended to use sulfoxaflor, pirimicarb and flonicamid for controlling pymetrozine resistant green peach aphids. As shown in the present study, also the TRPV channel modulators pyrifluquinazon and afidopyropen seem to be still active against pymetrozine resistant *M. persicae* aphid populations. In contrast, the pyrethroid deltamethrin is not recommended for pymetrozine IRM strategies. Additionally, the insecticide acetamiprid is not useful, neither to control pymetrozine resistant, nor imidacloprid resistant *M. persicae* insects. In addition, sulfoxaflor is not recommended for controlling aphid strains carrying the R81T mutation in the β1-subunit of the nAChR and being resistant to neonicotinoids. Here, flonicamid could be an effective alternative as well. Imidacloprid resistance, as present in the IMDR strain analyzed, can be still controlled using pymetrozine, due to only minor cross-resistance levels detected in the respective strain analyzed. For an effective IRM strategy, the rotation of the recommended insecticides with different MoA is of high importance.

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Chapter 3

Molecular characterization of pyrethroid resistance in field-collected populations of the pea aphid, *Acyrthosiphon pisum*

Short title: Metabolic resistance to pyrethroids in pea aphids

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Abstract: *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) is a globally distributed and economically important aphid pest of leguminous crops including peas, broad beans, and alfalfa. Infestations weaken plants directly by feeding, and indirectly, by the transmission of yield-affecting plant viruses. Although pea aphid control strategies largely rely on the application of insecticides, cases of insecticide resistance under applied conditions have not yet been described. Here we investigated the molecular basis of high levels of pyrethroid resistance (up to >180-fold) in two strains of *A. pisum*, PYR-R1 and VR, collected in the field in France and Italy, respectively. Foliar bioassays revealed significant synergistic effects between deltamethrin and piperonyl butoxide (a cytochrome P450 (P450) inhibitor), and, to a lesser extent, S,S,S-tributyl phosphorotrithioate (an esterase inhibitor). P450 and esterase activities in both resistant strains were significantly higher compared to an insecticide susceptible reference strain SUS-1. Target-site resistance mutations in the voltage-gated sodium channel known to confer pyrethroid resistance in other aphid pest species were not detected in either resistant strain. However, transcriptome profiling identified the P450 gene *CYP6CY12* as highly over-expressed in the resistant PYR-R1 strain compared to the SUS-1 strain, and real-time quantitative PCR showed that this gene is also significantly overexpressed (>30-fold) in the VR strain. Functional expression of recombinant *CYP6CY12* revealed its catalytic capacity to hydroxylate deltamethrin, thus confirming its causal role in pyrethroid resistance. Our study uncovered a P450-mediated mechanism of pyrethroid resistance in *A. pisum* for the first time and will inform future resistance management tactics to control this pest.

Keywords: Pea aphid, insecticide resistance, cytochrome P450, detoxification, RNAseq, CYP6CY12

1 Introduction

The pea aphid, *Acyrthosiphon pisum* Harris 1776 (Hemiptera: Aphididae) is a globally distributed sucking pest of leguminous crops such as pulses, including peas, lentils and broad beans (Blackman, & Eastop, 2000; El Fakhouri et al., 2021; Sandhi & Reddy, 2020). *A. pisum* directly damages plants by feeding (Maiteki & Lamb, 1985; Sirur & Barlow, 1984; Wilkinson & Douglas, 1998), and indirectly as a vector of destructive plant viruses such as pea enation mosaic virus and bean leafroll virus (Ng & Perry, 2004; Paudel et al., 2018). To prevent crop losses due to high infestation levels, management tactics to control pea aphids usually rely on the foliar application of insecticides (Gavloski, 2018; Sandhi & Reddy, 2020). Several chemical classes of insecticides, and their combinations, belonging to different modes of action have been shown to be effective against pea aphids, including organophosphates, neonicotinoids and pyrethroids (Sadeghi et al., 2009; Taillebois et al., 2015; Taillebois & Thany, 2016). Pyrethroids such as deltamethrin are highly effective, rapidly acting, synthetic insecticides used against a broad range of agricultural and public health pests (Elliott, 1989; Khambay, 2002; Pulman, 2011). They are of particular importance for the control of pest aphids as virus vectors, because, due to their rapid knockdown activity, they prevent the transmission and spread of plant virus diseases (Gibson et al., 1982). Their rapid neurotoxic action is based on the binding to, and modulation of, insect voltage-gated sodium channels (VGSCs) located in the central nervous system (Soderlund, 2020). Structurally, VGSC are large ion channels with four repeat domains (DI-IV) each having six transmembrane spanning segments (S1-6), and are encoded by a single gene in most insects (Dong et al., 2014). However, in aphids such as *A. pisum*, *Myzus persicae* and *Aphis glycines* VGSCs are heterodimers encoded by two different genes (Amey et al., 2015; Pires Paula et al., 2021).

Pyrethroids were launched more than 40 years ago and their share of the global insecticides market is only exceeded by the neonicotinoid insecticides (Sparks & Nauen, 2015). However, frequent applications of this inexpensive, but effective chemical class of insecticides in a broad range of agricultural settings, facilitated the evolution of pyrethroid resistance in many crop pests, including aphids (Bass et al., 2014; Dong et al., 2014; Slater et al., 2011). Pyrethroid resistance in arthropod pests is mediated by two major mechanisms; 1) increased detoxification, most commonly driven by the constitutive overexpression and/or duplication of genes of metabolic enzymes, e.g. cytochrome P450-monooxygenases (P450s), and 2) target-site mutations in the VGSC interfering with pyrethroid binding (Liu et al., 2015; Nauen et al., 2022; Panini et al., 2016; Rinkevich et al., 2013; Scott, 2019). Other mechanisms of resistance are related to the cuticular penetration of insecticides and altered behavior of exposed pest insects. Of these, reduced insecticide penetration due to altered cuticular structure/composition (reviewed in Balabanidou et al., 2018) has been reported for some

pyrethroid resistant pests (Ahmad et al., 2006; Koganemaru et al., 2013). In contrast, convincing cases of behavioural insecticide resistance are rare and are often not appropriately validated (Zalucki & Furlong, 2017).

High levels of metabolic pyrethroid resistance exclusively linked to the overexpression of major detoxification enzyme families such as P450s appear to be uncommon in aphids, unlike in other pest insects (Elzaki et al., 2018; Gimenez et al., 2020; Nauen et al., 2022; Vontas et al., 2020; Zimmer et al., 2014). However, a few studies with pyrethroid resistant aphid species reported synergism with piperonyl butoxide (PBO), increased P450/carboxylesterase enzyme activity or elevated levels of detoxification gene transcripts, but almost always in combination with target-site resistance mediated by VGSC mutations (Chen et al., 2017; Panini et al., 2014; Wang et al., 2020). The most common amino acid substitutions in the VGSC conferring pyrethroid target-site resistance are L1014F and (L1014F+) M918T, referred to as *kdr* (knockdown resistance) and *skdr* (super-*kdr*) respectively when first described in house flies, *Musca domestica* (Williamson et al., 1996). The impact on pyrethroid binding of these and other mutations has been functionally validated by recombinant expression of mutated VGSCs in *Xenopus* oocytes (reviewed in Dong et al., 2014). In major aphid pests, high levels of pyrethroid resistance compromising field rates have been almost exclusively linked to mutations in the VGSC. The *kdr* and *skdr* alleles or variants thereof (e.g. M918L) have been described in important aphid pests including *M. persicae* (Bass et al., 2014; Fontaine et al., 2011; Roy et al., 2013), *Aphis gossypii* (Marshall et al., 2012), *Aphis glycines* (Pires Paula et al., 2021; Valmorbida et al., 2022), *Sitobion avenae* (Foster et al., 2014) and *Rhopalosiphum padi* (Wang et al., 2020). However, in *A. pisum* phenotypic pyrethroid resistance linked to *kdr/skdr* alleles has not been described to date. However, a recent genotyping study in Canada reported an unspecified *skdr* mutation (likely at position M918) in ten different aphid species sampled in potatoes, including *A. pisum*, albeit without bioassay validation (MacKenzie et al., 2018).

Interestingly, the Arthropod Pesticide Resistance Database (Mota-Sanchez & Wise, 2020) lists >1000 cases of insecticide resistance for 28 different aphid species, but strikingly not a single entry for *A. pisum*. In 2015 and 2018 we collected two strains of *A. pisum* from field peas that survived pyrethroid treatments under applied conditions in France and Italy, respectively. It was unclear if the observed failures were due to formulation/application issues or resistance. The aim of the present study was to investigate the pyrethroid susceptibility of both strains in comparison to a laboratory reference strain, and, in case pyrethroid resistance was confirmed, to characterize the molecular mechanisms conferring the first reported case of insecticide resistance in *A. pisum* globally.

2 Material and Methods

2.1 Insects

Two field strains of *A. pisum,* PYR-R1 and VR, were collected from field peas in Morbihan, France and near Verona, Italy, respectively. Strain PYR-R1 was originally collected in 2015 after pyrethroid failure and since 2019 maintained under selection pressure on three-week old *Pisum sativum* L. (Fabales: Fabaceae) plants treated with 40 mg/L deltamethrin (applied as a.i. in 0.02 % aqueous Triton X-100). Strain VR was originally collected in 2018 and received in 2020 under the Commission Delegated Regulation (EU) 2019/829 with the number SG06/20-DE-NW and maintained as described above. Both strains were tested for pyrethroid resistance before being kept under selection pressure. An insecticide susceptible reference strain, SUS-1, maintained on *P. sativum* without selection pressure for more than 30 years was kindly provided by Katz Biotech AG (Baruth, Germany). All strains were reared at 24±1°C, 50% relative humidity and a L16:D8 photoperiod and followed a parthenogenetic viviparous reproduction cycle of max. 10 days including four nymphal instars.

2.2 Chemicals

All chemicals used in this study, including the insecticides deltamethrin, λ-cyhalothrin, α-cypermethrin, *cis*-permethrin and *tau*-fluvalinate, were of analytical grade and purchased from Sigma-Aldrich (Munich, Germany) unless otherwise stated. Trizol reagent, 1-naphthyl butyrate and 7-benzyloxymethoxy resorufin (BOMR; CAS: 87687-02-3; Vivid™ P2951) was purchased from ThermoFisher Scientific (Waltham, MA, USA). 7-benzyloxymethoxy-4-trifluoromethylcoumarin (BOMFC; CAS: 277309–33-8; purity 95%) was synthesized by Enamine Ltd. (Riga, Latvia). 7-pentoxycoumarin and 4'OH-deltamethrin (CAS: 66855–89-8) were internally synthetized (Bayer AG, Leverkusen, Germany). All solvents were of HPLC grade.

2.3 Aphid bioassays

Pyrethroid susceptibility in *A. pisum* was tested according to IRAC method no. 019 (http://www.irac-online.org). Briefly: for full dose-response assays, pea leaves were dipped for 3 sec into nine different pyrethroid concentrations prepared in aqueous 0.02 % (w/v) Triton X-100. Leaves dipped into 0.02 % aqueous Triton X-100 served as a control. Leaves were then air-dried on filter paper for 20 min and subsequently placed onto 1.5 % agar in small ventilated petri dishes as described previously (Nauen & Elbert, 2003). Thirty 6-day old aphids (five aphids per petri dish) from synchronized populations were tested per concentration at 24±1 °C

and scored for mortality after 24 h. Aphids with irreversible symptoms of poisoning were scored as dead. Mortality figures were corrected for control mortality using Abbott's formula (Abbott, 1925). Lethal concentration (LC) values and 95 % confidence intervals (95 % CI) were calculated from log-dose probit-mortality regressions using Polo PC (LeOra Software, Berkeley, California). Resistance ratios were calculated by dividing the LC_{50} value of the field strains by the LC_{50} value of strain SUS-1.

Discriminating dose bioassays were conducted with α-cypermethrin, *cis*-permethrin and *tau*-fluvalinate at 200 mg/L – a concentration well above the labelled field rate for all these compounds – to check for pyrethroid cross-resistance using the same methodology as described above. All bioassays were replicated thrice. Mean percentage mortality values ± SD (n=30) was analyzed by Graph Pad Prism v8 (GraphPad Prism Inc., CA, USA).

Synergist bioassays with strain PYR-R1were conducted as described above, except that aphids were topically treated prior to deltamethrin exposure with 0.1 µL of an acetonic solution of either 0.03 µg S,S,S-tributyl phosphorotrithioate (DEF, an esterase inhibitor), 0.05 µg piperonyl butoxide (PBO, a cytochrome P450 inhibitor) or 1 µg diethylmaleate (DEM, interfering with glutathione S-transferases). One-hour after synergist application, aphids were placed on pea leaves treated with 40 mg/L deltamethrin, a concentration nontoxic to strain PYR-R1. Mortality was scored as described above and synergist tests with 10 aphids each were replicated thrice.

2.4 Detoxification enzyme activity measurements

Carboxylesterase activity assays were performed with slight modifications as described previously (Grant et al., 1989). Ten single aphids were homogenized each in 200 µL ice-cold 0.1 M sodium-potassium-phosphate buffer, pH 7.5 and centrifuged for 15 min at 10,000 *x g* and 4°C. Aliquots of 20 µL (0.1 aphid equivalents) were pipetted into a 384-well microplate. The reaction was started by adding 80 µL of substrate solution containing Fast Blue RR salt in sodium phosphate buffer (0.2 M, pH 6.0) and 1 mM 1-naphthyl butyrate as substrate. Enzyme activity was measured using at least four replicates per strain continuously for 10 min at 450 nm and 23 °C in a Tecan Spark® multimode reader (Tecan Trading AG, Männedorf, Switzerland). Buffer only served as control for the non-enzymatic reaction. Enzyme kinetic data were analyzed using Magellan Data Analysis Software (Tecan Trading AG) and GraphPad Prism v8 (GraphPad Prism Inc., CA, USA).

Glutathione S-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates according to previous protocols (Habig et al., 1974) with slight modifications. After homogenization of 20 pea aphids (four replicates

per strain) in 300 µL Tris-HCl buffer (0.05 M, pH 7.5) and subsequent centrifugation at 10,000 *x g* and 4°C for 5 min, 100 µL supernatant was used for GST activity measurement in a 96-well microplate. After adding CDNB (final concentration 0.4 mM; containing 0.1 % (v/v) ethanol), and GSH (final concentration 4 mM) in Tris-HCl buffer (0.05 M, pH 7.5) changes in absorbance were continuously measured for 5 min at 340 nm and 25 °C using the Spark® multimode microplate reader as described above. Buffer only served as control for the non-enzymatic reaction. GST activity was expressed as nmol CDNB conjugated/min/mg protein by means of the extinction coefficient ε_{340nm} = 9.6 mM^{-1} cm^{-1} of the resulting 2,4-dinitrophenyl-glutathione.

Cytochrome P450-monooxygenase (P450) activity in microsomal preparations of *A. pisum* strains was measured by the *O*-debenzylation of 7-benzyloxymethoxy resorufin (BOMR) as described elsewhere (Nolden et al., 2021) with slight modifications. Mass homogenates of 1500 mg pea aphids (stored at -80°C) were prepared using ice-cold 0.1 M KH_2PO_4 buffer (pH 7.6) containing 1 mM DTT, 1 mM EDTA and 200 mM sucrose employing a schuett homgen-plus semi-automatic homogenizer (schuett-biotec GmbH, Göttingen, Germany). After centrifugation steps of 5 min at 5,000 *x g* and 20 min at 15,000 *x g* at 4 °C, the resulting supernatant was centrifuged at 100,000 *x g* for 60 min at 4 °C. The resulting microsomal pellet was resuspended in 250 µL 0.1 M KH_2PO_4 buffer (incl. 1 mM DTT, 0.1 mM EDTA, 5% glycerol, pH 7.6), and protein concentration adjusted to 0.5 mg/mL. P450 activity measurement using BOMR as a substrate in the presence of NADPH was replicated four times and conducted as recently described (Nolden et al., 2021).

The amount of protein was determined according to Bradford (Bradford, 1976) using bovine serum albumin as a standard.

2.5 RNA extraction, sequencing, and transcriptome profiling

Transcriptome sequencing was undertaken on two *A. pisum* strains, PYR-R1 showing phenotypic pyrethroid resistance, and the susceptible reference strain SUS-1. Five biological replicates of pools of ten age-synchronized apterous aphids (6 d in age) were flash frozen in liquid nitrogen and homogenized with 3 mm stainless steel beads at 20 Hz, for two times for 10 s with a MM300 laboratory bead mill (Retsch GmbH, Germany). Total RNA was extracted with TRIzol® reagent (Invitrogen, USA) followed by RNA purification using RNeasy® Plus Universal Mini Kit (QIAGEN, Germany) according to manufacturer's instructions and RNA purity checked as recently described (Boaventura et al., 2021). Approximately 1 µg of purified RNA was shipped to Eurofins Genomics (Ebersberg, Germany) for mRNA poly(A) enriched library preparation, mRNA fragmentation, random primed cDNA synthesis, adapter ligation and

adapter specific PCR amplification, followed by paired-end sequencing of > 5 M read pairs (read length 2 x 150 bp) per replicate utilizing a HiSeq Illumina sequencing platform (Illumina Inc., CA, USA). All sequence data has been deposited and archived in GenBank under BioProject PRJNA826712 including the accession numbers SAMN27578316 – SAMN27578325 for the individual replicates sequenced. One replicate of strain SUS-1, SAMN27578320, was excluded from further analysis due to a sequencing error.

For transcriptome profiling Illumina raw reads were mapped to the *A. pisum* reference genome GCF_005508785.2 (Y. Li et al., 2019) using STAR v2.6.1d (Dobin et al., 2013) and transcripts were quantified with RSEM v1.3.1 (B. Li & Dewey, 2011). Alignment free quantification was performed using kallisto v0.45.0 (Bray et al., 2016). Transcript information was summarized at the gene level in R v3.6.3 using tximport v1.14.2 (Soneson et al., 2015). Statistical analysis was performed with DESeq2 v1.26.0 (Love et al., 2014) and differentially expressed genes were identified using cut-off criteria of fold change >2 and an adjusted p-value of <0.05. Functional annotation and GO (gene ontology) term enrichment analysis were performed as previously described (Boaventura et al., 2021).

2.6 Gene expression analysis by RT-qPCR

RNA was isolated from four biological replicates of 10 pooled pea aphids of each *A. pisum* strain using TRIzol® reagent (Invitrogen, USA) followed by RNA purification using RNeasy® Plus Universal Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, USA) according to the manufacturer’s instructions. RT-qPCR to analyze the expression level of *CYP6CY12* (GenBank: XM_001952415) and *FE4-like esterase* (GenBank: XM_008182475) was done using the primers described in table S1. RT-qPCR reactions were performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) according to the manufacture's protocol. Reaction mixtures (10 µL) contained 2.5 µL cDNA (2.5–5 ng), 5 µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA), 300 nM of reverse/forward primers (Table S2), and nuclease-free water. Reactions were run in triplicate on a CFX384™ Real-Time system (Bio-Rad) with no-template mixtures serving as negative controls. PCR conditions comprised: 3 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. A final melt-curve step was included post-PCR (ramping from 65 °C to 95 °C by 0.5 °C every 5 s) to check for non-specific amplification. Data were normalized by geometric averaging using four stable reference genes (NADH, RPL12, 16S rRNA and 18S rRNA; Table S1) (Yang et al., 2014). Gene expression analysis was carried out employing the Bio-Rad CFX Maestro 1.0 v. 4.0 software (Bio-Rad, Hercules, USA) followed by subsequent unpaired t-tests in qbase

(Biogazelle, Belgium) to compare for significant differences unless otherwise stated (Vandesompele et al., 2002).

2.7 Voltage-gated sodium channel (VGSC) target-site mutation analysis

Sequencing data of both strain PYR-R1 and SUS-1 were mapped to the *A. pisum* VGSC genes with particular emphasis on regions encompassing known *kdr* and *skdr* mutation sites and finally aligned to GenBank entry XP_029343808.1, a fully annotated *A. pisum* VGSC (2232 amino acids). Special consideration was given to the presence/absence of amino acid substitutions M918L/T/V (*skdr*), L932F, and L1014F (*kdr*) (numbered according to *Musca domestica* VGSC (GenBank X96668)). These are known VGSC target-site mutations associated with pyrethroid resistance in *M. persicae* (Bass et al., 2014). Furthermore, a pyrosequencing based genotyping assay was developed to target the most frequent M918L/T and L1014F mutations separately and performed across strains PYR-R1, VR and SUS-1 (10 individual aphids per strain). Primer pairs were designed using the Assay Design Software (QIAGEN, Hilden, Germany), based on *A. pisum* VGSC sequence data deposited at NCBI (GenBank XP_029343808.1). The PCR and pyrosequencing reaction was carried out as previously described (Boaventura et al., 2020), using a sequencing primer specific for every target-site mutation analyzed, according to table S1.

2.8 Cytochrome P450 phylogenetics

CYP gene sequences were curated from the *M. persicae* v2.0, *A. pisum* v3.0 and *Aphis gossypii* v1.0 genome assemblies deposited in AphidBase (Legeai et al., 2010) using the functional annotation available for each assembly and validated using the scaffolds provided. Amino acid sequences were imported into MEGA X (Kumar et al., 2018) and aligned using MUSCLE. The same software was subsequently used to determine the most reliable substitution and rate variation model for phylogenetic analysis, i.e., the Jones-Taylor-Thornton (JTT) model using a gamma-shape parameter (+G). Neighbour-joining phylogenetic trees were then created in MEGA with a bootstrap value of 1000. Visualization of phylogenetic trees was performed using FigTree v1.4.4 (A. Rambaut, UK).

2.9 Functional expression of candidate genes in insect cells and enzyme kinetics

CYP6CY12 and *FE4-like esterase* identified as candidate resistance genes by transcriptomic profiling in this study were recombinantly expressed in High-5 insect cells using a baculovirus

expression system exactly as previously described (Nolden et al., 2022a). The functional expression of *CYP6CY12* and *FE4-like esterase* was validated by their capacity to metabolize coumarin/resorufin substrates (in the presence of NADPH) and 1-naphthylbutyrate, respectively, along with mock cell preparations as a control.

Metabolism of different fluorogenic coumarin and resorufin substrates by functionally expressed CYP6CY12 for substrate profiling was measured in 384-well plates according to recently published protocols (Nolden et al., 2021; Zimmer et al., 2014). Michaelis Menten kinetics of CYP6CY12-mediated coumarin substrate metabolism and fluorescent probe competition assays using increasing concentrations of deltamethrin were conducted as recently described (Haas & Nauen, 2021), except that 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC) was replaced by 7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin (BOMFC) and the assay time reduced from 60 min to 20 min. Substrate saturation kinetics of the *O*-debenzylation of BOMFC resulting in the formation of 7-hydroy-4-(trifluoromethyl)-coumarin (HC) was analyzed by non-linear regression using GraphPad Prism v8 assuming Michaelis-Menten kinetics. Michaelis-Menten kinetics of the formation of 1-naphthol using 1-naphthylbutyrate as a substrate by recombinantly expressed FE4-like esterase followed the biochemical assay procedure described above (2.4). K_m and V_{max} values were calculated using GraphPad Prism v8.

2.10 Deltamethrin metabolism assay utilizing UPLC-MS/MS

UPLC-MS/MS analysis of deltamethrin metabolism using *A. pisum* microsomes (40 µg protein) as well as recombinantly expressed CYP6CY12 and FE4_6921 respectively (80 µg protein) was carried out as previously described (Nolden et al., 2022a), except that enzyme preparations were incubated with deltamethrin for 120 min instead of 60 min. Chromatographic analysis was carried out on an Agilent 1290 Infinity II, a Waters Acquity HSS T3 RP18 column (2.1 × 50 mm, 1.8 µm) with 2 mM ammonium-acetate in methanol and 2 mM ammonium-acetate in water as the eluent. Ion transitions were recorded on a Sciex API6500 Triple Quad after positive electrospray ionization. Deltamethrin and the resulting metabolites 4'OH deltamethrin and 3-phenoxybenzoic acid were measured in positive ion mode (ion transitions: deltamethrin 523 > 281; 4'OH deltamethrin 539 > 281; 3-phenoxybenzoic acid 214.9 > 152.9). The linear ranges for quantitation of deltamethrin, 4'OH deltamethrin and 3-phenoxybenzoic acid were 0.5–100 ng/mL, 0.1–200 ng/mL and 0.5-200 ng/mL, respectively. The experiments were replicated thrice.

3 Results

3.1 Detection of phenotypic pyrethroid resistance and synergism in *A. pisum*

Leaf-dip bioassays with deltamethrin and λ-cyhalothrin revealed high levels of phenotypic pyrethroid resistance in two field-collected strains of *A. pisum*, PYR-R1 and VR, when compared to a susceptible reference strain, SUS-1 (Table 1). The highest resistance ratios (RR) of >183- and 115-fold were obtained in strain PYR-R1 for deltamethrin and λ-cyhalothrin, respectively. Subsequent single discriminating dose bioassays with cypermethrin, *cis*-permethrin and *tau*-fluvalinate – at a concentration exceeding field recommended label rates – revealed significant cross-resistance against different pyrethroid chemotypes in strains PYR-1 and VR compared to strain SUS-1 (Fig. 1A). Next, we selected strain PYR-R1 for synergist bioassays with known detoxification enzyme inhibitors, because PYR-R1 exhibited pyrethroid cross-resistance at a consistently higher level than strain VR. We detected significant synergism of deltamethrin toxicity at a fixed dose in aphids pre-exposed to nonlethal doses of PBO (a common P450 inhibitor) and DEF (an esterase inhibitor), but not DEM (a GSH depleting agent affecting GST activity), suggesting a role for P450s and esterases in pyrethroid resistance in strain PYR-R1 (Fig. 1B).

Table 1. Log-dose probit-mortality data for deltamethrin and lambda-cyhalothrin against 6d old adults of different strains of *Acyrthosiphon pisum* in a leaf-dip assay (24 h).

Insecticide	Strain	LC_{50} [mg L^{-1}]	95% CI[a]	Slope ± SE	RR[b]
Deltamethrin	SUS-1	5.46	2.05-14.8	2.22 ± 0.18	
	PYR-R1	>1000	-	-	>183
	VR	559	318-1250	0.93 ± 0.09	102
λ-cyhalothrin	SUS-1	1.65	1.33-2.05	1.79 ± 0.15	
	PYR-R1	190	106-320	1.60 ± 0.14	115
	VR	68.2	19.7-251	1.82 ± 0.14	43

[a] 95% confidence interval (95% CI); [b] Resistance ratio (RR) = LC_{50} PYR-R1 or VR divided by LC_{50} SUS-1

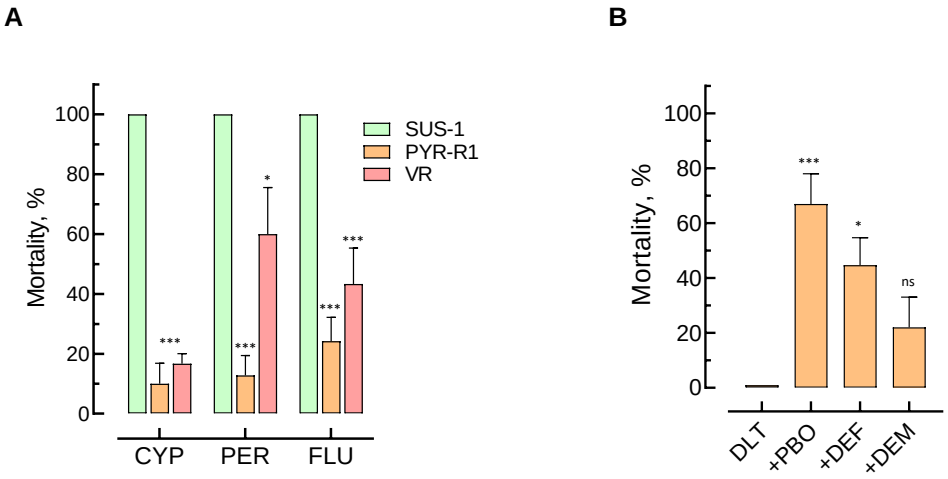

Fig 1. (A) Efficacy of a discriminating rate (200 mg L-1) of alpha-cypermethrin (CYP), cis-permethrin (PER), and tau-fluvalinate (FLU) against 6d old adults of different strains of *Acyrthosiphon pisum* in leaf-dip assays (24h). **(B)** Synergistic effects of piperonyl butoxide (PBO), S,S,S-tributyl phosphorotrithioate (DEF), and diethyl maleate (DEM) on the efficacy of foliarly applied deltamethrin (DLT, 40 mg L-1) against 6d old adults of *A. pisum* strain PYR-R1 in a leaf-dip assay (24h). Significant differences (one-way ANOVA; post hoc Tukey comparison) are denoted by *** ($P < 0.001$), * ($P < 0.05$) and ns (not significant). Data are mean values ± SD (n=3).

3.2 Elevated levels of detoxification enzyme activity in pyrethroid resistant *A. pisum*

Microsomal preparations of strains PYR-R1 and VR showed significantly higher P450 activity compared to strain SUS-1, thus supporting the synergist bioassay data obtained with PBO (Fig. 2A). Likewise, esterase activity in cytosolic fractions in both pyrethroid-resistant strains was significantly higher than in strain SUS-1 (Fig. 2B), whereas no difference in GST activity was detected between strains (Fig. 2C).

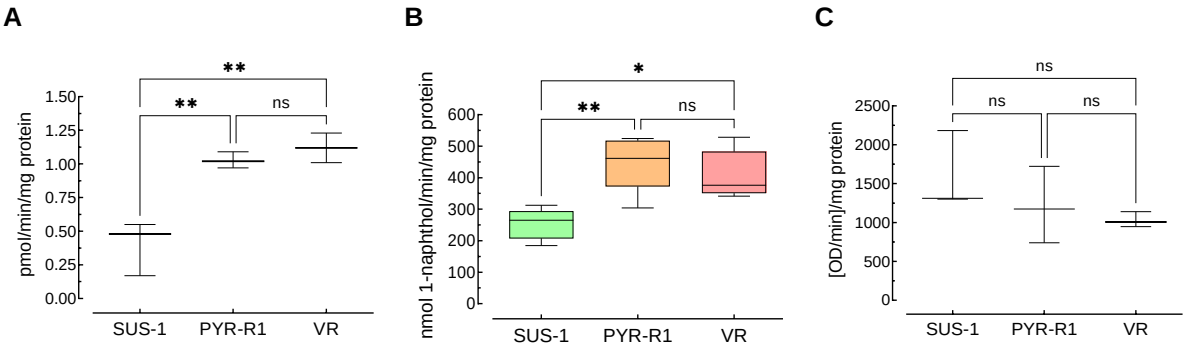

Fig. 2. Enzyme activity of **(A)** cytochrome P450s (substrate: 7-benzyloxymethoxy-resorufin, BOMR), **(B)** esterases (substrate: 1-naphthyl butyrate), and **(C)** glutathione S-transferases (substrate: 1-chloro-2,4-dinitrobenzol, CDNB) in homogenates of different strains of *Acyrthosiphon pisum*. Significant differences (one-way ANOVA; post hoc Tukey comparison) are denoted by ** ($P < 0.01$), * ($P < 0.05$) and ns (not significant). For details on enzyme activity measurement refer to the materials and methods section.

3.3 Hydroxylation of deltamethrin by microsomal preparations of *A. pisum*

Next, we incubated microsomal preparations of all *A. pisum* strains with deltamethrin and subsequently analyzed the resulting fractions by UPLC-MS/MS for the presence of two metabolites, 4´OH-deltamethrin and 3-phenoxybenzoic acid. We failed to detect 3-phenoxybenzoic acid, suggesting that microsomal esterases of *A. pisum* are unlikely to hydrolyze deltamethrin. In contrast, we detected significant quantities of 4´OH-deltamethrin when microsomal preparations of all strains were incubated in the presence of NADPH (Fig. S1). In addition, we found a significantly higher rate of 4´OH-deltamethrin formation with microsomes from strains PYR-R1 and VR in comparison to SUS-1, suggesting a role of microsomal P450s in pyrethroid resistance.

3.4 Transcriptome profiling reveals overexpression of genes encoding detoxification enzymes

Transcriptome sequencing of five independent biological replicates for both strain PYR-R1 and SUS-1 resulted in between 17.92 M and 24.15 M sequences, which were submitted to NCBI and archived under GenBank BioProject accession number PRJNA826712 (Table S2). One biological replicate of strain SUS-1 could not be properly analyzed due to a sequencing error and was excluded from the analysis. A comparative analysis of the normalized differential expression level of genes revealed distinct gene expression profiles between strains PYR-R1 and SUS-1 (Fig. 3A), which are well separated in principal component analysis (Fig. 3B). Subsequent analysis revealed 1614 differentially expressed genes (DEGs) between PYR-R1 and SUS-1 ($P \leq 0.01$, $\log_2FC \geq 1$ and $\log_2FC < 1$): 921 up- and 693 down-regulated in strain

PYR-R1 compared to SUS-1 (Fig. S2). Functional annotation and gene ontology (GO) term enrichment analysis of differentially expressed genes ($P \leq 0.01$, ≥ 5 regulated genes) revealed significant enrichment in several GO terms including those attributed to detoxification processes such as “oxidation-reduction process” and “oxidoreductase activity” (Fig. S2). A closer analysis of individual genes upregulated in strain PYR-R1 revealed only a few candidate detoxification genes (Table S3), including *CYP6CY12*, which showed a $\log_2$FC of 3.98 based on differences in FPKM (Fragments Per Kilobase Million) between SUS-1 (37.1 ± 1.7) and PYR-R1 (732 ± 2.5). The next two *CYP* genes on the list of those significantly overexpressed in strain PYR-R, *CYP380C1* and *CYP6CY5*, showed $\log_2$FC values of 1.60 and 1.26, respectively. Another *CYP* gene, annotated as methyl-farnesoate epoxidase-like *CYP15A3P,* was also overexpressed in PYR-R1, but FPKM values of this pseudogene were rather low, so it was excluded from subsequent follow-up work. Since DEF showed some synergism of deltamethrin toxicity in bioassays, we also analyzed the transcriptome for upregulated esterase genes, however, only a few candidate genes with a significant increase in $\log_2$FC in PYR-R1 were identified (Table S3). In addition, we identified two significantly overexpressed UDP-glycosyltransferase (UGT) genes, as well as one GST and one ATP-binding cassette transporter gene (Table S3). Next, we validated the expression level of the most upregulated P450 and esterase gene, *CYP6CY12* and *FE4-like esterase* (LOC100166921), respectively by RT-qPCR and obtained results consistent with the DEG analysis (Fig. 4A, B). Interestingly both genes were also upregulated in strain VR (Fig. 4B), thus suggesting a potential role in pyrethroid resistance in *A. pisum*.

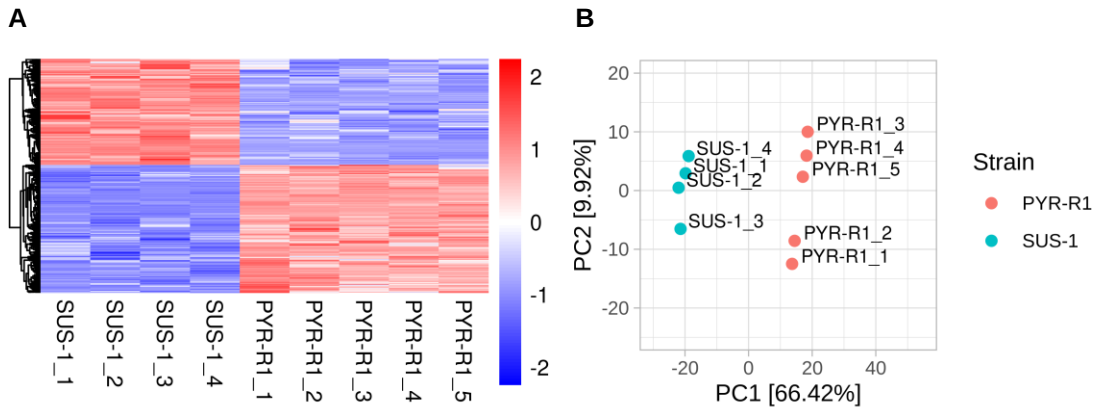

Fig. 3. (A) Heatmap showing normalized differential expression level for the top 1300 genes (adjusted P-value ≤ 0.05) between *Acyrthosiphon pisum* strains SUS-1 and PYR-R1 based on minimal average expression across samples of 100 based on variance stabilizing transformation implemented in the DESeq2 package. **(B)** Principal component analysis (PCA) of RNA-Seq data obtained for strains SUS-1 and PYR-R1.

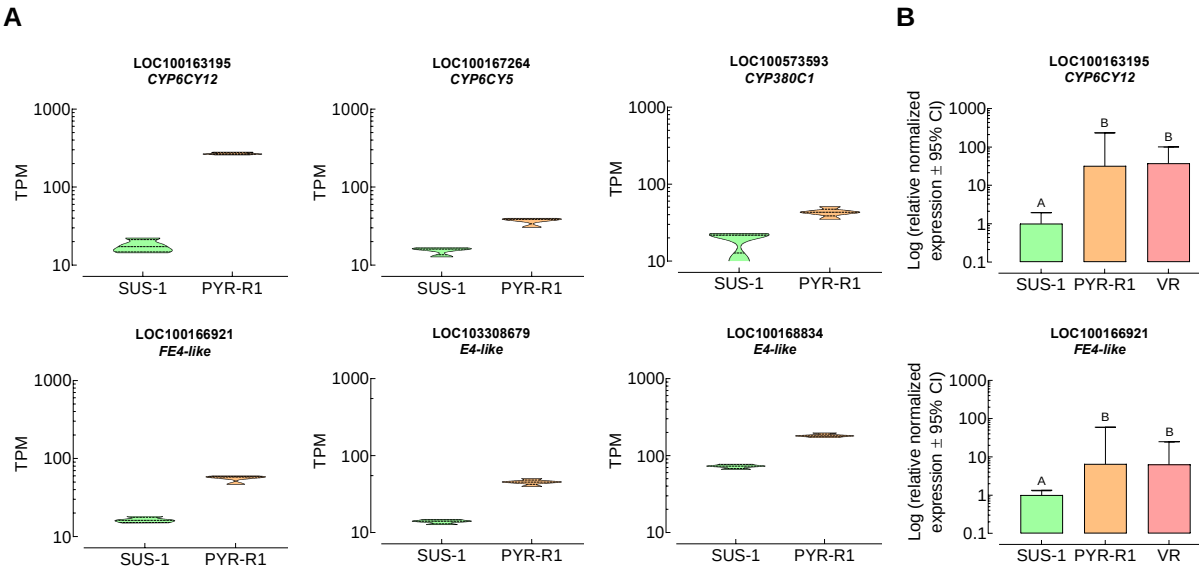

Fig. 4. (A) Violin plots of top 3 differentially expressed P450 (upper panel) and esterase genes (lower panel) between two strains (SUS-1 and PYR-R1) of *Acyrthosiphon pisum* analyzed by RNAseq (TPM, transcripts per million). **(B)** Validation of RNAseq analyses by RT-qPCR for the most differentially expressed P450 (*CYP6CY12*) and esterase gene (*FE4-like esterase*) between the SUS-1 strain and two pyrethroid-resistant strains, PYR-R1 and VR. Data are mean values ± CI95% (n =4). Different letters denote a significant difference (One-way ANOVA; post hoc Tukey comparison, P < 0.05).

3.5 Lack of VGSC target-site mutations in pyrethroid-resistant *A. pisum*

To further investigate the molecular origin of pyrethroid resistance in strain PYR-R1 we mapped sequencing reads of strains SUS-1 and PYR-R1 to the *A. pisum* VGSC genes (see methods) with special reference to known mutation sites previously described in aphids, particularly M918 (*skdr*) and L1014 (*kdr*), and rare mutations between these sites. We did not find any homozygous non-synonymous mutations in the assembled PYR-R1 VGSC when compared to the assembled VGSC of the pyrethroid susceptible strain SUS-1, i.e., we failed to detect commonly known VGSC target-site alterations explaining phenotypic pyrethroid resistance in strain PYR-R1 (Fig. S3). As we lacked RNAseq data for strain VR we designed a pyrosequencing assay to reiterate the findings of the transcriptomic analysis for individual aphids from strain PYR-R1, and additionally, to confirm the absence of non-synonymous mutations in VGSCs from individuals of strain VR. VGSC genotyping by pyrosequencing failed to detect the presence of *kdr* and *skdr* mutations in individuals of strains PYR-R1, VR and SUS-1, and all analyzed individuals were homozygous susceptible at these positions (Fig. S4). Accordingly, pyrethroid resistance in strains PYR-R1 and VR is not associated with known target-site mutations at residues M918 and L1014 or both, respectively.

3.6 Functionally expressed CYP6CY12, but not FE4-like esterase has the capacity to metabolize deltamethrin

Based on the transcriptome analysis and RT-qPCR data we selected two promising candidate detoxification genes differentially expressed in PYR-R1 and VR, *CYP6CY12* and *FE4-like esterase* (LOC100166921), for recombinant expression in High-5 cells to further characterize them and functionally validate their capacity to metabolize deltamethrin *in vitro*.

Functional expression of *CYP6CY12* in microsomal membranes of High-5 cells was confirmed by its competence to metabolize various coumarin and resorufin substrates in the presence of NADPH, whereas mock cells were inactive. The highest reaction rates were obtained for the *O*-debenzylation of OOMR and BOMFC, followed by the *O*-deethylation of EFC (Fig. 5A). Next we employed a recently developed coumarin probe competition assay (Haas & Nauen, 2021) with functionally expressed CYP6CY12 using BOMFC, which revealed the highest reaction rates among six tested coumarin substrates. The *O*-debenzylation of BOMFC by CYP6CY12 follows Michaelis-Menten kinetics and revealed a K_m value of 28.1 µM (CI95%: 22.2–35.7) and a V_{max} of 78.3 pmol HC/min x mg protein^{-1} (CI95%: 72.4-84.9) (Fig. 5B). CYP6CY12 co-incubation of BOMFC with increasing concentrations of deltamethrin up to 100 µM strongly interferes with the *O*-debenzylation of BOMFC, resulting in a significantly increased K_m value of 400 µM (CI95%: 181-2570) and an unchanged V_{max} of 91.1 pmol HC/min x mg protein^{-1} (CI95%: 54-443), suggesting competitive inhibition. Next, we incubated recombinantly expressed CYP6CY12 with deltamethrin and detected by UPLC-MS/MS the formation of 4´OH-deltamethrin, which follows Michaelis-Menten kinetics (Fig. 5C), clearly indicating the capacity of CYP6CY12 to bind and metabolize deltamethrin (K_m value 6.26 µM; CI95%: 4.32-9.14; Vmax value 11.3 pmol DLT-OH/min x mg protein-1 (CL95%: 9.96-12.9)). Thus, providing clear evidence of a causal role of overexpressed CYP6CY12 in pyrethroid resistance in strains PYR-R1 and VR.

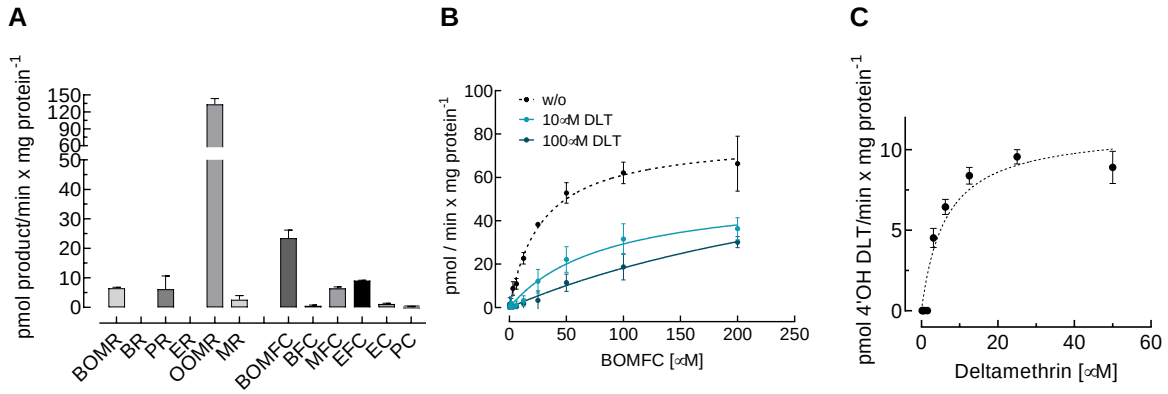

Fig. 5. (A) Metabolism of different fluorogenic coumarin and resorufin substrates by recombinantly expressed CYP6CY12 of *Acyrthosiphon pisum*. Data are mean values ± SD (n=3). **(B)** Effect of increasing deltamethrin concentrations on the O-debenzylation of 7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin (BOMFC) by recombinantly expressed *A. pisum* CYP6CY12. Data are mean values ± SD (n=3). **(C)** Saturation kinetics of the formation of 4-hydroxy deltamethrin (4´OH DLT) by recombinantly expressed CYP6CY12 of *A. pisum*. Data are mean values ± SD (n=3).

Abbreviations: BFC, 7-benzyloxy-4-trifluoromethyl coumarin; MFC, 7-methoxy-4-trifluoromethyl coumarin; EFC, 7-ethoxy-4-trifluoromethyl coumarin; BOMFC, 7-benzyloxymethoxy-4-trifluoromethyl coumarin; PC, 7-n-pentoxy coumarin; EC, 7-ethoxy coumarin; MC, 7-methoxy coumarin; BOMR, 7-benzyloxymethoxyresorufin; ER, 7-ethoxyresorufin; BOR, 7-benzyloxyresorufin; MR, 7-methoxyresorufin; OOMR, octyloxymethoxyresorufin; PR, 7-n-pentoxyresorufin.

Functional expression of *FE4-like esterase* was confirmed by the hydrolysis of 1-naphthylbutyrate and the formation of 1-naphthol following Michaelis-Menten kinetics (Fig. S5 A). In contrast to the results obtained for CYP6CY12, we detected no differences in the metabolism of deltamethrin by the formation of 3-phenoxybenzoic acid between mock cells and those expressing the hydrolytic enzyme (Fig. S5 B). Thus, suggesting no obvious role of the overexpressed esterase gene in pyrethroid resistance.

4 Discussion

The present work reveals for the first-time significant levels of field-relevant pyrethroid resistance in *A. pisum* populations collected in France and Italy. Unlike in other major aphid crop pests, pyrethroid resistance levels compromising recommended field-rates (e.g., 37.5 g/L for deltamethrin) have not been described to date for *A. pisum*. The LC_{50}-values calculated for deltamethrin against strain PYR-R1, and VR are >27- and 15-fold above the recommended label rate in field peas, respectively. This explains the observed field failure of pyrethroid applications at the sites in France and Italy where the populations were collected. The high

levels of resistance against deltamethrin and λ-cyhalothrin observed in full-dose response bioassays in *A. pisum* were accompanied by cross-resistance to other pyrethroids such as *tau*-fluvalinate and *cis*-permethrin, suggesting a mechanism of resistance affecting different pyrethroid chemotypes. The most prominent mechanism of resistance known to confer cross-resistance against a broad range of pyrethroids is associated with VGSC target-site mutations, of which many have been described in insect pests, including aphids (Dong et al., 2014; Rinkevich et al., 2013). As detailed in the introduction, target-site mutations at the VGSC, particularly L1014F, M918T and allelic variations thereof, play a major role in pyrethroid resistance in *M. persicae* (Bass et al., 2014; Roy et al., 2013), and have been linked to phenotypic pyrethroid resistance in at least four other aphid pest species. However, we have shown in the present study that the pyrethroid resistant *A. pisum* strain PYR-R1 lacks any known mutations in the VGSC previously described to confer pyrethroid resistance levels compromising recommended field rates in other aphid species. The lack of VGSC mutations such as *kdr* (L1014F) and *skdr* (M918T/L) was also confirmed in the VR strain by pyrosequencing the amplified region of interest, thus suggesting that VGSC mutations do not contribute to the observed levels of pyrethroid resistance in either the PYR-R1 and VR strains. However, other mechanisms than *kdr/skdr* conferring nerve insensitivity cannot be ruled out as recently demonstrated by neurophysiological recordings in pyrethroid resistant fall armyworm (McComic et al., 2020).

Synergist bioassays often provide a first line of evidence for the presence of metabolic resistance mediated by elevated levels of detoxification enzymes. Here we showed that the application of PBO and DEF, known as P450 and esterase inhibitors, respectively, increased the toxicity of deltamethrin in strain PYR-R1. Previous studies with other aphid species exhibiting resistance to pyrethroids revealed similar findings, e.g., in *A. gossypii* and *A. glycines* (Xi et al., 2015; Zeng et al., 2021). Significantly increased levels of P450 and esterase activity in both strain PYR-R1 and VR supported the results of the synergist bioassays. P450s are known to play a key role in xenobiotic metabolism and insecticide resistance (Dermauw et al., 2020; Nauen et al., 2022), by catalyzing a broad spectrum of oxidative reactions (Esteves et al., 2021). An additional line of evidence for P450s contributing to pyrethroid resistance in *A. pisum* was revealed by incubations of deltamethrin with aphid microsomes in the presence of NADPH, resulting in the formation of 4´OH-deltamethrin, but not 3-phenoxybenzoic acid (e.g., formed by microsomal esterases). Hydroxylation is the primary reaction of P450 enzymes, and has been shown to be one of the major routes of oxidative pyrethroid metabolism in crop and public health pests (Nolden et al., 2022a; Stevenson et al., 2011; Zimmer et al., 2014), and also vertebrates (Anand et al., 2006). Although we observed synergistic effects with the esterase inhibitor DEF, pyrethroid hydrolysis by esterases seems more common in vertebrates than in insects (Bhatt et al., 2020). Related to this, in our study the DEF-mediated synergism

of deltamethrin toxicity was less pronounced (and significant) when compared to PBO synergism, suggesting a more prominent role for an oxidative metabolic fate rather than hydrolysis.

Despite its status as a major legume pest, *A. pisum* is also widely known as a model aphid species (Brisson & Stern, 2006). The pea aphid was the first aphid to have its genome sequenced and was recently assembled to chromosome-level (Consortium, 2010; Y. Li et al., 2019). Its DETOXome has been analyzed (Ramsey et al., 2010), and shown to comprise more than 80 *CYP* genes encoding for P450 enzymes representing four major clans (CYP2, 3, 4, and mito), with lineage-specific *CYP* gene family expansions in the subfamilies *CYP6CY* and *CYP380C* belonging to clans CYP3 and CYP4, respectively (Fig. 6). Based on the multiple lines of evidence for oxidative metabolism as a major mechanism of pyrethroid resistance in *A. pisum*, we were particularly interested in the expression of *CYP* genes in our resistant strains. Transcriptome profiling revealed *CYP6CY12* as highly overexpressed in the PYR-R1 strain compared to the susceptible strain, with RT-qPCR analysis showing it is also upregulated in the VR strain. Interestingly, members of the *CYP6CY* subfamily – which is aphid specific – have been previously implicated in the detoxification of xenobiotics in other aphid species, e.g., CYP6CY3 in *M. persicae* (Troczka et al., 2021), CYP6CY13 and CYP6CY22 in *A. gossypii* (Hirata et al., 2017).

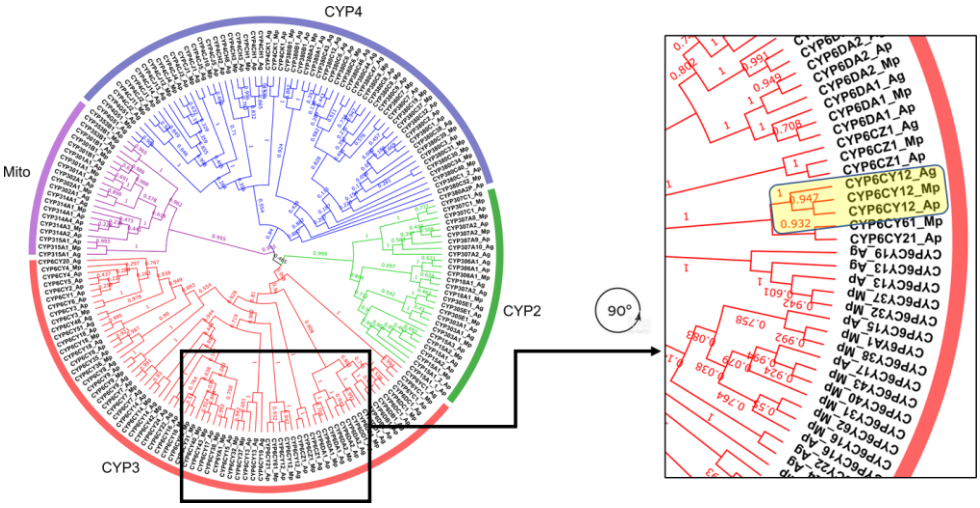

Fig. 6. Neighbour-joining consensus tree of cytochrome P450 sequences from *Acyrthosiphon pisum* (Ap), *Myzus persicae* (Mp) and *Aphis gossypii* (Ag) divided into 4 clades: CYP2, CYP3, CYP4 and mitochondrial (mito). The inset highlights the relationship between *A. pisum CYP6CY12* and its orthologs in *M. persicae* and *A. gossypii*. Branch values denote bootstrapping values from 1000 replicates, given as a decimal.

Recombinant expression of CYP6CY12 demonstrated its functional capability to metabolize a range of coumarin and resorufin substrates in the presence of NADPH. To date, only a few other aphid CYP6CY subfamily members have been functionally expressed, e.g., CYP6CY3, CYP6CY4 and CYP6CY23 from *M. persicae* (Bass et al., 2013; Singh et al., 2020), and CYP6CY13 and CYP6CY22 from *A. gossypii* (Hirata et al., 2017) but none of these were characterized for their coumarin and resorufin substrate profile. We used the coumarin based fluorescent probe assay to assess a potential interaction of deltamethrin with CYP6CY12 by inhibiting the *O*-debenzylation of BOMFC. Indeed, we detected a competitive inhibition of CYP6CY12-mediated BOMFC metabolism by deltamethrin, suggesting a direct interaction of deltamethrin with the catalytic site of CYP6CY12 and indicating its potential to possibly metabolize deltamethrin. Similar assays have been previously described to be predictive for insecticide metabolism with other insect P450s such as *Bemisia tabaci* CYP6CM1 (Hamada et al., 2019), *Apis mellifera* CYP9Q3 (Haas & Nauen, 2021), and *Anopheles funestus* CYP6P9a/b (Nolden et al., 2022b). UPLC-MS/MS analysis of deltamethrin samples incubated with recombinantly expressed CYP6CY12 supported the fluorescent probe assay results and confirmed its capacity to hydroxylate deltamethrin. Together, these results provide clear functional evidence of the role of CYP6CY12 in deltamethrin resistance in strains PYR-R1 and VR of *A. pisum*. It is likely that CYP6CY12 also confers cross-resistance to the other tested pyrethroids in *A. pisum*, but additional studies in the future are necessary to confirm this. Interestingly, CYP6CY12 is the fourth P450 out of the aphid CYP6CY subfamily which has been confirmed to confer insecticide resistance. Others include CYP6CY3 of *M. persicae* which has been shown to metabolize nicotine and neonicotinoids (Bass et al., 2013), and CYP6CY13 and CYP6CY22 of *A. gossypii* which have been demonstrated by functional expression in *Drosophila* S2 cells to metabolize neonicotinoids (Hirata et al., 2017). Phylogeny (Fig. 6) revealed that *M. persicae* and *A. gossypii* have *CYP6CY12* orthologs showing >80% sequence similarity, thus it would be interesting in future studies to investigate if they principally have the same capacity to metabolize deltamethrin. However, such a metabolic mechanism of pyrethroid resistance in these two species may be redundant considering the high levels of resistance already conferred by the presence of VGSC target-site mutations as discussed above.

Based on our bioassay results with the esterase inhibitor DEF, we also selected the most highly expressed esterase gene in strain PYR-R1 (LOC100166921) for recombinant expression in order to biochemically assess its capacity to hydrolyze deltamethrin. Despite the successful functional expression of this esterase, we did not observe any capacity for it to hydrolyze deltamethrin when compared to mock cell preparations. Thus, the role, if any, of this esterase in resistance to pyrethroids in *A. pisum* remains unclear.

In conclusion we describe the first case of pyrethroid cross-resistance in *A. pisum* that is sufficiently potent to compromise control using recommended field rates. Furthermore, we demonstrate that resistance is not associated with target-site resistance in the VGSC but is linked to the marked overexpression of a single P450, CYP6CY12. This P450 has the capacity to efficiently hydroxylate the pyrethroid insecticide deltamethrin, thus confirming its causal role in pyrethroid resistance in *A. pisum* field populations collected in France and Italy. Elevated levels of CYP6CY12 may serve as a diagnostic marker in future resistance monitoring campaigns to detect pyrethroid resistance in field samples of *A. pisum*. Rotation of insecticides with different modes of action is warranted to conserve the efficacy of pyrethroids for the control of *A. pisum* under applied condition and to prevent the global spread of resistance.

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Chapter 4

Ketoenol resistance in the cabbage whitefly *Aleyrodes proletella* (Homoptera: Aleyrodidae) is associated with a target-site mutation, A2083V, in the CT domain of Acetyl-CoA carboxylase (ACC)

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Abstract

Ketoenol insecticides are a new chemical class of compounds being active against sucking insect pests, such as aphids, mites and whiteflies. First cases of ketoenol resistance have been described in 2012, increasing the struggle of controlling these important pest species. While the resistance mechanism of the greenhouse whitefly *Trialeurodes vaporariorum* against spiromesifen remains elusive, a target-site mutation, A2083V, in the carboxyltransferase (CT) domain of acetyl-CoA carboxylase (ACC) was shown to confer spiromesifen resistance in the cotton whitefly, *Bemisia tabaci*. However, the cabbage whitefly *Aleyrodes proletella* is an emerging pest that causes crop losses globally. Till now, there was only pyrethroid resistance described in this species. Here, the first cases of ketoenol insensitivity of several collected strains from Europe could be identified. In bioassay studies, one population from Belgium, 5/19, and two from Germany, 2/20 and 6/20, showed high resistance levels to spiromesifen and spirotetramat. However, no cross-resistance to the pyrethroid λ-cyhalothrin, the neonicotinoid acetamiprid and the butenolide flupyradifurone, was observed. In a pyrosequencing approach, the different strains were analyzed for the presence of the A2083V mutation, formerly described in *B. tabaci*. It could be shown that the spiromesifen resistant *A. proletella* populations are carrying the A2083V mutation. Next, several other populations collected European wide and preserved in ethanol, were sequenced for the presence of the relevant mutation site. Homozygote resistant insects, carrying the A2083V polymorphism, were identified in strains from Switzerland, the Netherlands, Belgium and Germany, whereas heterozygous resistant whiteflies were also found in samples collected in Croatia, Poland and Spain. Reciprocal crossing experiments with a spiromesifen selected strain, SPI-5/19, and a susceptible reference strain, revealed an autosomal dominant trait.

Within this study, the A2083V mutation, that is causing high levels of ketoenol resistance in *B. tabaci*, was also detected in ketoenol resistant cabbage whiteflies of different origins in Europe. The presence of the polymorphism in an additional whitefly species, highlights the importance of integrated pest and resistance management strategies for whitefly control.

1 Introduction

The tetronic and tetramic acid derivates (ketoenols) such as spiromesifen and spirotetramat are lipid biosynthesis inhibitors active against a broad range of important pest species, such as aphids and whiteflies (Brück et al., 2009; Nauen et al., 2003; Nauen et al., 2005; Nauen et al., 2006; Nauen et al., 2008). The systemic pro-insecticides spiropidion and spirotetramat are transported through xylem and phloem in plants (Brück et al., 2009; Muehlebach et al., 2021). Since their introduction to the market, ketoenol insecticides are widely used for sucking pest control (Brück et al., 2009; Muehlebach et al., 2021; Nauen et al., 2003; Nauen et al., 2005).

First cases of spiromesifen resistance have been described approximately ten years ago in the greenhouse whitefly *Trialeurodes vaporariorum* (Karatolos et al., 2012b). The observed resistance has been associated with a mutation, E645K, in the enzyme targeted by ketoenols, acetyl-CoA carboxylase (ACC). However, a later study failed to link the E645K mutation in *T. vaporariorum* to spiromesifen resistance in Greek field populations (Kapantaidaki et al. 2018). The widespread pest species feeds on a large range of vegetables and ornamental crops cultivated in greenhouses (Brødsgaard & Albajes, 1999). In 2019, lack of spiromesifen and spirotetramat toxicity against field samples of the tobacco whitefly *Bemisia tabaci* has been reported in Spain (Bielza et al., 2019). Resistance of the species against spiromesifen and spirotetramat has been shown to be conferred by an amino acid substitution in ACC, A2083V (Lueke et al., 2020).

The highly invasive cabbage whitefly *Aleyrodes proletella* has not yet been reported resistant against ketoenol insecticides (Mota-Sanchez & Wise, 2022). The pest species is spreading in European fields and is also dispersing rapidly around the world (CABI, n.d.; CABI, 2022; CABI Data Mining, n.d.; Dale et al., 1976; De Barro & Carver, 1997; Finch & Thompson, 1992; Jansen & Ivanova, 2018; Malumphy et al., 2009; Malumphy & Ostrauskas, 2013; Koca & Kütük, 2020; Seebens et al., 2017). Next to ketoenols, are also other insecticides of different chemical classes including pyrethroids, neonicotinoids and butenolides used for controlling whiteflies (IRAC 2019). In 2012 the first and only resistance case to pyrethorids in British *A. proletella* strains was detected (Springate & Colvin, 2012).

Damage through whiteflies is caused by adults and nymphs that suck plant sap out of the phloem cells. Furthermore, nymphs can cause direct damage to their host plants through the removal of chlorophyll and starch, which ends in leaf fall as well as reduced growth of the plants (Brødsgaard & Albajes, 1999). Indirect damage is mainly caused in cultivations with low whitefly infestation rates. The excreted honeydew from adults and nymphs can also stimulate growths of sooty moulds. Furthermore, *B. tabaci* is for example known to transmit serious plant pathogenic virus diseases (Brødsgaard & Albajes, 1999).

In this study, the first ketoenol resistance case in the cabbage whitefly *A. proletella* was investigated in detail. The aim of the study was the identification of the relevant resistance mechanism present in the pest species. After the first screening of ketoenol insecticides in bioassays, the resistant strains were sequenced by pyrosequencing for the presence of the previously confirmed target-site mutation in spiromesifen resistant *B. tabaci*, A2083V (Lueke et al., 2020). Followed by an ribonucleic acid (RNA)-sequencing (RNAseq) approach for the detection of other mechanisms of resistance such as enhanced expression levels of detoxification genes. Cabbage whitefly field samples have been genotyped in order to get an idea on the extent and spread of ketoenol resistance across Europe.

2 Materials and methods

2.1 Whitefly insects

For studying resistance of *A. proletella*, strains of different European countries were reared in the laboratory (Table 1) or preserved in 70 % ethanol (Table S1). Latter were stored at 4 °C and used for molecular diagnostic studies. The living insects were cultivated on untreated savoy cabbage plants *Brassica oleracea* L. convar. Capitata (L.) Alef. Var. sabauda L.. The ketoenol resistant strains 5/19 (SPI-5/19), 2/20 (SPI-2/20) and 6/20 (SPI-6/20), were also maintained on cabbage plants treated with 200 ppm spiromesifen (the names of the strains are given in brackets). All living populations were evaluated in bioassays as well as molecular diagnostic studies and were maintained at 23 ± 1 °C, 50 % relative humidity and a photoperiod of L16:D8.

Table 1: Field-collected populations of *Aleyrodes proletella* (Name, Year, Country, Venue, Host) maintained under lab conditions for bioassay work.

Name	Year	Country	Venue	Host
1/19	2019	France	Richebourg	Cauliflower
2/19	2019	Croatia	Virovitičko-Podkavska Country	Savoy cabbage
3/19	2019	Croatia	Varazdin Country	Green cabbage
4/19	2019	France	Warrem	White cabbage
5/19	2019	Belgium	Borgworm	White cabbage

Name	Year	Country	Venue	Host
SPI-5/19	2019	Belgium	Borgworm	5/19 selected: 200 ppm spiromesifen
6/19	2019	Belgium	Lier	White cabbage
1/20	2020	Germany	Helse	Unknown
2/20	2020	Germany	Blomberg	Green cabbage
SPI-2/20	2020	Germany	Blomberg	2/20 selected: 200 ppm spiromesifen
4/20	2020	Germany	Bardowick	Unknown
5/20	2020	Germany	Bardowick	Unknown
6/20	2020	Germany	Hannover	Green cabbage
SPI-6/20	2020	Germany	Hannover	6/20 selected: 200 ppm spiromesifen

2.2 Chemicals

Spiromesifen, Oberon 240 SC, and spirotetramat, Movento 150 OD, were used as commercial formulations provided internally. Acetamiprid, λ-cyhalothrin and flupyradifurone were of analytical grade. Latter was provided internally. Acetamiprid and λ-cyhalothrin as well as chloroform and Triton X-100 were purchased from Sigma-Aldrich. Trizol Reagent was provided by Invitrogen, Thermo-Fisher Scientific (Waltham, MA, USA).

2.3 *Aleyrodes proletella* ketoenol toxicity bioassays

All toxicity bioassays were conducted according to the IRAC susceptibility test method 016 (IRAC International, 2022) as well as to Lueke et al. (2020) with slight modifications. Two-week-old cabbage plants were cut for having two leaves per plant. Each leaf represented one replicate. In total, each treatment consists of four replicates, resulting in two cabbage plants. Moreover, the plants were infested with adult whiteflies for 24 h. Afterwards, the insects were removed for letting their oviposition develop. After 13 days at 24 ± 1 °C, 50 % relative humidity and a photoperiod of L16:D8, the infested plants were treated with a purpose-built spraying device. The ketoenol insecticides spiromesifen and spirotetramat were applied at

concentrations of 0.32 to 1,000 ppm diluted with 0.02 % aqueous Triton X-100. Control plants were not treated with any insecticide. Ten days after insecticide application, all leaves were evaluated for alive, dead or symptomatic whitefly nymphs. Alive insects have further developed to their fourth nymph stage close to emerge. Symptomatic whiteflies stayed in their second instar. All data were corrected for control mortality (Abbott, 1925) and analyzed with Graph Pad Prism v8 (GraphPad Prism Inc., CA, USA) and Polo PC (LeOra Software, Berkeley, California) software afterwards.

2.4 *Aleyrodes proletella* cross-resistance bioassays

Cross-resistance experiments using the pyrethroid λ-cyhalothrin, the neonicotinoid acetamiprid and the butenolide flupyradifurone, were conducted according to Rauch & Nauen (2003) and Elbert et al. (2008). Discs of two-week-old cabbage plants were dipped for 3 s in insecticide concentrations ranging from 0.128 to 2,000 ppm, diluted with 0.02 % aqueous Triton X-100. The leaves were dried on their adaxial surface on a filter paper for approximately 20 min and each leaf was placed in one well of 6-well plates (Greiner) filled with 1.5 % agar previously. Each insecticide concentration included three replicates, resulting in four replicates in total. *A. proletella* adults were anaesthetized with carbon dioxide (CO_2) briefly and placed on the sprayed leaves. The 6-well plates were closed with a porous foil. Leaves that were only treated with 0.02 % Triton X-100 served as control. The insects were evaluated for symptomatic, dead and alive cabbage whitefly adults after three days. The calculated data was corrected and statistically analyzed as mentioned above (2.3).

2.5 Pyrosequencing diagnostic study for genotyping A2083V mutation in ACC of *Aleyrodes proletella*

The genomic DNA of individual *A. proletella* (n=10) whiteflies were isolated using the DNAdvanced (Beckman Coulter) kit according to the manufacturer's instructions. After quality confirmation with the Infinity M200Pro plate reader (Tecan Trading AG), deoxyribonucleic acid (DNA) was inserted in a polymerase chain reaction (PCR) reaction containing 25 µL mixture in total. As polymerase served 2x JumpStartTM Taq Ready Mix (Sigma-Aldrich). Also, 10 µM primer dilutions were added to the reaction mix before the PCR started with 3 min at 95 °C. For A2083V genotyping, 45 rounds at 95 °C for 30 s, 58 °C at 30 s and 72 °C at 3 s followed. After the final step for 5 min at 72 °C, PCR was completed. Pyrosequencing was conducted using the PyroMark Q96 ID (Qiagen) and PyroMark Gold Q96 Reagent Kit (Qiagen) according to the manufacturers' instructions as well as the ACCA2083Vseq primer (Table S2). The resulting pyrograms were analyzed with the PyroMark Q96 ID Software 2.5 (Qiagen).

2.6 RNAseq of *Aleyrodes proletella* ACC

The susceptible *A. proletella* strains 3/19, 4/19, 6/19 and 5/20 as well as the ketoenol resistant populations 2/20 and SPI-2/20 were selected for an RNAseq approach. RNA of ten *A. proletella* adults per replicate was extracted with Trizol reagent (Invitrogen, Thermo-Fisher Scientific). Therefore, the insects were flash frozen and homogenized with two 3 mm steel beads at 20 Hz for 2 x 10 s with the MM300 laboratory bead mill (Retsch). After Trizol was added to the crushed insects, the samples were incubated for 5 min at room temperature. Then, 100 µL chloroform was added before samples were inverted for 15 s and incubated for three additional minutes. After a centrifugation step of 15 min at 10.000 *x g* and 4 °C, the aqueous phase was used for the following RNA purification step using the RNAdvanced (Beckman Coulter) kit according to the manufacturer's instructions. The quality and concentration of RNA was determined using the Infinity M200Pro plate reader (Tecan Trading AG) and revealed high quality. Degradation was evaluated using the 2100 Expert Bioanalyzer (Agilent) and confirmed best quality RNA. After preparation of the strand-specific complementary DNA (cDNA) library, an Illumina approach with 5 M reads (2 x 150 bp) was performed. Afterwards, all sequencing data reads of the four replicates per population were assembled using Trinity 2.8.5 (Grabherr, et al., 2011) and further consolidated with TransDecoder 5.3.0 (https://github.com/TransDecoder/TransDecoder/releases) using blast results versus SwissProt 2021_4 as of 2022-Mar-2 and Pfam 34 as of june 2021 alignments. ACC sequences were identified by selecting full length blast hits using *B. tabaci* QJQ31013.1 as the query. The *T. vaporariorum* ACC sequence was constructed using an alignment of *B. tabaci* coding sequence (MN567040.1) vs. contig VMOF01000024.1 of the genomic assembly ASM1176424v1 by GMAP (Wu & Watanabe, 2005) as a guideline. Multiple alignment was performed using clustal omega v.1.2.3 (Sievers, et al., 2011).

2.7 Reciprocal crossing experiments of *Aleyrodes proletella*

Crossing experiments were conducted according to Lueke et al. (2020) with slight modifications. Leaf discs with nymphs close to emergence were placed on non-infested cabbage leaves in petri dishes. Of the two populations selected, the susceptible 6/19 and the resistant SPI-5/19, one female and one male were placed together in a petri dish. The sex of the nymphs was identified due to their size. Male nymphs are known in being smaller than females (Horowitz, 1983; Horowitz, et al., 2003). Each cross was repeated five times to obtain the following populations: susceptible 6/19 ♀ x resistant SPI-5/19 ♂ and SPI-5/19 ♀ x 6/19 ♂. The nymph development to adult as well as mating and oviposition happened inside the petri dishes. After seven additional days, the leaves with developed eggs (dark brown) close to the first nymph stadium, were placed on non-infested and non-treated cabbage plants. One plant

for each cross was used. Generally, first instar whitefly nymphs are mobile and can move to new plant material (van Roermund & van Lenteren, 1992; Weber, 1931). After six additional days, old leaf discs were discarded and the now infested plants were applied with 200 ppm spiromesifen, Oberon 240 SC, formulation using a purpose-built spraying device. Ten days afterwards, the nymphs' vitality was evaluated as described previously (s. 2.3).

3 Results

3.1 *Aleyrodes proletella* nymph bioassays with ketoenols

Toxicity studies with spiromesifen revealed eight susceptible populations reared in the laboratory, the 1/19-4/19, 6/19, 1/20, 4/20 and 5/20 strains (Table 2). Affected nymphs were smaller or non-developed and showed deformations. Besides, three strains were found in being spiromesifen resistant with resistance ratios (RR) of 27 (strain 5/19), >35 (strains 6/20 and 2/20). All three strains were subsequently maintained on 200 ppm spiromesifen treated savoy cabbage plants (resulting in selected strains SPI-5/19, SPI-2/20, SPI-6/20). An additional set of bioassays was conducted testing spirotetramat for cross-resistance (Table 2). The studies revealed spirotetramat cross-resistance in all spiromesifen resistant strains. The populations that were susceptible against spiromesifen applications, were also sensitive against spirotetramat treatments.

Table 2. Log-dose probit-mortality data for the ketoenol insecticides spiromesifen and spirotetramat against 2nd instar nymphs of different strains of *Aleyrodes proletella* in a bioassay using a purpose-built spraying device (10 d).

Insecticide	Strain	LC_{50} [mg L^{-1}]	95% CI[a]	Slope ± SE	RR[b]
Spiromesifen	1/19	3.96	3.39-4.63	3.42 ± 0.33	0.69
	2/19	50.5	31.9-84.2	1.88 ± 0.17	8.86
	3/19	2.76	1.71-4.49	1.87 ± 0.15	0.48
	4/19	4.33	1.74-11.6	2.29 ± 0.19	0.76
	5/19	154	45.4-1235	0.9 ± 0.07	27
	SPI-5/19	>200			>35.1
	6/19	5.7	3.42-9.33	2.1 ± 0.17	
	1/20	13.8	8.88-21.3	2.24 ± 0.2	2.42
	2/20	>200			>35.1
	SPI-2/20	>200			>35.1
	4/20	9.85	5.23-20.8	2.82 ± 0.26	1.73
	5/20	4.13	2.34-7.42	1.78 ± 0.14	0.72

Insecticide	Strain	LC_{50} [mg L^{-1}]	95% CI[a]	Slope ± SE	RR[b]
Spiromesifen	6/20	>200			>35.1
	SPI-6/20	>200			>35.1
Spirotetramat	1/19	11.3	6.36-20.9	2.42 ± 0.21	2.67
	2/19	3.24	0.26-15.7	0.98 ± 0.08	0.76
	3/19	6.95	5.92-8.17	3.18 ± 0.34	1.64
	4/19	6.15	4.53-8.41	2.66 ± 0.25	1.45
	5/19	57.8	37.2-91	1.57 ± 0.12	13.6
	SPI-5/19	202	136-350	1.13 ± 0.13	47.6
	6/19	4.24	1.98-9.43	2.48 ± 0.22	
	1/20	3.58	3.08-4.16	3.67 ± 0.34	0.84
	2/20	>200			>47.2
	SPI-2/20	>200			>47.2
	4/20	3.7	1.69-7.79	3.26 ± 0.3	0.87
	5/20	3.63	1.7-8.17	2.55 ± 0.22	0.86
	6/20	123	103-146	2.76 ± 0.26	29
	SPI-6/20	>200			>47.2

[a] 95% confidence interval (95% CI); [b] Resistance ratio (RR) = LC_{50} of each strain divided by LC_{50} susceptible 6/19.

3.2 *Aleyrodes proletella* cross-resistance adult bioassays

Moreover, cross-resistance to the pyrethroid λ-cyhalothrin, the neonicotinoid acetamiprid and the butenolide flupyradifurone was examined afterwards. However, no relevant resistance levels could be observed for all populations (Table 3).

Table 3. Log-dose probit-mortality data for acetamiprid, λ-cyhalothrin and flupyradifurone insecticides against one week old adults of different strains of *Aleyrodes proletella* in a leaf-dip assay (72 h).

Insecticide	Strain	LC_{50} [mg L^{-1}]	95% CI[a]	Slope ± SE	RR[b]
Acetamiprid	3/19	40.5	7.81-255	1.21 ± 0.08	1.07
	4/19	37.8	8.03-244	1.1 ± 0.07	
	5/19	75.1	61.3-92.2	1.93 ± 0.15	1.99
	SPI-5/19	42.1	35.8-49.5	3.09 ± 0.3	1.11
	6/19	99.6	47.8-220	1.72 ± 0.13	2.63
	6/20	60.7	31.6-123	1.86 ± 0.14	1.61
Λ-cyhalothrin	3/19	52.7	12.6-299	1.29 ± 0.08	1.89
	4/19	27.9	3.59-387	1.02 ± 0.06	
	5/19	137	95.1-195	2.1 ± 0.18	4.91

Insecticide	Strain	LC_{50} [mg L^{-1}]	95% CI[a]	Slope ± SE	RR[b]
Λ-cyhalothrin	SPI-5/19	223	100-460	2.24 ± 0.18	7.99
	6/19	174	142-212	1.98 ± 0.16	6.24
	6/20	94.8	40.1-245	1.2 ± 0.08	3.4
Flupyradifurone	3/19	50.1	23.1-108	1.27 ± 0.08	4.07
	4/19	50.1	30.2-81.1	2.17 ± 0.18	4.07
	5/19	23.2	13.6-38.6	1.87 ± 0.15	1.89
	SPI-5/19	50.4	27.2-90.1	1.87 ± 0.14	4.1
	6/19	30	22-40.8	2 ± 0.16	2.44
	6/20	12.3	10.2-14.7	2.46 ± 0.22	

[a] 95% confidence interval (95% CI); [b] Resistance ratio (RR) = LC_{50} of each strain divided by LC_{50} of most susceptible strain.

3.3 RNAseq of *Aleyrodes proletella* ACC

Full length ACC transcript and protein sequences could be assembled from all six populations. The principal component analysis (PCA) pictured a separation of the SPI-2/20 strain to almost all other strains analyzed (Fig. 1). PC1 is separating the spiromesifen susceptible strains to the resistant ones, although 2/20 was represented in all clusters. This explains 62.2 % variance. PC2 is explaining 17 % of variance and is clustering all samples in treated and untreated. Moreover, multiple alignment of the protein sequences showed that only SPI-2/20 and 2/20 (not shown) carried the A2083V alteration (Fig. 2, S1 and S2). *B. tabaci*, *M. persicae* and *T. vaporariorum* sequences were aligned for comparison.

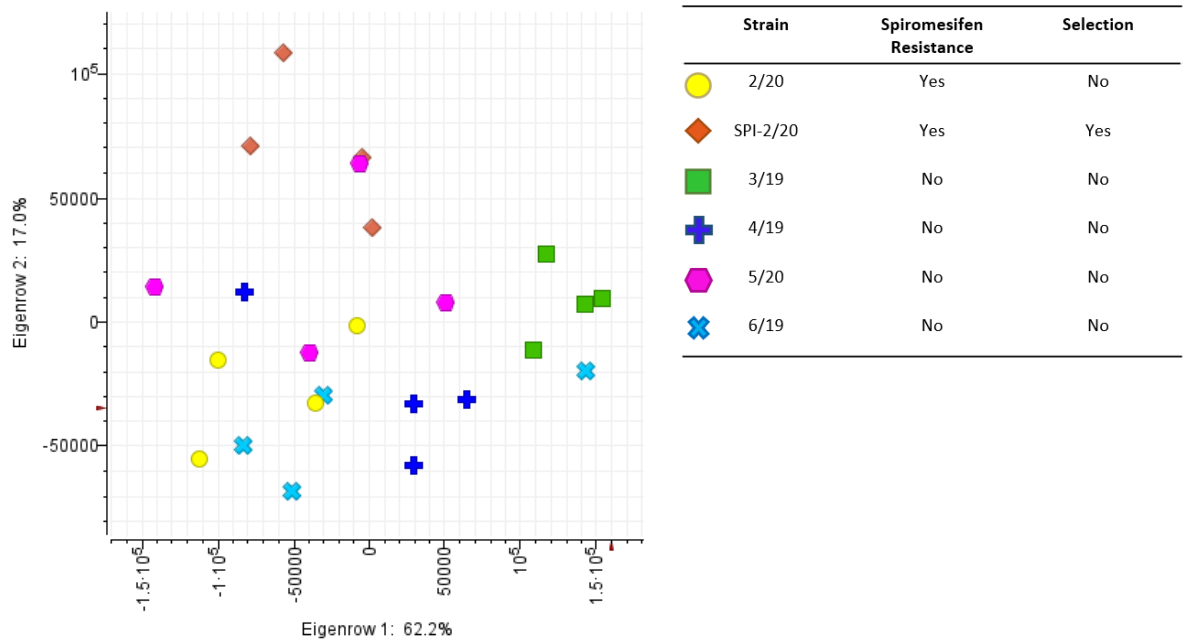

Fig. 1: Principal component analysis (PCA) of RNAseq data of six samples of *Aleyrodes proletella* of different origin. PC1 is displayed as “Eigenrow 1” on the x-axis, while PC2 is named “Eigerow 2” as y-axis. Each data point represents one sample. Population 2/20 is marked as yellow points, SPI-2/20 as orange transversal squares, the 3/19 as green squares, the 4/19 as blue plus signs, the 5/20 as pink hexagon and the 6/19 as light blue multiply signs.

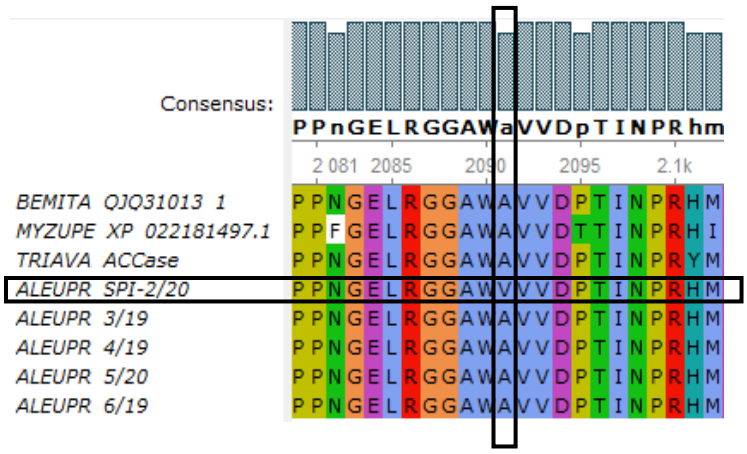

Fig. 2: Amino acid alignment of a partial sequence of the *Aleyrodes proletella* (ALEUPR) ACC carboxyltransferase (CT) domain encompassing the mutation site A2083V. The sequences from *Bemisia tabaci* (BEMITA) (GenBank accession no. MN567040), *Trialeurodes vaporariorum* (TRIAVA) (excerpt of ASM1176424v1 / Contig VMOF01000024.1 based on alignment of *B. tabaci* CDS) and *Myzus persicae* (MYZUPE) (XP_022181497.1) were aligned for comparison. The ALEUPR strain 2/20 was similar as its selected SPI-2/20 and is due to that not listed in the amino acid alignment.

3.4 Pyrosequencing diagnostic to genotype A2083V mutation in ACC of *Aleyrodes proletella*

In pyrosequencing diagnostic studies, the A2083V mutation was also found in several other *A. proletella* strains collected in European fields (Fig. 3). First studies made in 2019 and 2020, confirmed observations made in bioassays. Insects of the three living *A. proletella* populations, 5/19, 2/20 and 6/20, that showed high levels of resistance against spiromesifen and spirotetramat, were found having the known A2083V mutation in ACC (Table S3), as described for *B. tabaci* (Lueke, et al., 2020). In 2019, 10 % of the tested insects of the 5/19 population from Belgium were homozygote resistant for the A2083V mutation. Twenty percent were heterozygously resistant, having one allele containing the mutation and one wildtype allele. Later on, the selected 5/19 strain, SPI-5/19, was measured with 80 % homozygote resistant and 20 % heterozygote resistant whiteflies. Additionally, one strain, 2/19 from Turnasica, Croatia, showed susceptibility in bioassays (3.1, Table 2), had 20 % of insects being heterozygously resistant. The two strains from Germany, 2/20 and 6/20, that were spiromesifen resistant in bioassays (3.1, Table 2), were found having the A2083V mutation as well. The 2/20 insects were measured being 30 % homozygously and 60 % heterozygously resistant. Ten percent were evaluated in being homozygote susceptible (Table S3). The selected 2/20 strain, SPI-2/20, was measured with 90 % homozygously resistant and 10 % homozygously susceptible insects afterwards. The same data was collected for the 6/20 insects. Approximately 70 % of the spiromesifen resistant insects were homozygously resistant, i.e., having the A2083V mutation, while around 30 % were wildtype. All spiromesifen selected SPI-6/20 insects contained the A2083V mutation. The presence of the polymorphism in Germany (Fig. 3) was confirmed in further pyrosequencing studies with samples collected in 2021 (Table S1 and S3). Furthermore, in 2019 a population from Kallnach in Zollikofen, Switzerland (Fig. 3, Table S1), was found having 100 % homozygote resistant insects containing the A2083V polymorphism. In addition, insects of a strain from Warsaw, Poland, showed 10 % heterozygote resistance (Fig. 3, Table S3). All other insects that were received in ethanol in 2019, were homozygote susceptible. Furthermore, especially the strains collected from the Netherlands were found having the A2083V mutation in ACC (Fig. 3). Only two strains were found in being homozygote susceptible in 2021. All other populations tested between 2020 and 2021 were heterozygote or homozygote resistant (Table S3). Besides, insects collected in Spain were mostly susceptible. Just two strains sampled in 2021 showed a heterozygote resistance for A2083V (Fig. 3, Table S3).

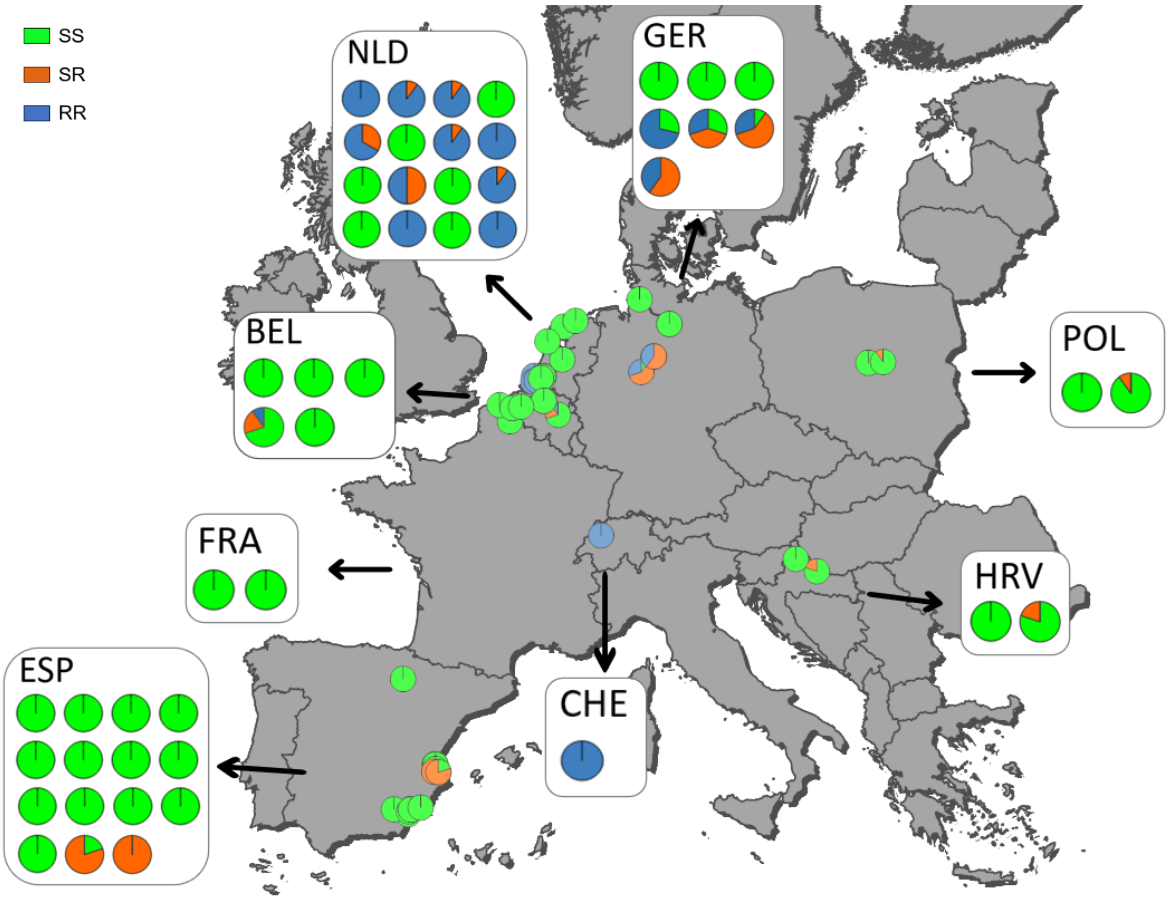

Fig. 3: Genotyping of ACC A2083V ketoenol target-site resistance alleles in adults of 49 *Aleyrodes proletella* populations collected from different geographies across Europe (A2083V genotyping data of individual samples are displayed in table S3). Pie charts display the proportion of genotypes assigned to RR resistant homozygotes (blue), SR heterozygotes (orange) and SS susceptible homozygotes (green).

3.5 Single-dose bioassays with the F1 generation of reciprocal crossed resistant and susceptible *Aleyrodes proletella* strains

Second instar nymphs of the F1 generation of susceptible 6/19 ♀ x resistant SPI-5/19 ♂ and SPI-5/19 ♀ x 6/19 ♂ were treated with a single-dose of 200 ppm spiromesifen. The F1 generations exhibited the same resistance level as its parent resistant SPI-5/19 population (Fig. 4). Ten percent of the offspring of the resistant SPI-5/19 ♀ x susceptible 6/19 ♂, died after 200 ppm spiromesifen treatment. Also, 2.5 % of the F1 generation of SPI-5/19 ♂ x 6/19 ♀ died after the application with the insecticide. Overall, the results suggest an autosomal dominant resistance pattern.

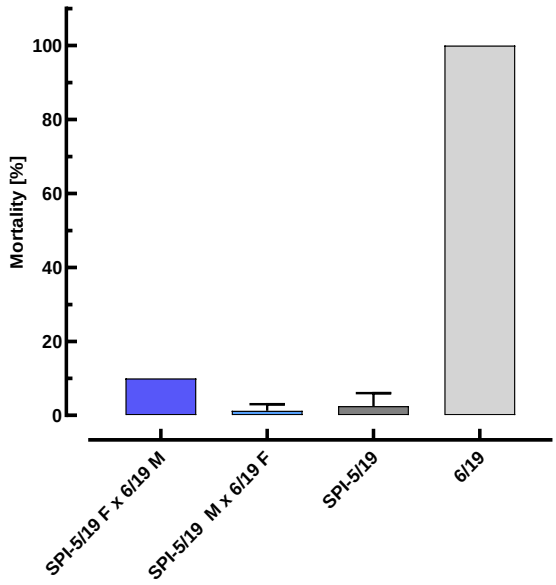

Fig. 4: Genetics of ketoenol resistance in *Aleyrodes proletella*. Reciprocal crossings and subsequent testing of the F1 offspring of the spiromesifen resistant SPI-5/19 and the susceptible reference strain 6/19 revealed an autosomal dominant inheritance of the resistance allele.

4 Discussion

4.1 Ketoenol resistance in *A. proletella*

In this study, the first case of ketoenol resistance in different *A. proletella* strains from Europe was investigated. Three out of eleven *A. proletella* strains reared in the laboratory were shown to have a high spiromesifen and spirotetramat resistance in bioassay studies. However, no cross-resistance to other insecticides such as λ-cyhalothrin, acetamiprid or flupyradifurone was detected in any *A. proletella* strain tested. Furthermore, the known A2083V mutation, which was found previously in being responsible for *B. tabaci* ketoenol resistance (Lueke et al., 2020), was also detected in the resistant *A. proletella* whitefly strains 5/19, 2/20 and 6/20 as well as in several other strains preserved in ethanol and collected from 2019 to 2021.

The importance of the mutation site was previously described in *B. tabaci* by Lueke et al. (2020). In synergism studies, only the inhibitor piperonyl butoxide (PBO) increased the ketoenol efficacy against resistant cotton whiteflies at very low level, contributing to the assumption that metabolic resistance could not be the major factor of ketoenol resistance in *B. tabaci* (Bielza et al., 2019). The A2083V target-site mutation is located in a strongly conserved region of ACC and was functionally confirmed to confer ketoenol resistance. In testing the insecticide toxicity to a genome edited *Drosophila* strain carrying the relevant mutation, high ketoenol resistance levels were measured. The observed spirodiclofen,

spiromesifen and spirotetramat resistance of the flies, confirmed that the A2083V mutation is the major mechanism for ketoenol resistance. Furthermore, the polymorphism was also found in being dominant when being passed to the F1 generation of reciprocally crossed resistant and susceptible *B. tabaci* populations (Lueke et al., 2020). The same results were obtained here in reciprocal crosses of resistant SPI-5/19 and susceptible 6/19 *A. proletella* strains. When second instar nymphs of SPI-5/19 ♀ x 6/19 ♂ and SPI-5/19 ♂ x 6/19 ♀ were exposed to 200 ppm spiromesifen, low mortality rates of 10 % and 2.5 % were measured, suggesting an autosomal dominant inheritance pattern (Fig. 4). The dominance of the A2083V mutation, shows its importance for future whitefly control strategies. Because of that, strains that were found in being heterozygously resistant for A2083V were of same relevance as homozygously resistant insects carrying two resistance alleles. In addition, a second amino acid substitution, the A2151V, was found in ketoenol resistant *B. tabaci* strains in Spain. However, the mutation is located in a less conserved region, suggesting no direct influence on ketoenol insecticide resistance (Lueke et al., 2020). Due to that, the analyzed *A. proletella* populations were not evaluated for the presence of the A2151V mutation in this study. In addition, previously in *T. vaporariorum* spiromesifen resistance has been associated to a E645K mutation site (Karatolos et al., 2012b). However, it is known, that this polymorphism is not located in the whitefly ACC CT domain, but between the BC and BCC domain. Due to that the ketoenol insecticides cannot interact with the respective E645K amino acid site (Lueke et al., 2020; Lümmen et al., 2014). Additionally, in a study with *T. vaporariorum* populations from Greek, the frequency of the mutation connected to spiromesifen resistance was analyzed by sequencing and Taqman allelic discrimination assays. Although the polymorphism was found in 20 whitefly populations with around 38 % frequency, it was not possible to confirm the relationship to the relevant phenotype, causing spiromesifen resistance in the populations from Greek (Kapantaidaki et al. 2018). The same was observed for the A1079T mutation in *Tetranychus urticae* which was linked to spirodiclofen resistance. Due to the fact, that the polymorphism is located outside the conserved ACC CT domain, the involvement in spirodiclofen resistance is unlikely (Khajehali, 2010; Lümmen et al., 2014; Wybouw et al., 2019). The mutation was also engineered into a *Drosophila* line which was tested for its ketoenol resistance in toxicity bioassays. However, there was no statistically significant difference detected in spirodiclofen susceptibility of the A1079T *Drosophila* transgenic strain and the wildtype line (Lueke et al., 2020).

4.2 Whitefly control strategies

As recommended by the Insecticide Resistance Action Committee (IRAC), insecticides with different mode of action (MoA) should be rotated in sucking pest control strategies (Fig. 5).

During an insect generation of approximately 30 days (= one window), just compounds of the same MoA should be applied. In the following window, another MoA class should be used. The same as for the third application window. The fourth application window, nearly at the end of the cultivation cycle, can include either the first or second used MoA class again, because consecutive generations are not directly exposed to the same MoA (IRAC, 2020) (Fig. 5). As shown in this study, the insecticides λ-cyhalothrin, acetamiprid or flupyradifurone could be used for an effective cabbage whitefly control strategy, because of the lack of cross-resistance to ketoenols. However, the usage of the pyrethroid λ-cyhalothrin should be considered carefully, as cabbage whiteflies were found having evolved resistance against the insecticide previously (Springate & Colvin, 2012). Additionally, pyrethroid resistance is known in *T. vaporariorum* as well as *B. tabaci*, making the insecticide class less attractive for an effective IRM strategy (Karatolos et al., 2012a; Roditakis et al., 2006). Even though acetamiprid resistance levels were reported for cotton whitefly and greenhouse whitefly (Elbert & Nauen, 2000; Karatolos et al., 2010), it was shown that the insecticide is still effective for cabbage whitefly control. The same recommendation can be given for flupyradifurone usage. The insecticide can be still recommended for cabbage and greenhouse whitefly control. In *B. tabaci* resistance was already detected, but with RR of 4 to 16.3 (Smith & Nagle, 2014) and 22 to 29 (Wang et al., 2020), however, these resistance ratios are unlikely to compromise recommended label rates. Moreover, for an overview of described insecticide resistance cases in insect pests, the 'Arthropod Pesticide Resistance Database' (APRD, http://www.pesticideresistance.org) as well as the IRAC MoA classification schemes, teaching material and guidelines (https://irac-online.org) can be recommended. Besides, the integrated usage of pest management strategies, as crop rotation, natural enemies and the consideration of the economic threshold can effectively reduce pest infestations (Andrews & Kassam, 1976; Horowitz et al., 2020; Kenis et al., 2019; Koch et al., 2018; Lopes et al., 2016).

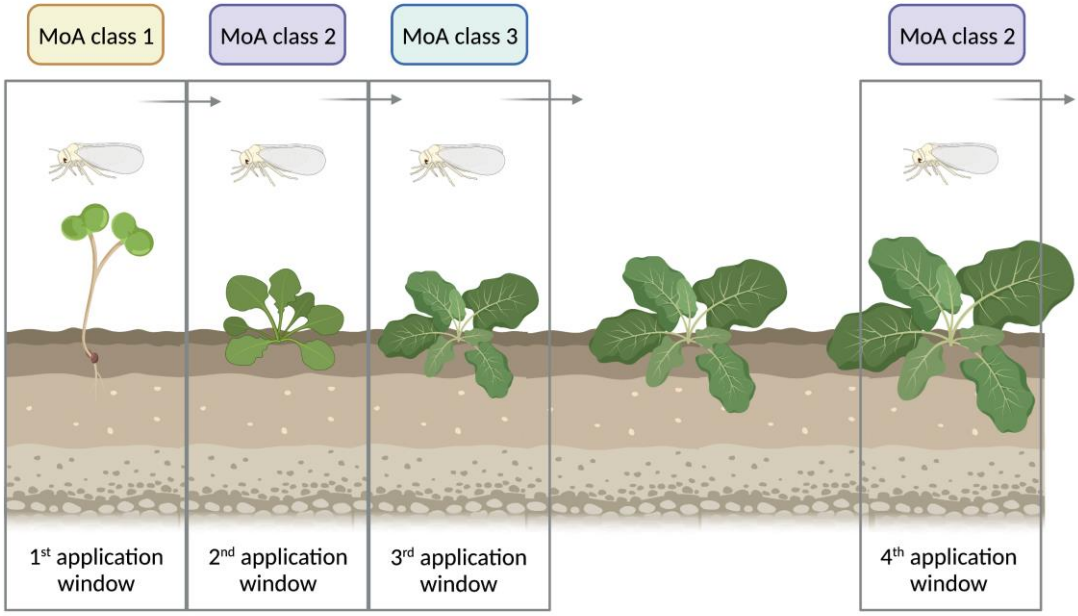

Fig. 5: Resistance management strategy using a mode of action treatment window approach for sustainable *Aleyrodes proletella* control in cabbage (Created with BioRender.com; modified after IRAC, 2020).

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Chapter 5

General Discussion

Modern agriculture faces major challenges: feeding a growing number of humans despite fewer resources and minimizing the effects of climate change (Foley et al., 2011; Handler, 1970; Knipling, 1979; and references cited therein). Sustainable production is not only important for addressing these challenges, but it is also relevant by reducing the risk of insecticide resistance (Foley et al., 2011; Sparks et al., 2021). Insecticide resistance is the reduced sensitivity of insect pests which feed on cultivated plants, reducing the plants quality, and lowering their yields, furthermore insect pests are no longer successfully controllable with insecticides (IRAC, 2021). In fact, aphids and whiteflies belong to the most challenging pest species. The sucking insects are mostly distributed worldwide, often polyphagous and develop in complex life cycles (Gullan & Martin, 2009).

5.1 Insecticide resistance in Hemipteran crop pests

The use of insecticides of different MoA is the basis for an effective control strategy for sucking pests such as aphids and whiteflies. One of the insecticide classes being recommended for an effective aphid control are chordotonal organ TRPV channel modulators like pymetrozine (IRAC, 2018). Because of its selectivity, pymetrozine was described as an optimal insecticide for IPM strategies. It is highly effective against pest aphid populations but harmless to non-target arthropods which are beneficial for IPM (Margaritopoulos et al., 2010; Sechser et al., 2002). This insecticide was shown to effectively control green peach aphids that are resistant to various insecticide classes (Foster et al., 2002) and pymetrozine resistance has not been found in any aphid species yet (Mota-Sanchez & Wise, 2022). After this thesis' examination and detection, it can be stated that pymetrozine resistance in green peach aphids with RR up to 403 could be proven for the first time and was presumably caused by upregulated esterases and P450 genes (chapter 2). However, Pymetrozine resistance is by far not as widespread as pyrethroid resistance in Hemipteran crop pests such as aphids. The first cases of pyrethroid insecticide resistance in green peach aphids were described in the early 80s. Next to organophosphates and carbamates, low to moderate levels of pyrethroid resistance were found to be linked to overexpressed carboxylesterase E4 and FE4 genes (Devonshire & Moores, 1982; Devonshire et al., 1983; Needham & Sawicki, 1971). Another important mechanism of pyrethroid resistance is mediated by *kdr* and *skdr* polymorphisms in the VGSC (Eleftherianos et al., 2008; Fontaine et al., 2011; Martinez-Torres et al., 1997; Martinez-Torres et al., 1999). Pyrethroid resistance has been described in several hemipteran pests including whitefly species such as *B. tabaci* (Cahill et al., 1995) and was investigated in pea aphids *A.*

pisum in this study, too. The resistance was linked to an upregulated P450 gene, *CYP6CY12* (chapter 3). There is no other documented insecticide resistance case of pea aphids described yet (Mota-Sanchez & Wise, 2022). Furthermore, pyrethroid resistance was identified in cabbage whiteflies *A. proletella* but the mechanism of resistance was not further investigated (Springate & Colvin, 2012). Whitefly species were known to be effectively controlled with ketoenol insecticides as spiromesifen and spirotetramat (IRAC, 2019). Field relevant levels of resistance to ketoenols are not widespread yet and are restricted to the whitefly species *B. tabaci* (Bielza et al., 2019) and *T. vaporariorum* (Karatolos et al., 2012). In the greenhouse whitefly *T. vaporariorum* ketoenol resistance seemed to be linked to an E645K mutation in ACC (Karatolos et al., 2012). However, later work did not confirm the relevance of the mutation for ketoenol resistance (Kapantaidaki et al., 2018). Another target-site mutation, A2083V, was discovered in ketoenol-resistant strains of *B. tabaci* and was functionally linked to high levels of spiromesifen resistance (Lueke et al., 2020). The mutation was found in a highly conserved region of the ACC CT domain, the binding site of ketoenol insecticides (Lueke et al., 2020; Lümmen et al., 2014). The authors employed a reverse genetic approach to functionally validate the A2083V mutation in the model insect *D. melanogaster* which is a wildtype sensitive to ketoenol insecticides. 'Clustered regularly interspaced short palindromic repeats' (CRISPR)-Cas9 genome-edited transgenic *Drosophila* flies carrying an A2083V mutation were highly resistant to the ketoenols spiromesifen, spirotetramat and spirodiclofen when compared to wildtype flies (Lueke et al., 2020). In this thesis, the A2083V mutation was also identified in *A. proletella* whiteflies which showed high levels of spiromesifen and spirotetramat resistance in cultivated fields as well as in greenhouses studies.

The investigation of insecticide resistance is of great importance for effective IRM and thereby improved IPM strategies.

5.2 Resistance diagnostic studies

5.2.1 Bioassay approaches

To uncover resistance mechanisms in insect species, a wide range of methods and experiments have been performed. Molecular-, biochemical- and sequencing-approaches are required for gaining a complete understanding of the detected resistance case. The IRAC is providing easy to conduct standard bioassays for a validated test result and monitoring of insect resistance across the world (IRAC International, 2022). Within this thesis, *M. persicae* and *A. pisum* aphids as well as *A. proletella* whiteflies were evaluated in their resistance to various insecticides using IRAC test method 019 (chapter 2 and 3) and 016 (and others, chapter 4) (IRAC International, 2022).

Pymetrozine resistant green peach aphids showed cross-resistance to the neonicotinoid acetamiprid and the pyrethroid deltamethrin. Cross-resistance patterns occur when insecticides are influenced of the same resistance mechanism or when having the same target-site. In most cases, the affected insecticides have the same MoA and are often structural relatives (FAO, 2012). Multiple resistance is visible in organisms showing more than one resistance mechanism which might affect different classes of insecticides (FAO, 2012). Neonicotinoid cross resistance with pymetrozine was previously investigated in greenhouse and cotton whiteflies (Elbert & Nauen, 2000; Karatolos et al., 2010). Low levels of pymetrozine (up to 18-fold) and deltamethrin (up to 4.7-fold) resistance were investigated in cotton whitefly strains from West Africa (Houndété et al., 2010). Next to others, non effectiveness of pymetrozine, the pyrethroid etofenprox and the carbamate isoprocarb were previously observed on the whitebacked planthopper *Sogatella furcifera*. All three insecticides were not recommended for future *S. furcifera* control (Li et al., 2020). No cross-resistance cases to other TRPV channel modulators, neither to afidopyropen, nor pyrifluquinazon were found in the pymetrozine resistant green peach aphids *M. persicae* (chapter 2, Table 2). The obtained results are comparable to previous studies. Afidopyropen and pymetrozine were found having no cross-resistance in *B. tabaci* as well (Zhang et al., 2021). Additionally, pyrifluquinazon as well as flonicamid, the chrodotonal organ modulator with an undefined target-site, were analyzed for their effectiveness against green peach aphids. The former is not known for resistance to insect pests yet (Mota-Sanchez & Wise, 2022). Flonicamid cross-resistance was previously described in a neonicotinoid resistant *A. gossypii* strain (Koo et al., 2014). Cross-resistance between flonicamid and pymetrozine has not been found in any insect species to date (Mota-Sanchez & Wise, 2022).

Bioassays were also conducted for investigating pyrethroid resistance in pea aphids (chapter 3). Next to the analyzed deltamethrin resistance in the pea aphid populations PYR-R1 and VR, the pyrethroids λ-cyhalothrin, tau-fluvalinate, α-cypermethrin and cis-permethrin were non effective against the insects. The same pattern was observed in previous studies where pyrethroid resistance was investigated in the cabbage whitefly *A. proletella*. The insects showed resistance against λ-cyhalothrin, deltamethrin and cypermethrin. Furthermore, *A. proletella* strains were found having evolved defenses against bifenthrin (Springate & Colvin, 2012). Correlation in ineffectiveness of pyrethroids was also monitored in resistance evaluations of the aphid species *Rhopalosiphum padi* and *S. avenae*. Both defended the toxic effect of the pyrethroids bifenthrin and beta-cypermethrin (Gong et al., 2021). Non toxicity of this chemical class was also studied as being correlated to organophosphate resistance in insect species (Ahmad et al., 2002; Cahill et al., 1995). However, in chapter 3 of this thesis, cross-resistance in pyrethroid resistant pea aphids to organophosphates could not be confirmed. In previous studies, the P450 gene *CYP6CY12*, which was linked to pyrethroid

resistance in pea aphids in this study, was already detected in other insecticide resistant insects. In the cotton aphid *A. gossypii* upreagulated P450 genes, including *CYP6CY12*, were detected and were putative drivers for cyantraniliprole and pyrethroid cross-resistance (Zeng et al., 2021). Cross-resistance of pea aphids to pyrethroids and RyR insecticides were not evaluated in the present thesis (chapter 3). Additionally, the upregulation of the *CYP6CY12* gene in another *A. gossypii* strain was linked to neonicotinoid resistance (Wu et al., 2018). There are already a number of cases known in which neither pyrethroids nor neonicotinoids were effective in controlling insect pests (Darriet & Chandre, 2013; Erdogan et al., 2021; Gong et al., 2021; Liu & Yue, 2000; Romero & Anderson, 2016). A future influence of the *CYP6CY12* in neonicotinoid resistance in pea aphids can be expected (Wu et al., 2018). Cross-resistance of pyrethroid insecticides and flupyradifurone has not been published yet (Mota-Sanchez & Wise, 2022). However, a low cross-resistance to the butenolide flupyradifurone was found in the pyrethroid resistant *A. pisum* strain PYR-R1 analyzed in this thesis (chapter 3).

Flupyradifurone cross-resistance could not be detected in the spiromesifen resistant *A. proletella* strains analyzed in chapter 4. As the butenolide flupyradifurone, the pyrethroid *λ*-cyhalothrin and the neonicotinoid acetamiprid were effective in controlling the ketoenol resistant cabbage whiteflies. The previously detected pyrethroid resistance in cabbage whiteflies could not be confirmed in the strains analyzed within this thesis (Springate & Colvin, 2012). In addition, the reported results made within this study are not correlating with cross-resistance issues identified in the cotton aphid *A. gossypii*. In *A. gossypii* high levels of resistance to the ketoenol spirotetramat were accompanied by cross-resistance to the pyrethroids α-cypermethrin and bifenthrin. Although the underlying mechanisms remained elusive, the involvement of a P450 gene, named CYP6A2, is indicated (Peng et al., 2016). A similar pattern was observed in *T. vaporariorum* strains with resistance to imidacloprid and spiromesifen (Kapantaidaki et al. 2018). In contrast, cross resistances between spiromesifen and spirotetramat were identified in the cabbage whiteflies being analyzed in this study. The same results were obtained in *B. tabaci* which was highly resistant to spiromesifen but was also evaluated with levels of resistance to spirotetramat and spiropidion (Bielza et al., 2019; Lueke et al., 2020).

5.2.2 Target-site resistance

In a following set of molecular diagnostic studies, all analyzed insect strains were evaluated for the presence of known mutation sites causing target-site resistance towards insecticides. The evaluation of known mutations which cause resistance against various insecticides is a necessary step to inform resistance management strategies.

After the detection of high pyrethroid resistance in pea aphids in vivo, the insects were further analyzed for the presence of known mutations present in the VGSC (chapter 3). However, the *kdr* and *skdr* polymorphisms which are known for being responsible for pyrethroid resistance (Rinkevich et al., 2013 and references cited therein), were neither detected by pyrosequencing nor by RNAseq approaches. As for the pea aphids, *A. proletella* and *M. persicae* populations were also checked for the presence of known mutations by pyrosequencing method. Due to its sensitivity to detect less than two percent differences between the allele frequency of pools, the sequencing device is a standard (Gruber et al., 2002). The technique was useful for evaluating target-site mutations in the ryanodine receptor being addressed by diamide insecticides in the fall armyworm *Spodoptera frugiperda* and diamondback moth *Plutella xylostella* (Boaventura et al., 2020a; Troczka et al., 2012). The pyrosequencing approaches being conducted in this study were successful as well. The method uncovered the target-site resistance in spiromesifen resistant *A. proletella* strains which is based on an amino acid exchange A2083V in the CT domain of ACC. The polymorphism is also conferring ketoenol resistance in *B. tabaci* (Lueke et al., 2020). *M. persicae* strains analyzed in chapter 2 were screened for known mutation sites in the VGSC, including *kdr* and *skdr*, but also R81T in the nAChR ß1-subunit that causes resistance to nAChR competitive modulators (Bass et al., 2011; Bass et al., 2014), as well as the S431F polymorphism which is responsible for insect resistance to AChE inhibitors (Andrews et al., 2002; Nabeshima et al., 2003). The pyrosequencing results can be reviewed in chapter 2, table 4.

Next to pyrosequencing, the RNA of the analyzed insect strains was sequenced in Illumina technology approaches. With NGS like Illumina sequencing a high throughput technology with reduced costs was developed in the past. The HiSeq2000 machine of Illumina can evaluate up to 50 Gb of sequences within one day. The technology is low-cost and performs with a great coverage of sequences (Caporaso et al., 2012). Today, the Illumina technology has been used for sequencing several insect populations for the detection of resistance mechanisms, e.g., with the approach, mutations such as the L1014F (*kdr*) and overexpressed detoxification genes, for example the *CYP6BQ23* in pollen beetle *Meligethes aeneus*, have been evaluated (Zimmer et al., 2014). The RNAseq approach in chapter 3 uncovered upregulated detoxification genes and was the basis for further examination of the *CYP6CY12* gene and is responsible for pyrethroid resistance in pea aphids. In addition, the technology was used for screening the ACC full length sequence in *A. proletella* (chapter 4). In chapter 2 of this thesis, a comparative study was performed to evaluate the preciseness of the new NGS method ONT in comparison to Illumina. ONT was already an effective tool in studies with limited resources, e.g., for detection of the Ebola virus disease (Quick et al., 2016). However, future development activities are needed to reduce high error rates and mismatches.

5.2.3 Metabolic resistance

RNAseq studies are not only useful for investigating relevant mutation sites in insect pests but also expression levels of DEGs in comparison to susceptible reference strains. In the two RNAseq approaches with Illumina and ONT in chapter 2, several induced and repressed DEGs were detected by both sequencing methods. In general, Illumina has detected more genes than ONT. However, a high sequencing expression of genes increased the probability of detection by ONT. A correlation of sequencing results of the two technologies was also visible in previous studies (Quick et al., 2016). Both technologies have uncovered upregulated esterases and P450 genes but their relevance in pymetrozine resistance in *M. persicae* needs to be investigated in future studies.

Generally speaking, first evidence of upregulated detoxification genes which could be responsible for insecticide resistance can be given by biochemical enzyme assays which were conducted with homogenates prepared from resistant insect pests in comparison to a susceptible strain. Based on such assays (chapter 3 of this thesis), metabolic resistance to pyrethroids in pea aphids were assumed. Homogenized insects were analyzed in their CEST and GST expression levels while the microsomes of the pea aphid strains were evaluated for upregulated P450 activity. Esterases and P450 genes were overexpressed, while GSTs were downregulated in the pyrethroid resistant pea aphid strains. As confirmed in previous studies, evaluations of expression levels of detoxification genes are an important step in analyzing resistance mechanisms of insect pests (e.g., in Boaventura et al., 2020b; Stumpf & Nauen, 2002).

However, at that point of the study, the specific genes which are responsible for the higher enzyme activity of P450 and CEST were not clear. Therefore, the RNAseq as well as the additional RT-qPCR confirmation of upregulated genes are of high importance. The pyrethroid resistant pea aphids' (chapter 3) were found with a series of upregulated P450s and esterases genes as well as UGT and ABC transporter proteins in RNAseq (appendix B, Table S3). In chapter 3 of this thesis, the detected overexpressed genes were also evaluated in their expression levels in RT-qPCR approaches. The method is useful to confirm upregulated genes which were measured in RNAseq studies, or which were already detected in being involved in insecticide resistance previously (Boaventura et al., 2020b). The downregulated GST genes were not of interest for this study and were therefore not further examined.

The recombinant expression of genes in insect cells is often a step following RNAseq and RT-qPCR. Here, the upregulated P450 *CYP6CY12* and *FE4-like esterase* (LOC100166921) genes in pea aphids were selected for a recombinant expression in insect cells which allowed their evaluation in pharmacogenetic studies (chapter 3). UPLC-MS/MS analysis was employed to uncover parent compound depletion, e.g., by hydroxylation during incubation with functionally

expressed P450 gene. Also, steady state kinetics show the binding of the analyzed gene with the substrate conjugation and is used as an effective tool of metabolic resistance approaches in various studies (Haas et al., 2021; Haas & Nauen, 2021; Nolden et al., 2021; Pavlidi et al., 2015).

5.3 Outlook on insecticide usage

5.3.1 Green peach aphid *Myzus persicae*

With the resistance diagnostic studies conducted, the newly emerged resistance mechanisms of the insect pests *M. persicae*, *A. pisum* and *A. proletella* were analyzed and uncovered. Based on the results obtained, recommendations for the selection of insecticide against each insect pest can be given.

For effective management of aphids and whitefly species the usage of insecticides of the same classes should be minimized (Bass et al., 2014; Horowitz et al., 2020). For green peach aphid management, the TRPV channel modulator pymetrozine is no longer recommended to be used against aphids from regions where resistance cases have been reported (chapter 2 of this thesis). However, the TRPV channel modulator pyrifluquinazon has only shown minor RR of 2.02 to 5.12 against the very same strains and could be used as an alternative in future aphid management strategies. No insect pest species were found to have evolved pyrifluquinazon resistance till now (Mota-Sanchez & Wise, 2022). Not only were green peach aphids published to be effectively controlled by pyrifluquinazon but also greenhouse whiteflies *T. vaporariorum* (Kang et al., 2012; McLeod & Rashid, 2014). In addition, the green peach aphids analyzed in chapter 2 of this thesis showed low resistance against the TRPV channel modulator afidopyropen (RR of 16 to 36). However, there are no resistance cases to that insecticide described till now (Mota-Sanchez & Wise, 2022). Afidopyropen was still useful for controlling aphid species (Koch et al., 2020; Vafaie & Grove, 2018). Moreover, when pymetrozine resistance is visible in field populations, flonicamid can also be useful for controlling *M. persicae*. However, if neonicotinoid resistance is obtained as in the strain IMDR, there could be a low cross-resistance to flonicamid obtained (RR of 15.9 in IMDR) (chapter 2). A correlation between imidacloprid and flonicamid resistance was previously reported in *A. gossypii*. Nevertheless, the resistance ratio of 62.4 is considerably higher than the 15.9 RR to flonicamid of the imidacloprid resistant *M. persicae* strain IMDR (chapter 2) (Koo et al., 2014). Besides, flonicamid is recommended to be used in an application program next to sulfoxaflor for controlling thiamethoxam (another neonicotinoid) resistant *A. gossypii* (Gore et al., 2013). However, the imidacloprid resistant *M. persicae* insects analyzed within this thesis showed high cross-resistance (184 RR) to sulfoxaflor (chapter 2). Due to that, the application of both

insecticides in rotation is not recommended for resistance management strategies. In contrast, the highly pymetrozine resistant strain PYM-R1 exhibited only minor cross-resistance to sulfoxaflor (RR of 17). The compound was effective (100 % mortality) against most of the green peach aphid strains tested in bioassays with discriminating rates (appendix A, Table S3). Thus, sulfoxaflor can be used in pymetrozine resistance management strategies against green peach aphids. Sulfoxaflor effectiveness is, however, expected to diminish in the upcoming years as studies with *A. gossypii* have shown increasing levels of resistance to sulfoxaflor after being under a continuous selection pressure of the insecticide (Ma et al., 2019; Wang et al., 2021).

Acetamiprid insecticide is not recommended to include in IPM strategies as resistance cases of insect pests against this compound are outstanding (chapter 2, Table 3) (Cai et al., 2021; Karatolos et al., 2010; Wang et al., 2007; Zhang et al., 2020). Additionally, green peach aphid populations from Greece have evolved resistance against the butenolide flupyradifurone (Papadimitriou et al., 2022) which was also detected in the strains evaluated in this thesis. The pymetrozine resistant green peach aphids showed cross-resistance ratios of 5.34 to 63.7 to the butenolide. Because of that, this insecticide is not recommended to be include in IPM strategies to control neonicotinoid resistant pests. Moreover, as a pyrethroid representative, deltamethrin was also shown to contribute to cross-resistance in analyzed pymetrozine resistant insects, as already identified in whiteflies *B. tabaci* (Houndété et al., 2010). Thus, making it difficult to control multi-resistant populations with a rather limited arsenal of mode of action in some regions, e.g. Europe.

5.3.2 Pea aphid *Acyrthosiphon pisum*

As for *M. persicae*, the variety of effective pyrethroids on pea aphids is rather low. Investigated in chapter 3, etofenprox could be an effective compound controlling not only *A. pisum* but also *M. persicae*. There are no studies published yet in which etofenprox was not effective in controlling aphids (Mota-Sanchez & Wise, 2022). In addition, studies have not yet shown that pea aphids have evolved resistance against any insecticide compound (Mota-Sanchez & Wise, 2022). The pyrethroid resistance found within this thesis was not linked to resistance cases against other insecticide classes (appendix B, Table S4): both, the butenolide flupyradifurone and the neonicotinoid thiacloprid were effective against pea aphids. In addition, ketoenol insecticides such as spirotetramat controlled pyrethroid resistant pea aphid species. All three insecticides are recommended for IPM of pyrethroid resistant pea aphid populations.

5.3.3 Cabbage whitefly *Aleyrodes proletella*

The cabbage whitefly *A. proletella*, being examined in chapter 4, has evolved resistance against the ketoenol insecticides spiromesifen and spirotetramat. The same was observed for *T. vaporariorum* as well as for *B. tabaci* previously (Lueke et al., 2020; Karatolos et al., 2012). Cross-resistance studies of *A. proletella* strains uncovered that ketoenol resistant insects have not evolved *λ*-cyhalothrin, flupyradifurone and acetamiprid resistance yet. However, resistance against the pyrethroid *λ*-cyhalothrin has been described in *A. proletella* and due to that is not recommended to be included in cabbage whitefly management strategies in those regions where the resistance allele is present (Springate & Colvin, 2012). Acetamiprid resistance has been studied in *B. tabaci* since 2000 and is also present in the greenhouse whitefly *T. vaporariorum* in lower rates (Elbert & Nauen, 2000; Karatolos et al., 2010). Due to that, cabbage whiteflies will likely develop acetamiprid resistance within the next years as well. Moreover, flupyradifurone is not known as being ineffective against greenhouse whiteflies but has shown lower susceptibility against Chinese and American *B. tabaci* (Smith & Nagle, 2014; Wang et al., 2020).

5.4 Future studies

For being prepared for the evolving resistance pattern in the future, studies for enhancing knowledge are essential for successful IRM and IPM strategies. To develop new pymetrozine resistance monitoring strategies, it is important to uncover the resistance mechanism of *M. persicae* against the TRPV channel modulator pymetrozine (chapter 2). Upregulated genes which were detected in RNAseq should be analyzed in their expressions in a RT-qPCR approach. Another point of interest is the usage of a digital PCR (dPCR) which is performing an absolute quantification of low resistance allele frequency in pooled samples. No standard curve needs to be determined as a calibration step, as is obligatory with RT-qPCR. dPCR samples are evaluated in an endpoint analysis (Gürtler & Gerdes, 2014). The method is particularly useful for the detection of rare mutations, for example in tumor diagnostic studies (Pohl & Shih, 2004). If a significantly upregulated gene in the resistant strains is detected within the future studies, the gene should be further evaluated in pharmacogenetic studies contributing to a final elucidation of the *M. persicae* resistance mechanism to pymetrozine.

In the future, a monitoring service for analyzing green peach aphid strains of different geographies would increase the knowledge about the spread of pymetrozine resistance. Such sequencing service will also be beneficial when studying *A. pisum* resistance and was already developed for European cabbage whitefly monitoring in ketoneol resistance in chapter 4. The screening of the presence of the A2083V mutation in ACC should be continued in future years.

Because the cabbage whitefly is dispersing rapidly all over the world (chapter 1, Fig. 8), globally sampled insects should be sequenced. Analyzing samples of different regions in their insecticide susceptibility will be helpful for advising IPM and IRM strategies. Of high interest is the usage of an allelic discrimination assay instead of the previously used pyrosequencing technique to detect target-site mutations. This method is based on a PCR approach and is of low labor costs. The high-throughput and rapid assay performs well with no failed reactions (Jones, et al., 2008; Salvi et al., 2001). It was also found to be an effective tool to confirm pyrosequencing results and to help monitor resistance cases in, e.g., fall armyworm populations (Boaventura et al., 2020a). The resistance investigation of other aphid and whitefly species to insecticides analyzed within this thesis, e.g., populations of the greenhouse whitefly *T. vaporariorum*, is also of great importance. New active ingredients against hemipteran crop pests should be involved in bioassays which are important for informing IRM strategies.

Also 'RNA interference' (RNAi) and CRISPR will be of interest in future IPM and IRM strategies. Gene knockdown via RNAi was already effective with marker genes of *A. pisum* (Jaubert-Possamai et al., 2007). The method is also useful for obtaining plant resistance to pests, e.g., in wheat against the grain aphid *S. avenae* (Zhao et al., 2018) or in breeding resistant plant varieties against whiteflies (Ibrahim et al., 2017). Besides, the CRISPR technology could become an important tool for future pest management strategies (Islam, 2019). For example, it was examined that watermelon vacuolar sugar transporter mutants decrease aphid feeding and can be included in gene editing strategies of crops (Li et al., 2022). Following these recommendations, will contribute to a successful IRM in the future.

5.5 Summary

The aim of this thesis was the investigation and characterization of resistance mechanisms in hemipteran crop pests against insecticides of different chemical classes. The cotton whitefly *B. tabaci* is the pest species with the highest described number of resistance cases followed by the green peach aphid *M. persicae* (chapter 1, Table 2) (Mota-Sanchez & Wise, 2022). The global relevance of aphids and whiteflies underlines the importance developing strategies for monitoring resistance.

The pymetrozine resistance in *M. persicae* that was examined in chapter 2 is the first published resistance case of green peach aphids against this active ingredient. Employing leaf-dip bioassays, cross-resistance to other chemical classes were detected making the resistance case of high importance. In further studies, several *M. persicae* strains were analyzed by pyrosequencing for the presence of target-site mutations. Known polymorphisms in the VGSC, nAChR as well as MACE were detected and helped to understand the genetic differences

between aphids with pymetrozine or imidacloprid resistance. The RNAseq Illumina approach was compared with a direct RNA sequencing via ONT and uncovered overexpressed detoxification genes in the analyzed green peach aphids. ONT was also estimated in its sequencing quality and preciseness as a portable solution for resistance diagnostics in areas with limited resources. Compared to ONT, which revealed a higher error rate, the standard method Illumina obtained more reliable sequencing results. Within this study, the sequencing suitability of ONT in delivering comparable results to Illumina whilst having limited resources was explored.

Furthermore, in the second study (chapter 3), another important aphid species was examined in its resistance mechanisms against pyrethroid insecticides. It is the first case of insecticide resistance reported in *A. pisum* and is therefore of high importance for future pea aphid management strategies. Leaf-dip bioassays revealed pyrethroid cross-resistance. In a pyrosequencing approach the pea aphid populations were analyzed for the presence of relevant mutations sites in the VGSC conferring pyrethroid resistance but no polymorphisms could be detected. Synergism bioassays suggested metabolic pyrethroid resistance and biochemical activity assays confirmed enhanced P450 and CEST activity in pyrethroid resistant pea aphids. Upregulated detoxification genes in the pyrethroid resistant pea aphids were detected within an RNAseq approach and further measured in their expressions by RT-qPCR. Due to that, the enhanced P450 gene *CYP6CY12* and the *FE4-like esterase* gene were selected for recombinant expressions in insect Sf9 cells. In pharmacogenetic studies, CYP6CY12 could finally be shown to hydroxylate the pyrethroid deltamethrin. Experiments with the functionally expressed FE4-like esterase (LOC100166921) gene failed to demonstrate its capacity to detoxify deltamethrin by hydrolysis. The results obtained are an important cornerstone for pea aphid management strategies.

Whitefly management is of great concern and was investigated in cabbage whiteflies resistant to ketoenols in this thesis (chapter 4). The cabbage whitefly is an emerging pest species which is rapidly dispersing around the world. Studies on its resistance to insecticides are relevant to inform future IPM and IRM strategies for controlling this pest. The ketoenol resistance investigated in this study was comparable to ketoenol resistance which recently evolved in *B. tabaci* (Lueke et al., 2020). It was possible to uncover the resistance in *A. proletella*, which is based on the same amino acid exchange A2083V being located in ACC as it was previously described for the cotton whitefly (Lueke et al., 2020). Utilizing a pyrosequencing approach, several samples collected in different European countries were analyzed for the presence of the A2083V polymorphism. With the establishment of a sequencing service, the dispersion of the mutation in European cabbage whiteflies was explored for over three years. The aim of this study, namely the uncovering of the resistance mechanisms of cabbage whiteflies against

ketoenol insecticides, went along with networking, monitoring and suggestions of IRM strategies to farmers and crop protection advisors.

In this thesis, a wide range of methods for the characterization of insecticide resistance was used. Next to standard bioassays and pyrosequencing-based diagnostics, new and complex assays, such as for example the ONT approach in chapter 2, were investigated and executed. Furthermore, the recombinant expression of detoxification genes as well as subsequent pharmacogenetic approaches, Michaelis-Menten kinetics or the expression analysis of genes by RT-qPCR helped to unravel a novel P450-based mechanism of resistance in pea aphids underlining the importance of molecular tools. This thesis characterizes diverse resistance mechanisms found in important hemipteran pest species and the obtained results will help to inform IPM and IRM strategies to sustainably control these pests.

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Appendix A: Supporting material of Chapter 2

Whole genome sequencing study of pymetrozine resistant green peach aphids *Myzus persicae* comparing Illumina Sequencing and Oxford Nanopore Technology

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Table S1. Primer pairs used for pyrosequencing of known mutation sites in green peach aphids *Myzus persicae*. Abbreviations: bp, base pair; VGSC, voltage-gated sodium channel; nAChR, nicotinic acetylcholine receptor; MACE, modified acetylcholinesterase; for., forward; rev., reverse; seq., sequencing, btn, biotin.

Target	Mutation	Primer	Sequence (5‘ to 3‘)	Length [bp]
VGSC	L1014F	for.	[btn]ATTATGTGGCGAATGGATAGAATC	24
		rev.	GCCCCGAGTAGTACATATTTATCA	24
		rev. seq.	AGTACTTATACATACCACG	19
	M918L/T	for.	GCCCACACTGAATCTTTTAA	20
		rev.	[btn]CATACCCATGACGGCAAATA	20
		for. seq.	TGAATCTTTTAATATCCAT	19
nAChR	R81T	for.	[btn]GCCTGCAGCTATTAAAATATCCAA	24
		rev.	GGCATTAGTCGTTTATGCGGTAG	23
		rev. seq.	TTAAGTAGGTTACTCACAAG	20
MACE	S431F	for.	[btn]TATACTCATGGGTAGTAACTCCG	23
		rev.	CAAAATTTTCACGAGACACCA	22
		rev. seq.	GCTCCGTCAAATAAT	15

Table S2. Primer pairs used for amplicon sequencing of nicotinic acetylcholine receptor (nAChR) as well as the protomers Inactive (IAV) and Nanchung (NAN) of the transient receptor potential vanilloid (TRPV) channel in green peach aphids *Myzus persicae*. Abbreviations: bp, base pairs; for., forward; rev., reverse; seq., sequencing, btn, biotin.

Target gene	Amplicon number	Primer	Sequence (5’ to 3’)	Primer length [bp]	Amplicon length [bp]
nAChR	1	for.	AGGATGTGGGTGGTCAGGTA	20	2,580
		rev.	CAACCTAACCTTCAGCGGGT	20	
IAV	1	for.	AGACTACTCGTATCTCCGTTCA	22	4,199
		rev.	CCGTTTGGTTTCCGCAACAA	20	
	2	for.	TCGGCGTCACTGATTCACAA	20	6,422
		rev.	TTTAGCGACCGGAGAAGACG	20	
	3	for.	CGTAGATTAGAAGGCACTGGC	21	4,011
		rev.	CCAAAAGACAATTTCAATGCTACGG	25	
NAN	1	for.	GAGCTACGGTACTGAGCTGC	20	6,665

Target gene	Amplicon number	Primer	Sequence (5' to 3')	Primer length [bp]	Amplicon length [bp]
NAN	1	rev.	TCGCCGGGCTTAAGTTCAAT	20	
	2	for.	TCTCACGCAACTATGACGCT	20	6,484
		rev.	TTGGGCGTTTCACACCGATA	20	
	3	for.	TCGTTCTCTGGAAACCGTCG	20	6,439
		rev.	CGATTTCGACCGAGTGTGGA	20	
	4	for.	AGAGCAGGCGTATTCGTTGT	20	6,700
		rev.	TGGGTTTCAAACACGGAGCT	20	
	5	for.	TTTACATCTCCACGGGGCAC	20	7,059
		rev.	TGGCTCCACCGTTTGAGTTT	20	
	6	for.	CGAAATACAGCAAGCGCACT	20	6,837
		rev.	CTGCCACCTCCTATCACGT	19	
	7	for.	CTTTTGACCCCCTTCCCCTC	20	6,755
		rev.	TGGCCAGTTATTGAACATTTAGCA	24	

Table S3. Single-dose (8 ppm) mortality data (±SEM) of three insecticides different classes against *Myzus persicae* adults (n=30) of different strains. Significant differences in mortality between all tested strains were measured (data not shown). Evaluation of affected peach aphids was done 72 h after leaf-dip bioassay application.

	Mortality (±SEM)		
Population	**Pymetrozine**	**Acetamiprid**	**Sulfoxaflor**
3/14	38.1 (12.6)	0 (0)	81.8 (31.5)
13/15	91 (8.4)	94 (5.22)	100 (0)
20/15	93 (6.08)	93.3 (11.6)	100 (0)
7/16	100 (0)	100 (0)	100 (0)
2/18	44.5 (25.9)	0 (0)	31.1 (20.1)
3/18	91.9 (7.08)	87.7 (12.5)	100 (0)
7/18	83.2 (29.2)	0.5 (35.5)	96 (7)
9/18	72.5 (16.3)	61.9 (57.5)	100 (0)
12/18	58.1 (29.6)	20.7 (1.28)	100 (0)
13/18	62.9 (14.8)	35.8 (60.5)	100 (0)

Table S4. Single-dose (10 and 100 ppm) mortality data in percent (±SEM) of imidacloprid insecticide against *Myzus persicae* adults (n=30) of different strains. Significant differences in mortality between all tested strains were measured (data not shown). Evaluation of affected peach aphids was done 72h after leaf-dip bioassay application.

Population	Imidacloprid mortality [%] (±SEM)	
	10 ppm	100 ppm
HS	100 (0)	100 (0)
IMDR	0 (0)	0 (0)
11/18	0 (0)	23.1 (24.8)

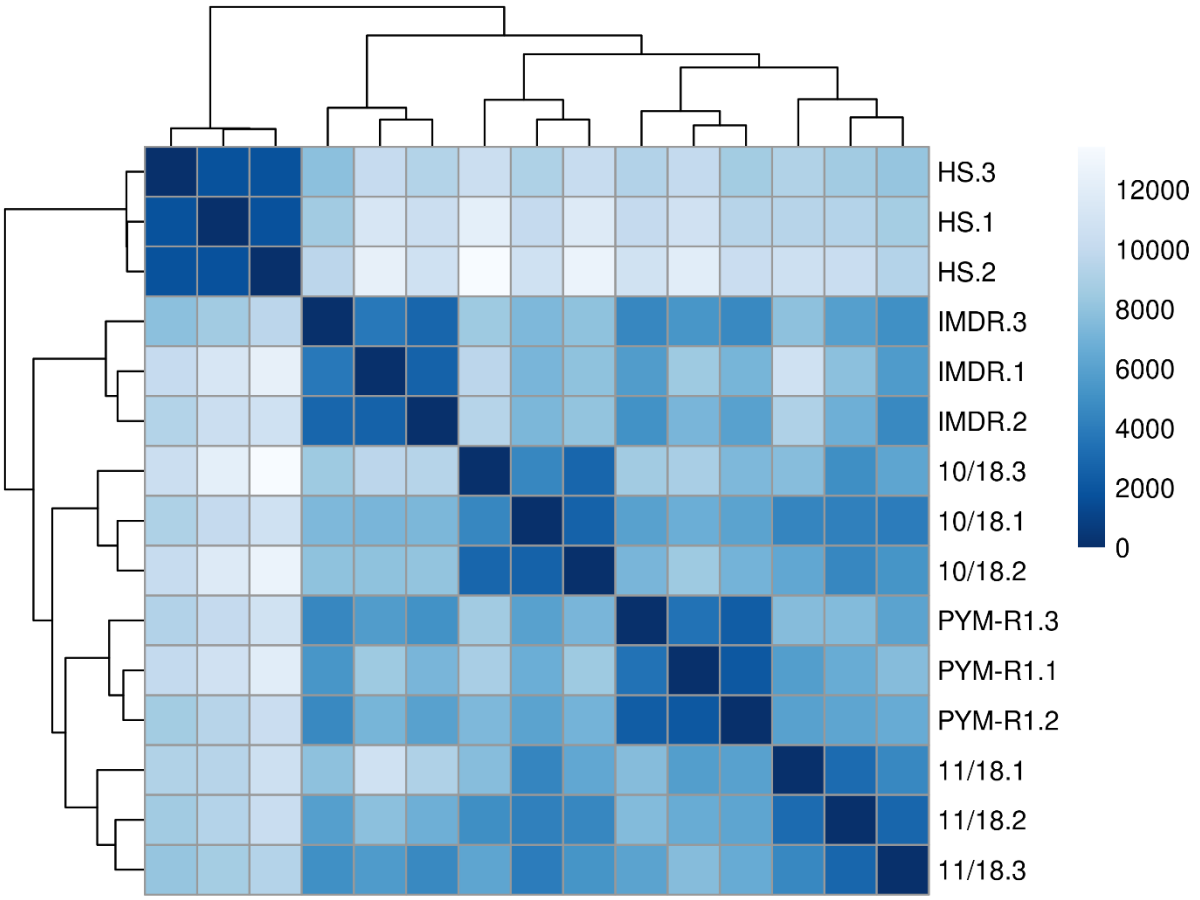

Fig. S1. Poisson distance pictures the differential expressed genes (DEG) in different *Myzus persicae* populations detected in the RNAseq approach by Illumina sequencing. The genes detected in the susceptible reference strain HS separates well from all other sequenced populations.

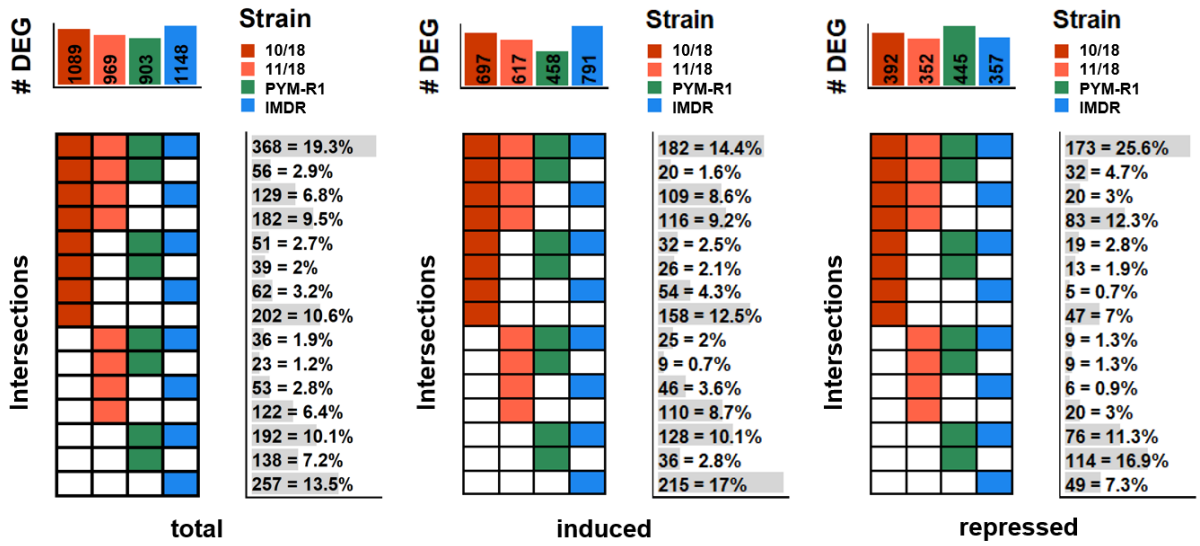

Fig. S2. Numbers of total, induced and repressed DEGs (log_2FC) in *M. persicae* populations when compared to the susceptible reference strain HS. Data in percentage depict the number of genes in comparison to all clustered 1,910 DEGs that were detected in strains 10/18, 11/18, PYM-R1 and IMDR (Fig. 2A).

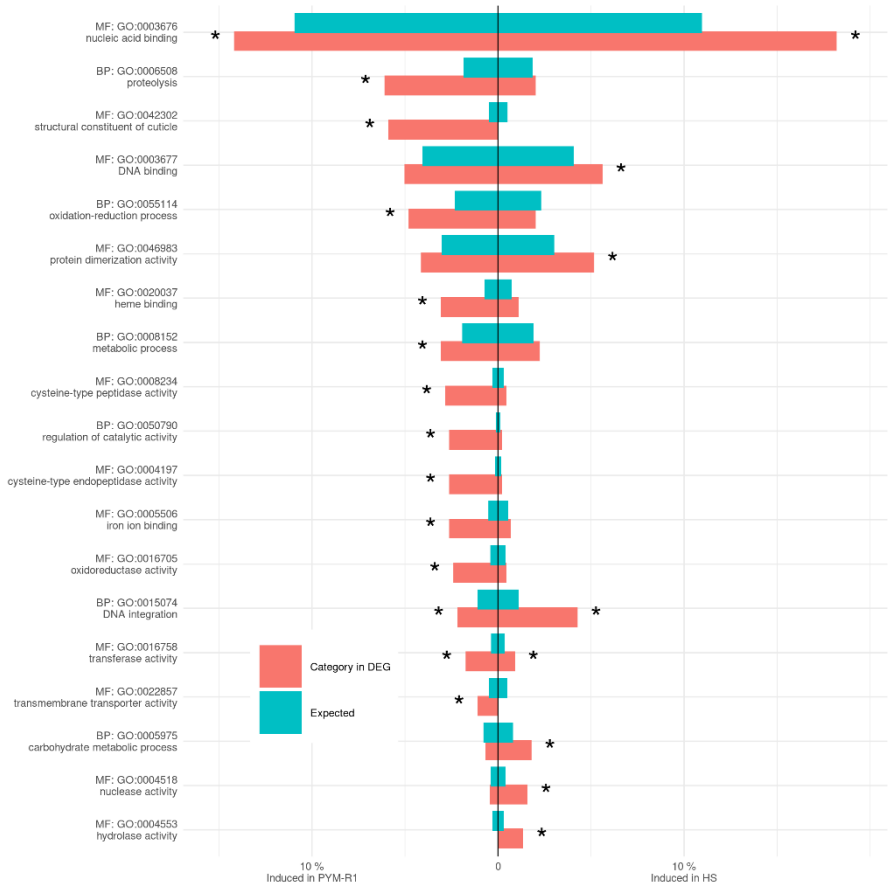

Fig. S3. Expression of differentially expressed genes (DEG) categorized by their function in the PYM-R1 *Myzus persicae* strain when compared to the susceptible reference strain HS. Blue bars are illustrating the proportion of all genes with corresponding gene ontology (GO) annotation in the genome. Red bars are picturing the proportion of genes with GO annotation in the DEG set.

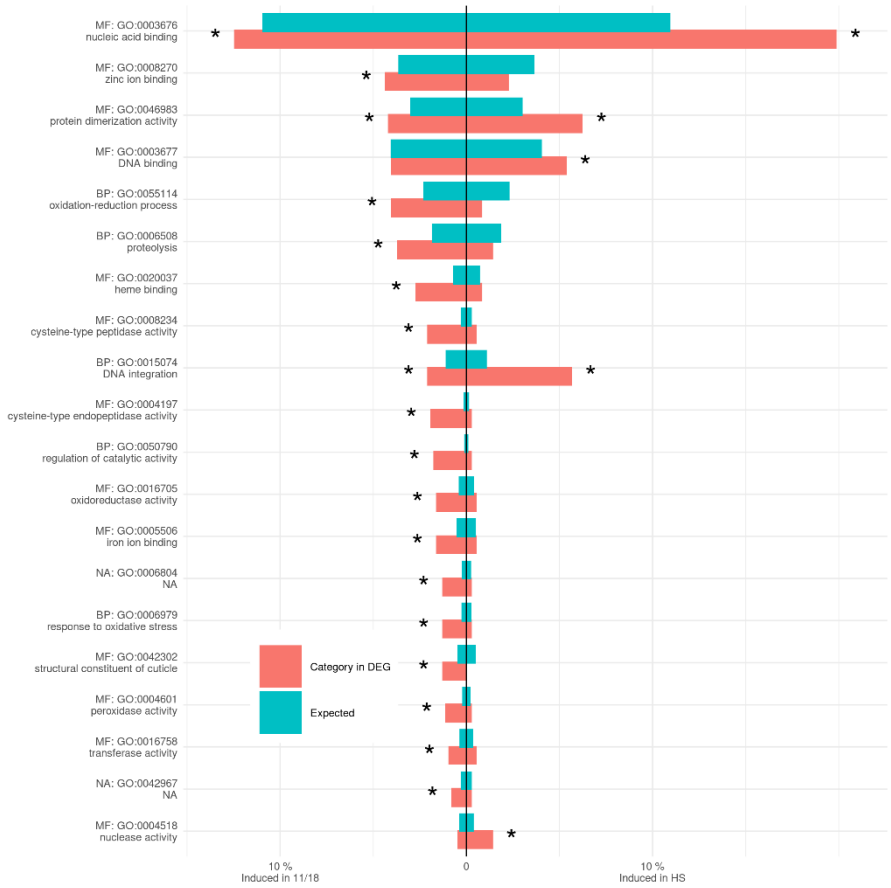

Fig. S4. Expression of DEGs categorized by their function in the 11/18 *Myzus persicae* strain when compared to the susceptible reference strain HS. Blue bars are illustrating the proportion of all genes with corresponding gene ontology (GO) annotation in the genome. Red bars are picturing the proportion of genes with GO annotation in the DEG set.

Fig. S5. Expression of DEGs categorized by their function when compared between the PYM-R1 and 11/18 *Myzus persicae* strains. Blue bars are illustrating the proportion of all genes with corresponding gene ontology (GO) annotation in the genome. Red bars are picturing the proportion of genes with GO annotation in the DEG set.

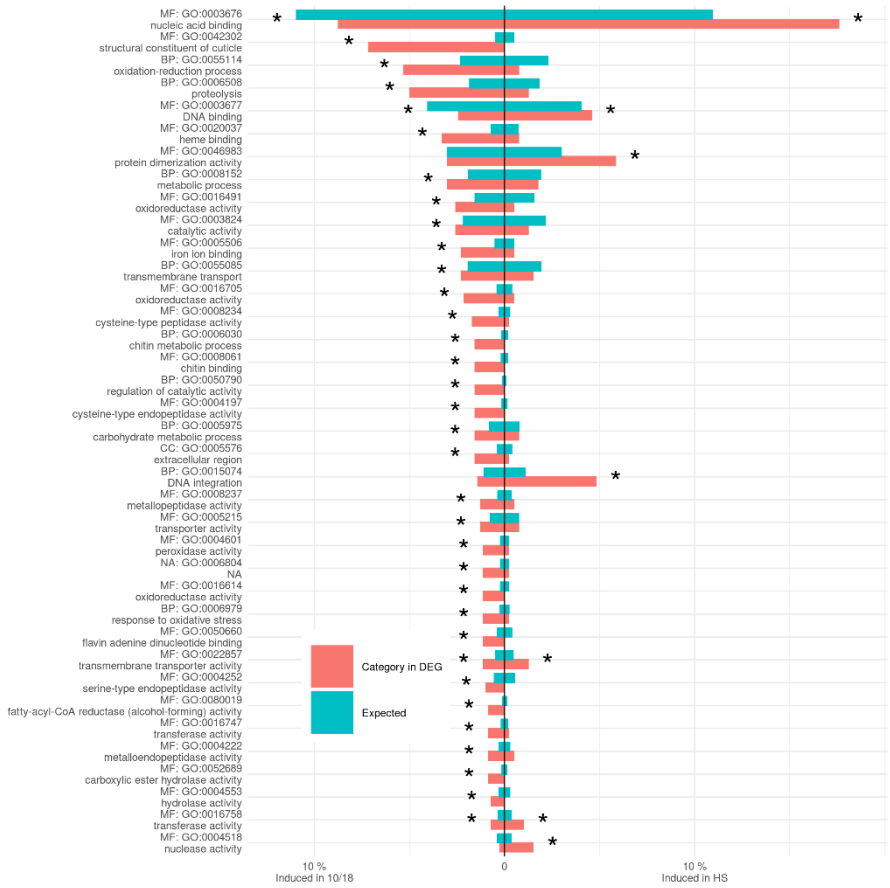

Fig. S6. Expression of DEGs categorized by their function in the 10/18 *Myzus persicae* strain when compared to the susceptible reference strain HS. Blue bars are illustrating the proportion of all genes with corresponding gene ontology (GO) annotation in the genome. Red bars are picturing the proportion of genes with GO annotation in the DEG set.

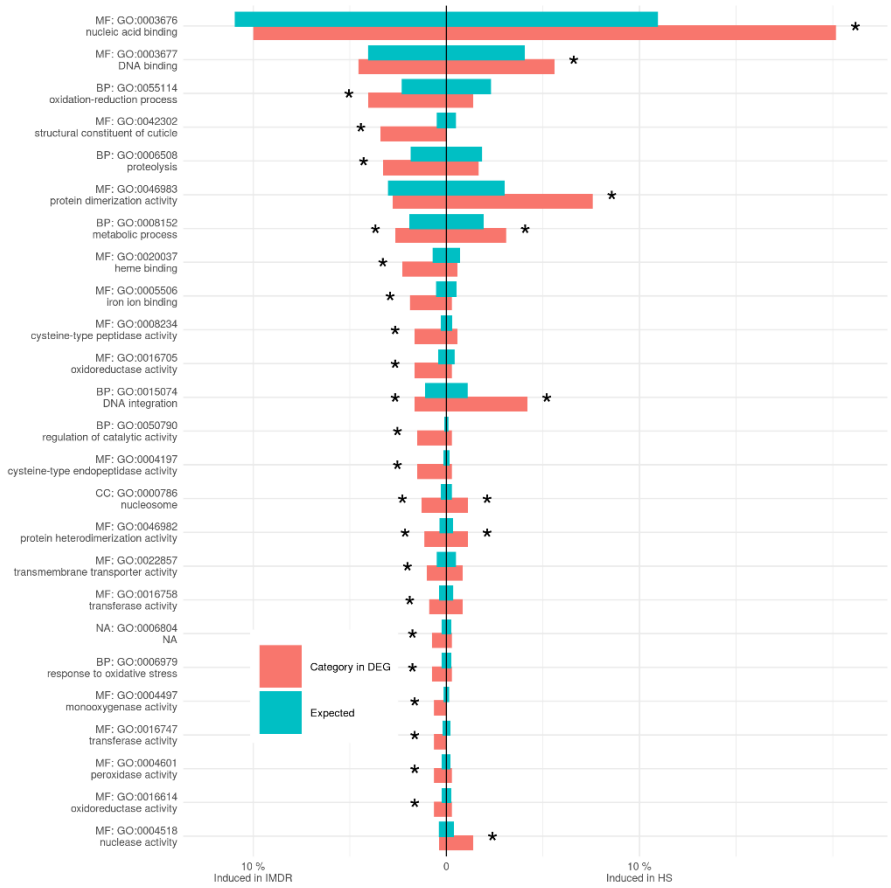

Fig. S7. Expression of DEGs categorized by their function in the IMDR *Myzus persicae* strain when compared to the susceptible reference strain HS. Blue bars are illustrating the proportion of all genes with corresponding gene ontology (GO) annotation in the genome. Red bars are picturing the proportion of genes with GO annotation in the DEG set.

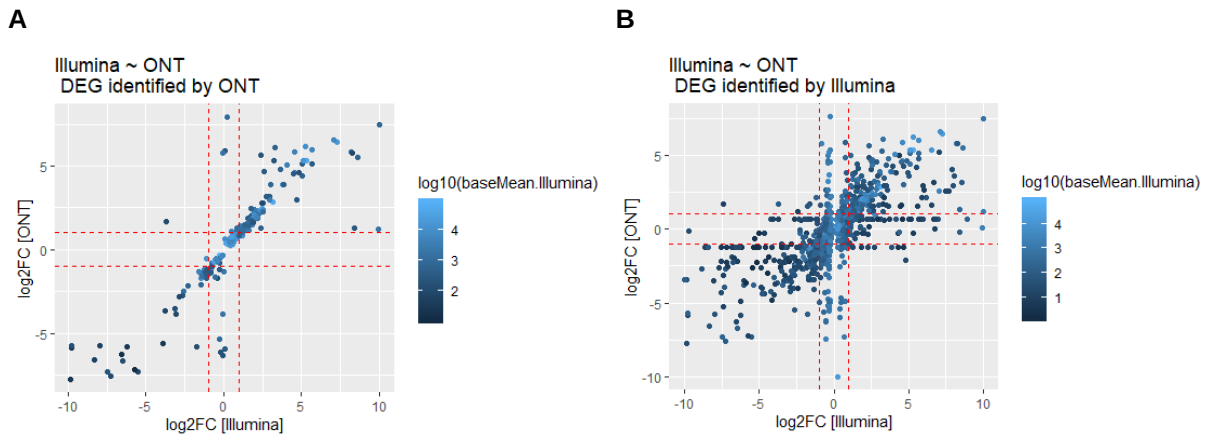

Fig. S8. Correlation of DEGs identified in RNAseq approaches with ONT **(A)** and Illumina **(B)** in the pymetrozine resistant PYM-R1 *Myzus persicae* population when compared to the susceptible reference strain HS.

Table S5. Detected genes found in a RNAseq approach with Illumina sequencing in the pymetrozine resistant PYM-R1 *Myzus persicae* population when compared to the susceptible reference strain HS. The “Base Mean Illumina” is explaining the number of detected gene sequences found with Illumina sequencing. “Base Mean ONT” with almost zero in all cases, describes that no gene sequences were found with ONT, meaning that the listed genes were not detected with ONT.

Gene ID	Base Mean Illumina	Base Mean ONT	Base Mean Ratio	Product
LOC111029202	1435,4	0,0		calcium/calmodulin-dependent protein kinase type II alpha chain, transcript variant X1
LOC111030824	1597,5	0,0		uncharacterized LOC111030824, transcript variant X2
LOC111031450	614,2	0,0		uncharacterized LOC111031450, transcript variant X4
LOC111032204	630,5	0,0		probable 28S ribosomal protein S26, mitochondrial
LOC111036055	1738,2	0,0		protein similar-like, transcript variant X1
LOC111036093	7374,4	0,0		glutathione S-transferase-like, transcript variant X1
LOC111037870	2255,6	0,0		Down syndrome cell adhesion molecule-like protein Dscam2, transcript variant X33
LOC111037941	825,5	0,0		voltage-dependent calcium channel type A subunit alpha-1, transcript variant X3
LOC111038250	2694,9	0,0		zinc finger protein OZF-like, transcript variant X3
LOC111039264	1135,9	0,0		transcript factor 12, transcript variant X6
LOC111043130	2828,5	0,0		oxidation resistance protein 1, transcript variant X7
LOC111040971	6328,6	0,5	12505,6	myb-like protein X
LOC111032815	1379,7	0,1	9713,0	neural-cadherin, transcript variant X3
LOC111026415	1079,9	0,1	7602,2	protein numb-like, transcript variant X3
LOC111042300	1170,1	0,2	7417,3	G protein-coupled receptor kinase 2
LOC111029847	1096,8	0,2	6952,8	uncharacterized LOC111029847
LOC111041303	1963,2	0,3	6667,3	remodeling and spacing factor 1
LOC111039407	780,6	0,1	5301,9	uncharacterized LOC111039407, transcript variant X3
LOC111037407	4973,5	0,9	5254,6	chromodomain-helicase-DNA-binding protein 7-like, transcript variant X5
LOC111037931	745,7	0,1	5249,4	cyclin-dependent kinase 5 activator 1
LOC111037699	796,6	0,2	5204,6	poly(rC)-binding protein 3-like, transcript variant X2
LOC111033838	2810,0	0,6	4812,4	esterase FE4-like
LOC111027297	792,5	0,2	4550,4	uncharacterized LOC111027297

Appendix B: Supporting material of Chapter 3

Molecular characterization of pyrethroid resistance in field-collected populations of the pea aphid, *Acyrthosiphon pisum*

Short title: Metabolic resistance to pyrethroids in pea aphids

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This manuscript might slightly differ to the published version.

Table S1. List of primers used for the validation of differentially expressed genes by RT-qPCR analysis and the detection of mutations in the voltage-gated sodium channel.

Gene	GenBank Accession number	Forward primer (5' to 3')	Reverse primer (5' to 3')	Source
NADH	NM_001162323	CGAGGAGAACATGCTCTTAGAC	GATAGCTTGGGCTGGACATATAG	Yang et al. 2014
RPS (RPL12)	NM_001126171	AAGGCTACATCTGACTGGAAAG	ACCAATGATGATGCAGAAGGA	Yang et al. 2014
16S rRNA	FJ411411	AGAAACCAACCTGGCTTACAC	TTGCGACCTCGATGTTGAATTA	Yang et al. 2014
18S rRNA	X62623	CCGCGAAACCGTCATTAAATC	GGAACTCTGTCGGCATGTATTA	Yang et al. 2014
CYP6CY12	XM_001952415	CACCAGTTGACCACCATCGA	TGTTGATCAGCTCCGGATCG	This study
FE4-like	XM_008182475	CAAAGTGCCGGAAGTGCTTC	GCCCATCCTGGTGGAGATAC	This study

Target	Mutation	Primers	Sequence (5' to 3')
Voltage-gated sodium channel (*VGSC*)	L1014F	L1014F_forward	GTGGCGAATGGATCGAAT
		L1014F_reverse	[btn]AAATTCTTGGGCTGAGTAGTACA
		L1014F_sequence	ACCGTTGTCATCGGT
	M918L/T	M918L/T_forward	TGGCCAACATTGAATCTCTTAA
		M918L/T_reverse	[btn]CATACCCATAACGGCGAATATAAA
		M918L/T_sequence	TGAATCTCTTAATATCCAT

Table S2. Summary of RNAseq transcript quantification obtained from 6 d old Acyrthosiphon pisum strains SUS-1 and PYR-R1 by pseudo-alignment with kallisto (GenBank BioProject accession number: PRJNA826712).

Sample	Sequences	Pseudoaligned	Estimated average fragment length	Pseudoaligned (%)	Minimum one read	Minimum one read (%)	GenBank entry
SUS-1 #1	19 861 251	17 007 520	302	85.6	14 013	76.7	SAMN27578316
SUS-1 #2	17 963 355	14 989 852	313	83.5	14 084	77.1	SAMN27578317
SUS-1 #3	17 921 293	14 831 981	310	82.8	13 915	76.2	SAMN27578318
SUS-1 #4	21 965 796	18 214 867	299	82.9	14 074	77.1	SAMN27578319
SUS-1 #5	*Failed*						
PYR-R1 #1	22 143 203	17 330 101	280	78.3	13 977	76.5	SAMN27578321
PYR-R1 #2	18 719 853	14 660 650	311	78.3	13 944	76.4	SAMN27578322
PYR-R1 #3	24 154 953	19 011 083	309	78.7	14 088	77.1	SAMN27578323
PYR-R1 #4	20 163 674	15 476 084	279	76.8	13 945	76.4	SAMN27578324
PYR-R1 #5	19 810 717	15 398 620	277	77.7	13 848	75.8	SAMN27578325

Table S3. List of selected detoxification gene transcripts significantly upregulated in Acyrthosiphon pisum strain PYR-R1. Abbreviations: P450, cytochrome P450-monooxygenases; EST, esterases; GST, glutathione S-transferases; UGT, UDP-glycosyltransferases; ABC, ATP-binding cassette transporters; FPKM, fragments per kilobase million.

Gene ID	Annotation	FPKM (±SD) SUS-1	FPKM (±SD) PYR-R1	KALLISTO Log2 FoldChange (FC)	Description
P450					
100160402	CYP15A3P	0.08 (0.02)	2.3 (0.21)	4.43	methyl farnesoate epoxidase-like
100163195	CYP6CY12	37.1 (1.68)	732 (2.51)	3.98	probable cytochrome P450 6a13
100573593	CYP380C1	3.26 (0.12)	12.3 (0.43)	1.60	cytochrome P450 4C1-like
100167264	CYP6CY5	30.7 (1.05)	91.9 (1.42)	1.26	probable cytochrome P450 6a13
EST					
100166921	FE4-like	43.9 (0.94)	185 (3.71)	1.75	esterase FE4
103308679	E4-like	22.8 (0.45)	89.2 (1.47)	1.64	esterase E4
100573892	Ace-1	21.7 (0.33)	79.4 (3.01)	1.55	acetylcholinesterase-1
100168834	E4-like	142 (1.83)	443 (4)	1.32	esterase E4
GST					
100160859	GST-like	32.9 (1.98)	91.9 (0.91)	1.16	glutathione S-transferase-like
UGT					
100162688	UGT-2B2	5.2 (0.38)	62.4 (1.5)	3.27	UDP-glucuronosyl transferase 2B2
100169313	UGT-2B20	0.29 (0.09)	3.27 (0.29)	3.18	UDP-glucuronosyl transferase 2B20
ABC					
100569981	ABCG-like	1.2 (0.09)	3.87 (0.24)	1.38	ABC transporter G family 23-like

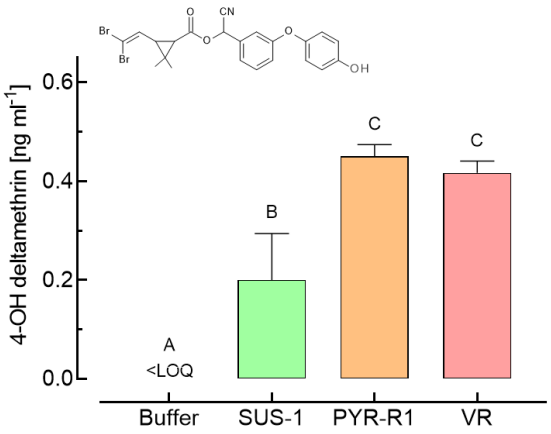

Fig. S1. UPLC-MS/MS determination of the metabolite 4-OH deltamethrin in microsomal preparations of adults of different strains of *Acyrthosiphon pisum* incubated with deltamethrin. Microsomal preparations incubated without NADPH were inactive (data not shown). Data are mean values ± SD (n=3); Different letters denote significant differences between microsomal preparations (one-way ANOVA, post-hoc Tukey comparison, $p < 0.05$). LOQ=Limit of quantitation.

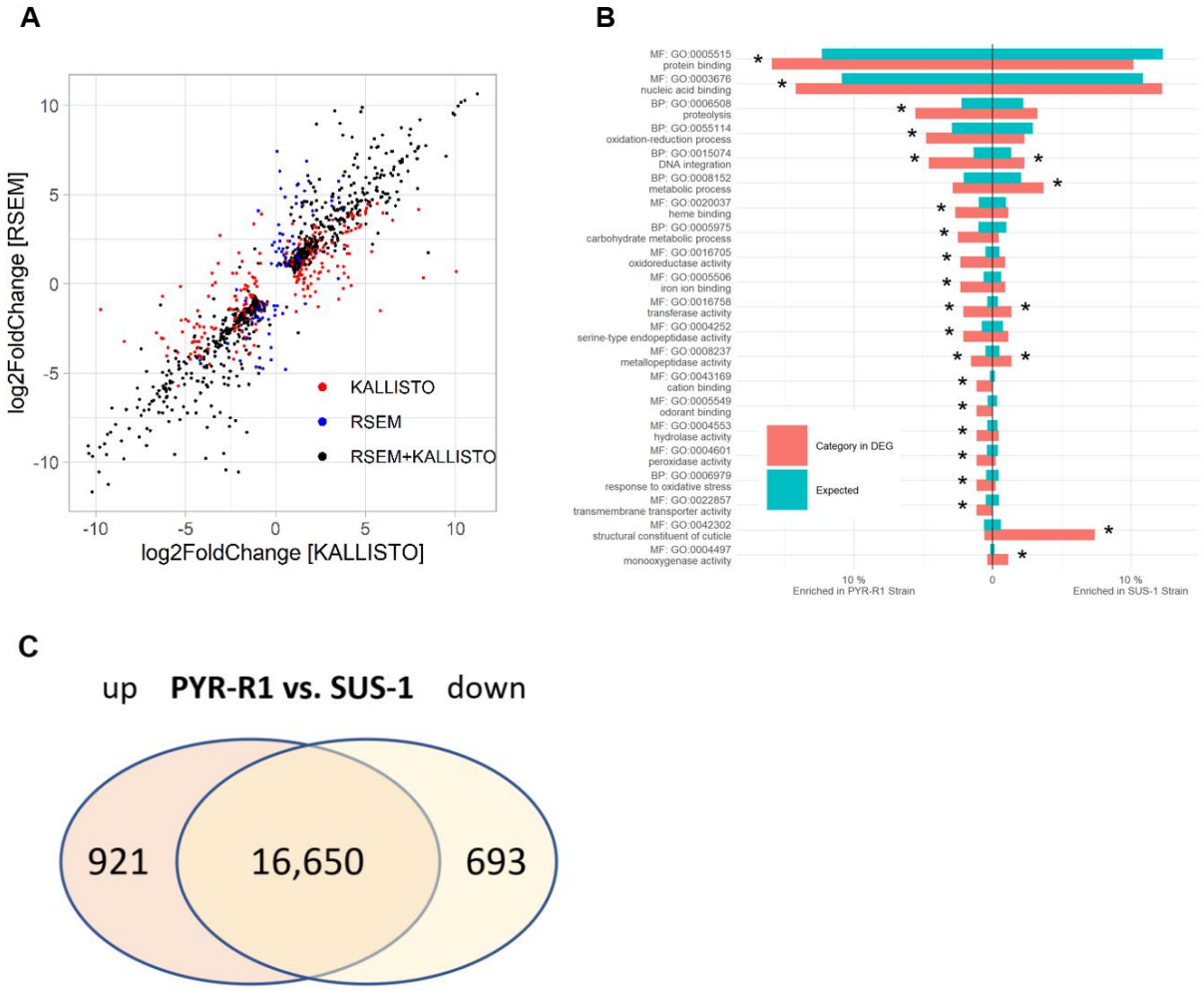

Fig. S2. (A) Comparison of the overall expression level of differentially expressed genes in *Acyrthosiphon pisum* analysed by KALLISTO and RSEM algorithms. Coloured dots indicate significantly expressed genes called after quantification by KALLISTO (red), RSEM (blue) or both methods (black). **(B)** Enriched gene ontology (GO) terms of differentially expressed genes (DEG) in *A. pisum* strains SUS-1 and PYR-R1 ($p \leq 0.05$ and DEG in category ≥5). Asterisks indicate significant enrichment of GO terms in the differentially induced gene sets (red) in PYR-R1 or SUS-1 strains compared to all genes expected in the respective set (blue). **(C)** Number of up- and down-regulated genes in strain PYR-R1 vs. SUS-1 based on log2 FC and $p<0.01$.

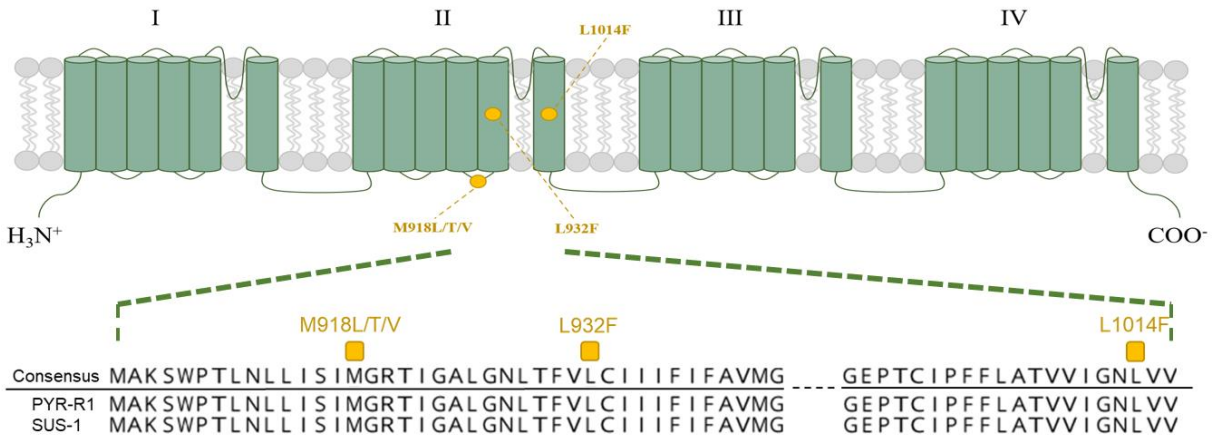

Fig. S3. Comparison of partially assembled stretches of the voltage-gated sodium channel (VGSC) of *Acyrthosiphon pisum* strains SUS-1 and PYR-R1 based on GenBank entry XP_029343808.1 (*A. pisum* VGSC) revealed no amino acid substitutions. Sequences were compared for the presence of M918L/T/V, L932F and L1014F VGSC target-site mutations known to be associated and conferring pyrethroid resistance in aphid pests such as *Myzus persicae* (numbering according to *Musca domestica* sodium channel (GenBank X96668)).

Acyrthosiphon pisum	L1014F			Genotype	[n]
	CTT Leu	C/T TT	TTT Phe		
Strain	**SS**	**SR**	**RR**		
SUS-1	100	0	0	susceptible	10
VR	100	0	0	susceptible	10
PYR-R1	100	0	0	susceptible	10

Acyrthosiphon pisum	M918L/T					Genotype	[n]
	ATG Met	A/CTG	AT/CG	CTG Leu	ACG Thr		
Strain	**SS**	**SR**	**SR**	**RR**	**RR**		
SUS-1	100	0	0	0	0	susceptible	10
VR	100	0	0	0	0	susceptible	10
PYR-R1	100	0	0	0	0	susceptible	10

Fig. S4. Genotyping by pyrosequening of adults of different strains of *Acyrthosiphon pisum* for the presence of the most frequent target-site mutations (L1014F and M918L/T) in aphid voltage-gated sodium channels conferring pyrethroid resistance

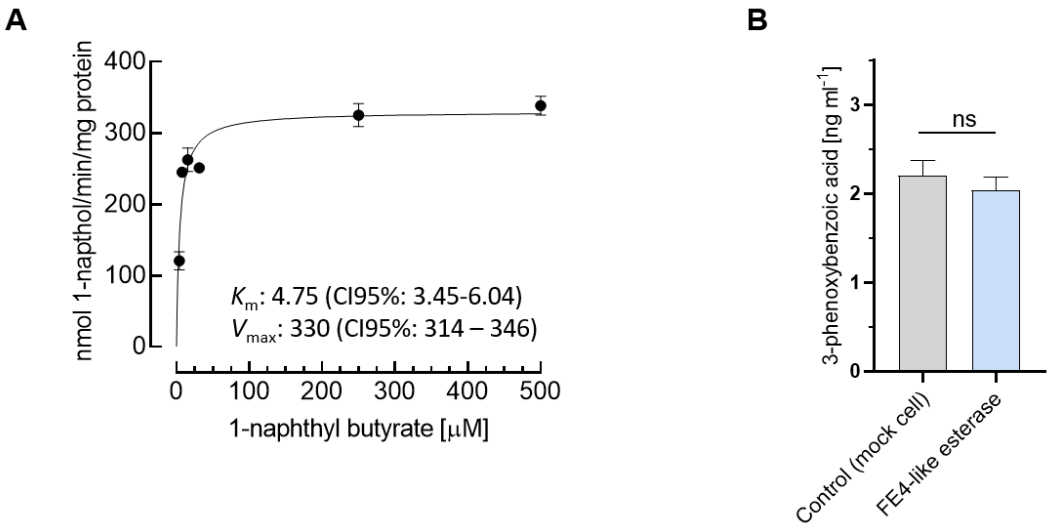

Fig. S5. (A) Michaelis-Menten kinetics of the formation of 1-naphthol using 1-naphthylbutyrate as a substrate by recombinantly expressed FE4-like esterase (LOC100166921) of *Acyrthosiphon pisum*. K_m- and V_{max}-values were calculated by non-linear regression analysis using GraphPad Prism v8.0 and expressed in µM and nmol 1-naphthol / min x mg protein^{-1}, respectively. Data are mean values ± SD (n=3). **(B)** Formation of 3-phenoxybenzoic acid by recombinantly expressed FE4-like esterase (LOC100166921) of *Acyrthosiphon pisum* in comparison to mock cells analyzed by UPLC-MS/MS after incubation with deltamethrin (30 °C, 2 h). Data are mean values ± SD (n=3) and not significantly different (ns, p>0.05, t-test).

Table S4. Log-dose probit-mortality data for insecticides different classes against 6d old *Acyrthosiphon pisum* adults (n=30) of different strains. Evaluation of affected pea aphids was done 24h after leaf-dip bioassay application.

Insecticide	Strain	LC_{50} [mg L^{-1}]	95% CI	Slope ± SE	RR
Flupyradifurone	SUS-1	< 0.32			
	PYR-R1	< 0.32			
Thiacloprid	SUS-1	0.73	0.53-1.02	2.62	
	PYR-R1	1.36	0.8-2.41	1	1.86
Spirotetramat	SUS-1	1	0.87-1.17	4.67	
	PYR-R1	2.44	1.27-4.9	1	2.44
147	SUS-1	0.39	0.27-0.52	1.57	
	PYR-R1	0.67	0.42-1.1	1	1.72

Appendix C: Supporting material of Chapter 4

Ketoenol resistance in the cabbage whitefly *Aleyrodes proletella* (Homoptera: Aleyrodidae) is associated with a target-site mutation, A2083V, in the CT domain of Acetyl-CoA carboxylase (ACC)

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Table S1: Field-collected populations of *Aleyrodes proletella* (Year, Sample-ID, Country, Venue, Host) preserved in ethanol for genotyping purposes.

Year	Sample-ID	Country	Venue	Host
2019	NAR2019-131	Netherlands	Mijnsherenland	Brussels sprouts
	NAR2019-132	Netherlands	Klaaswaal	Brussels sprouts
	NAR2019-148	Poland	Warsaw	Unknown
	NAR2019-149	Poland	Warsaw	Unknown
	NAR2019-150	Netherlands	Schagerbrug	Green cabbage
	NAR2019-151	Netherlands	Oosternijkerk	Broccoli
	NAR2019-162	Switzerland	Zollikofen	Brussels sprouts
	NAR2019-163	Spain	Andalucia	Broccoli
	NAR2019-164	Spain	Murcia	Broccoli
	NAR2019-165	Spain	Murcia	Broccoli
	NAR2019-166	Spain	Murcia	Broccoli
	NAR2019-183	Spain	La Rioja	Cauliflower
2020	NAR2020-119	Belgium	Roeselare	Brussels sprouts
	NAR2020-120	Belgium	Lier	Brussels sprouts
	NAR2020-121	Belgium	Kruishoutem (Kruisem)	Brussels sprouts
	NAR2020-122	Netherlands	Den Bommel	Brussels sprouts
	NAR2020-123	Netherlands	Oud-Beijerland	Brussels sprouts
	NAR2020-124	Netherlands	Heerjansdam	Brussels sprouts
2021	NAR2021-048	Spain	Picassent	
	NAR2021-049	Spain	Picassent	
	NAR2021-050	Spain	Alzira	
	NAR2021-051	Spain	Simat de la Valldigna	
	NAR2021-052	Spain	Algemesi	
	NAR2021-053	Spain	Alzira	
	NAR2021-054	Spain	Manuel	
	NAR2021-055	Spain	Simat de la Valldigna	

Year	Sample-ID	Country	Venue	Host
2021	NAR2021-060	Spain	Finca Las Cabecicas Lorca	
	NAR2021-061	Spain	Lorca	
	NAR2021-110	Netherlands	Den Bommel	Brussel sprouts
	NAR2021-111	Netherlands	Hellevoetsluis	Brussel sprouts
	NAR2021-112	Netherlands	Mijnsheerenland	Brussel sprouts
	NAR2021-113	Netherlands	Bruinisse	
	NAR2021-114	Netherlands	Zeewolde	
	NAR2021-115	Netherlands	Den Bommel	
	NAR2021-116	Netherlands	Hellvoetsluis	
	NAR2021-117	Netherlands	Mijnsheerenland	
	NAR2021-120	Netherlands	Sexbierum	
	ALTHFL_DEU21_0001	Germany	Hannover	Savoy cabbage
	ALEUPR_DEU21_0002	Germany	Hannover	Savoy cabbage

Table S2: Primer pairs used for acetyl-CoA carboxylase genotyping.

Method	Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon, bp
Pyrosequencing	ACC A2083V	TGTGGATGAGTTACGTGTTTACAA	[btn]GGGGTTAATGGTGGGATCAACTA	
	ACC A2083V_seq	AGAGGAGGAGCTTGG		106

Fig. S1: Partial nucleotide sequence of a cDNA fragment of the carboxyltransferase (CT) domain of *Aleyrodes proletella* ACC harboring the mutation site A2083 (red arrow). Annealing positions for primers for pyrosequencing diagnostics of the mutation site A2083V are indicated by light blue arrows.

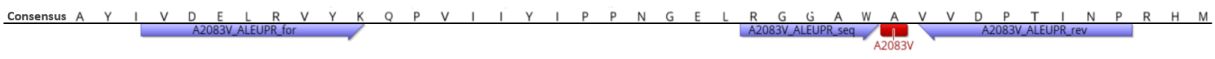

Fig. S2: Multiple alignment of predicted ACC amino acid sequences of assembled ACC genes of six strains of *Aleyrodes proletella*, 3/19 (319) (GenBank accession no. SAMN27777464), 4/19 (419) (SAMN27777465), 6/19 (619) (SAMN27777467), 2/20 (SAMN27777462), 5/20 (520) (SAMN27777466) and SPI-2/20 (220O) (SAMN27777463). The carboxyltransferase (CT) domain is highlighted in yellow (PF01039.18, AA 1658-2210). This Transcriptome Shotgun Assembly project has been deposited at DDBJ/ENA/GenBank under the accession GJYF00000000. The version described in chapter 4 is the first version, GJYF01000000.

```
                    1                                                   50
BEMITA_QJQ31013     MEGETGQKAN NVNFIVGDDG QDGEIPNGIN T..ISEEDER ERRENRDSFP
MYZUPE_XP_022181497 ~~~MSSETSG GVNFIVGDED GVDQAPAELV NGELMKALEA EKHENTDSFP
TRIAVA_ACC          ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
220O                MEGDTGPKTN NVNFIVGDDG QDGEIANGLI N..TI.SEED ERKENRDSFP
319                 MEGDTGPKTN NVNFIVGDDG QDGEIANGLI N..TI.SEED ERKENRDSFP
419                 MEGDTGPKTN NVNFIVGDDG QDGEIANGLI N..TI.SEED ERKENRDSFP
520                 MEGDTGPKTN NVNFIVGDDG QDGEIANGLI N..TI.SEED ERKENRDSFP
619                 MEGDTGPKTN NVNFIVGDDG QDGEIANGLI N..TI.SEED ERKENRDSFP

                    51                                                  100
BEMITA_QJQ31013     SGNG.PTGII SSSSSYKDMF GLAERRKRLR PSMSQGTVIH QRLLDKDFTV
MYZUPE_XP_022181497 LGKETRMGVT SNSSSYNNMF GLTEKRKRLR PSMSQGNVIH QRLTEKDFNV
TRIAVA_ACC          ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~MSQGTVIH QRLLDKDFTV
220O                SGNG.PTGLI SNSSSYNNMF GLAERRKRLR PSMSQGTVIH QRLLDKDFTV
319                 SGNG.PTGLI SNSSSYNNMF GLAERRKRLR PSMSQGTVIH QRLLDKDFTV
419                 SGNG.PTGLI SNSSSYNNMF GLAERRKRLR PSMSQGTVIH QRLLDKDFTV
520                 SGNG.PTGLI SNSSSYNNMF GLAERRKRLR PSMSQGTVIH QRLLDKDFTV
619                 SGNG.PTGLI SNSSSYNNMF GLAERRKRLR PSMSQGTVIH QRLLDKDFTV

                    101                                                 150
BEMITA_QJQ31013     GTPEEFVKRF KGTRVINKVL IANNGIAAVK CMRSIRRWSY EMFKNERAVR
MYZUPE_XP_022181497 STPEEFVKRF KGTRVINKVL IANNGIAAVK CMRSVRRWSY EMFRNERAVR
TRIAVA_ACC          GTPEEFVKRF KGTRVINKVL IANNGIAAVK CMRSIRRWSY EMFKNERAVR
220O                GTPEEFVKRF KGTRVINKVL IANNGIAAVK CMRSIRRWSY EMFKNERAVR
319                 GTPEEFVKRF KGTRVINKVL IANNGIAAVK CMRSIRRWSY EMFKNERAVR
419                 GTPEEFVKRF KGTRVINKVL IANNGIAAVK CMRSIRRWSY EMFKNERAVR
520                 GTPEEFVKRF KGTRVINKVL IANNGIAAVK CMRSIRRWSY EMFKNERAVR
619                 GTPEEFVKRF KGTRVINKVL IANNGIAAVK CMRSIRRWSY EMFKNERAVR

                    151                                                 200
BEMITA_QJQ31013     FVVMVTPEDL KANAEYIKMA DHYVPVPGGT NNNNYANVEL IVDIALRTQV
MYZUPE_XP_022181497 FVVMVTPEDL KANAEYIKMA DHYVPVPGGT NNNNYANVEL IVNIAIRSQV
TRIAVA_ACC          FVVMVTPEDL KANAEYIKMA DHYVPVPGGT NNNNYANVEL IIDIATRTQV
220O                FVVMVTPEDL KANAEYIKMA DHYVPVPGGT NNNNYANVEL IVDIALRTQV
319                 FVVMVTPEDL KANAEYIKMA DHYVPVPGGT NNNNYANVEL IVDIALRTQV
419                 FVVMVTPEDL KANAEYIKMA DHYVPVPGGT NNNNYANVEL IVDIALRTQV
520                 FVVMVTPEDL KANAEYIKMA DHYVPVPGGT NNNNYANVEL IVDIALRTQV
619                 FVVMVTPEDL KANAEYIKMA DHYVPVPGGT NNNNYANVEL IVDIALRTQV

                    201                                                 250
BEMITA_QJQ31013     QAVWAGWGHA SENPKLPELL HKNNIAFIGP PEKAMWALGD KIASSIVAQT
MYZUPE_XP_022181497 QAVWAGWGHA SENPELPKLL DKNKIAFIGP PEKAMFALGD KIASSIVAQT
TRIAVA_ACC          QAVWAGWGHA SENPKLPELL HKNNIAFIGP PEKAMWALGD KIASSIVAQT
220O                QAVWAGWGHA SENPKLPELL HKNNIAFIGP PEKAMWALGD KIASSIVAQT
319                 QAVWAGWGHA SENPKLPELL HKNNIAFIGP PEKAMWALGD KIASSIVAQT
419                 QAVWAGWGHA SENPKLPELL HKNNIAFIGP PEKAMWALGD KIASSIVAQT
520                 QAVWAGWGHA SENPKLPELL HKNNIAFIGP PEKAMWALGD KIASSIVAQT
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619                 QAVWAGWGHA SENPKLPELL HKNNIAFIGP PEKAMWALGD KIASSIVAQT

                    251                                                 300
BEMITA_QJQ31013     AEIPTLPWSG SELTAQYSGR KIKISSELYK RGCVSSVEEG LRSAQKIGFP
MYZUPE_XP_022181497 AEIPTLPWSG SGVVGHYSGK KIEIGPDLYK KGCVASIEEG LVSAEKVGYP
TRIAVA_ACC          AEIPT..... .......... ...ISSELYK KGCVQNVEEG LRSAQKIGFP
220O                AEIPTLPWSG SELTAQYSGK KIKISSDLYK KGCVSSVEEG LRSAHKIGFP
319                 AEIPTLPWSG SELTAQYSGK KIKISSDLYK KGCVSSVEEG LRSAHKIGFP
419                 AEIPTLPWSG SELTAQYSGK KIKISSDLYK KGCVSSVEEG LRSAHKIGFP
520                 AEIPTLPWSG SELTAQYSGK KIKISSDLYK KGCVSSVEEG LRSAHKIGFP
619                 AEIPTLPWSG SELTAQYSGK KIKISSDLYK KGCVSSVEEG LRSAHKIGFP

                    301                                                 350
BEMITA_QJQ31013     VMIKASEGGG GKGIRKVESS EEFPNLFRQV QSEVPGSPIF IMKLARCARH
MYZUPE_XP_022181497 IMIKASEGGG GKGIRKVENT EEFPNAYKQV QAEVPGSPIF IMKLAKCARH
TRIAVA_ACC          VMVKASEGGG GKGIRKVESS EEFPNLFRQV QAEVPGSPIF IMKLARCARH
220O                IMIKASEGGG GKGIRKVEAA EEFPNLFRQV QSEVPGSPIF IMKLARCARH
319                 IMIKASEGGG GKGIRKVEAA EEFPNLFRQV QSEVPGSPIF IMKLARCARH
419                 IMIKASEGGG GKGIRKVEAA EEFPNLFRQV QSEVPGSPIF IMKLARCARH
520                 IMIKASEGGG GKGIRKVEAA EEFPNLFRQV QSEVPGSPIF IMKLARCARH
619                 IMIKASEGGG GKGIRKVEAA EEFPNLFRQV QSEVPGSPIF IMKLARCARH

                    351                                                 400
BEMITA_QJQ31013     LEVQLLADQY GNAISLFGRD CSIQRRHQKI IEEAPAVIAE PEIFEDMEKA
MYZUPE_XP_022181497 LEVQLLADQY GNAISLFGRD CSIQRRHQKI IEEAPAVIAE PSVFEEMERA
TRIAVA_ACC          LEVQLLADQY GNAISLFGRD CSIQRRHQKI IEEAPAVIAE PEVFEAMEKA
220O                LEVQLLADQY GNAISLFGRD CSIQRRHQKI IEEAPAVIAE PDVFENMEKA
319                 LEVQLLADQY GNAISLFGRD CSIQRRHQKI IEEAPAVIAE PDVFENMEKA
419                 LEVQLLADQY GNAISLFGRD CSIQRRHQKI IEEAPAVIAE PDVFENMEKA
520                 LEVQLLADQY GNAISLFGRD CSIQRRHQKI IEEAPAVIAE PDVFENMEKA
619                 LEVQLLADQY GNAISLFGRD CSIQRRHQKI IEEAPAVIAE PDVFENMEKA

                    401                                                 450
BEMITA_QJQ31013     AVRLAKMVGY VSAGTVEYLY DPSEGQYYFL ELNPRLQVEH PCTEMVADVN
MYZUPE_XP_022181497 AVRIAKMVGY VSAGTVEYLY DT.DGNYYFL ELNPRLQVEH PCTEMVSDVN
TRIAVA_ACC          AVRLAKMVGY VSAGTVEYLY DPSEGQYYFL ELNPRLQVEH PCTEMVADVN
220O                AVRLAKMVGY VSAGTVEYLY DPSEGQYFFL ELNPRLQVEH PCTEMVADVN
319                 AVRLAKMVGY VSAGTVEYLY DPSEGQYFFL ELNPRLQVEH PCTEMVADVN
419                 AVRLAKMVGY VSAGTVEYLY DPSEGQYFFL ELNPRLQVEH PCTEMVADVN
520                 AVRLAKMVGY VSAGTVEYLY DPSEGQYFFL ELNPRLQVEH PCTEMVADVN
619                 AVRLAKMVGY VSAGTVEYLY DPSEGQYFFL ELNPRLQVEH PCTEMVADVN

                    451                                                 500
BEMITA_QJQ31013     LPAAQLQVAM GLQLHCIKDI RVLYGESPWG DSLIDFDQPR HKPQPWGHVI
MYZUPE_XP_022181497 LPAAQLQISM GLALNCIKDI RLLYSESAWG DSYIDFDAPR HKPHPWGHVI
TRIAVA_ACC          LPAAQLQIAM GLPLNCIKDI RLLYGESPWG DNLIDFDEPR HKPQPWGHVI
220O                LPAAQLQIAM GLQLNCIKDI RLLYGESPWG DNYIDFDEPR HKPQPWGHVI
319                 LPAAQLQIAM GLQLNCIKDI RLLYGESPWG DNYIDFDEPR HKPQPWGHVI
419                 LPAAQLQIAM GLQLNCIKDI RLLYGESPWG DNYIDFDEPR HKPQPWGHVI
520                 LPAAQLQIAM GLQLNCIKDI RLLYGESPWG DNYIDFDEPR HKPQPWGHVI
619                 LPAAQLQIAM GLQLNCIKDI RLLYGESPWG DNYIDFDEPR HKPQPWGHVI

                    501                                                 550
BEMITA_QJQ31013     AARITSENPD EGFKPSSGTV QELNFRSSKN VWGYFSVAAS GGLHEFADSQ
MYZUPE_XP_022181497 AARITSENPD EGFKPSSGTV QELNFRSSKN VWGYFSVAAS GGLHEFADSQ
TRIAVA_ACC          AARITSENPD EGFKPSSGTV QELNFRSSKN VWGYFSVAAS GGLHEFADSQ
220O                AARITSENPD EGFKPSSGTV QELNFRSSKN VWGYFSVAAS GGLHEFADSQ
319                 AARITSENPD EGFKPSSGTV QELNFRSSKN VWGYFSVAAS GGLHEFADSQ
419                 AARITSENPD EGFKPSSGTV QELNFRSSKN VWGYFSVAAS GGLHEFADSQ
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520                 AARITSENPD EGFKPSSGTV QELNFRSSKN VWGYFSVAAS GGLHEFADSQ
619                 AARITSENPD EGFKPSSGTV QELNFRSSKN VWGYFSVAAS GGLHEFADSQ

                    551                                                 600
BEMITA_QJQ31013     FGHCFSWGEN REQARENLVI ALKELSIRGD FRTTVEYLIT LLETESFQIN
MYZUPE_XP_022181497 FGHCFSWGEN REQARENLVI ALKELSIRGD FRTTVEYLIT LLETESFQSN
TRIAVA_ACC          FGHCFSWGEN REQARENLVI ALKELSIRGD FRTTVEYLIT LLETESFQIN
220O                FGHCFSWGEN REQARENLVI ALKELSIRGD FRTTVEYLIT LLETESFQIN
319                 FGHCFSWGEN REQARENLVI ALKELSIRGD FRTTVEYLIT LLETESFQIN
419                 FGHCFSWGEN REQARENLVI ALKELSIRGD FRTTVEYLIT LLETESFQIN
520                 FGHCFSWGEN REQARENLVI ALKELSIRGD FRTTVEYLIT LLETESFQIN
619                 FGHCFSWGEN REQARENLVI ALKELSIRGD FRTTVEYLIT LLETESFQIN

                    601                                                 650
BEMITA_QJQ31013     TIDTAWLDVL IAEKVQSEKP DILLGVMCGA LHIADRRVTD AFQNFQTSLE
MYZUPE_XP_022181497 TIDTAWLDLL ISERVQSEKP DIFLGVICGG LHIADRKISE SFQNFQTSLE
TRIAVA_ACC          TIDTAWLDVL IAQKVPSEKP DILLGVMCGA LHIADRKVTD AFQNFQTSLE
220O                TIDTAWLDLL ISERVQSEKP DVLLGVMCGA LHIADRKVCD AFQNFQTSLE
319                 TIDTAWLDLL ISERVQSEKP DVLLGVMCGA LHIADRKVCD AFQNFQTSLE
419                 TIDTAWLDLL ISERVQSEKP DVLLGVMCGA LHIADRKVCD AFQNFQTSLE
520                 TIDTAWLDLL ISERVQSEKP DVLLGVMCGA LHIADRKVCD AFQNFQTSLE
619                 TIDTAWLDLL ISERVQSEKP DVLLGVMCGA LHIADRKVCD AFQNFQTSLE

                    651                                                 700
BEMITA_QJQ31013     RGQIQGSNTL DHNVQVELIN DGLKYKVHAT KSGPNSYFLV MNGSFKEIEL
MYZUPE_XP_022181497 RGQVLSANTL DHHVSVELIN GGYKYKVQVT KSGLNSYFLI MNGSFKEIEV
TRIAVA_ACC          RGQIQGSNTL DHHLSVELIH DGLKYRVHAT KSGANSYFLV MNGSFKEIEL
220O                RGQIQGCNTL DHNVQVELIH DGLKYKVHAT KSGLNSYFLV MNGSFKEIEL
319                 RGQIQGCNTL DHNVQVELIH DGLKYKVHAT KSGLNSYFLV MNGSFKEIEL
419                 RGQIQGCNTL DHNVQVELIH DGLKYKVHAT KSGLNSYFLV MNGSFKEIEL
520                 RGQIQGCNTL DHNVQVELIH DGLKYKVHAT KSGLNSYFLV MNGSFKEIEL
619                 RGQIQGCNTL DHNVQVELIH DGLKYKVHAT KSGLNSYFLV MNGSFKEIEL

                    701                                                 750
BEMITA_QJQ31013     HRLSDGGILL SVDSSSFTTY MREEVDRYRI VIGNQTCVFE KENDPSLLRS
MYZUPE_XP_022181497 HRLSDGGILL SLDGSSFTTY MREEVDRYRI VIGNQTCVFD KENDPSLFRS
TRIAVA_ACC          HRLSDG.... .......... ...EVDRYRI VIGNQTCVFE KENDPSLLRS
220O                HRLSDGGILL SVDGSSFTTY MREEVDRYRI VIGNLTCVFE KENDPSLLRS
319                 HRLSDGGILL SVDGSSFTTY MREEVDRYRI VIGNLTCVFE KENDPSLLRS
419                 HRLSDGGILL SVDGSSFTTY MREEVDRYRI VIGNLTCVFE KENDPSLLRS
520                 HRLSDGGILL SVDGSSFTTY MREEVDRYRI VIGNLTCVFE KENDPSLLRS
619                 HRLSDGGILL SVDGSSFTTY MREEVDRYRI VIGNLTCVFE KENDPSLLRS

                    751                                                 800
BEMITA_QJQ31013     PSAGKLLSFL IEDGGHVSKG QAYAEIEVMK MVMTLTASEN GNVTFAKRPG
MYZUPE_XP_022181497 PSAGKLISFL IEDGGQVKKG QPYAEIEVMK MVMTLTATEN GRVYYSKRPG
TRIAVA_ACC          PSAGKLISFL IEDGGHVAKG AAYAEIEVMK MVMTLTANES GLVTFAKRPG
220O                PSAGKLISYL IEDGGHVSKG QAYAEIEVMK MVMTLTANES GLVTFAKRPG
319                 PSAGKLISYL IEDGGHVSKG QAYAEIEVMK MVMTLTANES GLVTFAKRPG
419                 PSAGKLISYL IEDGGHVSKG QAYAEIEVMK MVMTLTANES GLVTFAKRPG
520                 PSAGKLISYL IEDGGHVSKG QAYAEIEVMK MVMTLTANES GLVTFAKRPG
619                 PSAGKLISYL IEDGGHVSKG QAYAEIEVMK MVMTLTANES GLVTFAKRPG

                    801                                                 850
BEMITA_QJQ31013     AVLDAGSLIA TLELDDPSLV TKALDYKGQF PELDVSTPTV GEKLNHAHNH
MYZUPE_XP_022181497 AVLDAGSLIA TLELDDPSLV TKAIEYKGQF LELDGTSHIY GESLNNIHTC
TRIAVA_ACC          AVLDAGSLIA TLELDDISLV TKALDYKGQF PELDVSTPMV GDKLNHAHNH
220O                AVLDAGSLIG TLELDDPSLV SKALDYKGEF PELDVSTPMV GEKLNHAHNH
319                 AVLDAGSLIG TLELDDPSLV SKALDYKGEF PELDVSTPMV GEKLNHAHNH
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419                 AVLDAGSLIG TLELDDPSLV SKALDYKGEF PELDVSTPMV GEKLNHAHNH
520                 AVLDAGSLIG TLELDDPSLV SKALDYKGEF PELDVSTPMV GEKLNHAHNH
619                 AVLDAGSLIG TLELDDPSLV SKALDYKGEF PELDVSTPMV GEKLNHAHNH

                    851                                                 900
BEMITA_QJQ31013     YRQMLDNILA GYCLPDPYHL VRLREVIEKF MSSLRDPSLP LLELQEVISS
MYZUPE_XP_022181497 YRGMLDNILA GYCLPEPYHL VRLREVIEKF MNSLRDPSLP LLELQEVISS
TRIAVA_ACC          YRQMLDNILA GYCLPDPYHL VRLREVIEKF MSSLRDPSLP LLELQEVISS
220O                YRQMLDNILA GFCLPDPYHL VRLREVIEKF MSSLRDPSLP LLELQEVISS
319                 YRQMLDNILA GFCLPDPYHL VRLREVIEKF MSSLRDPSLP LLELQEVISS
419                 YRQMLDNILA GFCLPDPYHL VRLREVIEKF MSSLRDPSLP LLELQEVISS
520                 YRQMLDNILA GFCLPDPYHL VRLREVIEKF MSSLRDPSLP LLELQEVISS
619                 YRQMLDNILA GFCLPDPYHL VRLREVIEKF MSSLRDPSLP LLELQEVISS

                    901                                                 950
BEMITA_QJQ31013     ISGRIPISVE KKIRKLMTLY ERNITSVLAQ FPSQQIASVI DSHAATLQKR
MYZUPE_XP_022181497 ISGRIPKAVD KKIKSLMKLY ERNITSVLAQ FPSQQIAAII DGHAATLQKR
TRIAVA_ACC          ISGRIPISVE KKIRKLMTLY ERNITSVLAQ FPSQQIASVI DSHAATLQKR
220O                ISGRIPISVE KKIRKLMTLY ERNITSVLAQ FPSQQIAGVI DSHAATLQKR
319                 ISGRIPISVE KKIRKLMTLY ERNITSVLAQ FPSQQIAGVI DSHAATLQKR
419                 ISGRIPISVE KKIRKLMTLY ERNITSVLAQ FPSQQIAGVI DSHAATLQKR
520                 ISGRIPISVE KKIRKLMTLY ERNITSVLAQ FPSQQIAGVI DSHAATLQKR
619                 ISGRIPISVE KKIRKLMTLY ERNITSVLAQ FPSQQIAGVI DSHAATLQKR

                    951                                                1000
BEMITA_QJQ31013     SDRDGFFLTT QGIVQLVQRY RNGIRGRMKS AVHELLRQYY EVESQFQQGH
MYZUPE_XP_022181497 TDRDSFFQTT QGIVQLVQRY RNGIRGRMKS AVHELLKQYY EVESQFQHGS
TRIAVA_ACC          SDRDGFFLTT QGIVQLVQRY RNGIRGRMKS AVHELLRQYY EVESQFQQGH
220O                SDRDGFFLTT QGIVQLVQRY RNGIRGRMKS AVHELLRQYY EVESQFQQGH
319                 SDRDGFFLTT QGIVQLVQRY RNGIRGRMKS AVHELLRQYY EVESQFQQGH
419                 SDRDGFFLTT QGIVQLVQRY RNGIRGRMKS AVHELLRQYY EVESQFQQGH
520                 SDRDGFFLTT QGIVQLVQRY RNGIRGRMKS AVHELLRQYY EVESQFQQGH
619                 SDRDGFFLTT QGIVQLVQRY RNGIRGRMKS AVHELLRQYY EVESQFQQGH

                    1001                                               1050
BEMITA_QJQ31013     YDKCVTAIRE KFKDDMAAVT GTIFSHGQVA KKNMLVTMLI DHLWSNEPGL
MYZUPE_XP_022181497 YDKCATALRD RYKDDMAAVT STIFSHTQVA KKNMLVTMLI DHLWSNEPGL
TRIAVA_ACC          YDKCVTAIRE KFKDDMAAVV GTIFSHGQVA KKNMLVTMLI DHLWSNEPGL
220O                YDKCVMAIRE KFKDDMAAVT STIFSHGQVA KKNMLVTMLI DHLWSNEPGL
319                 YDKCVMAIRE KFKDDMAAVT STIFSHGQVA KKNMLVTMLI DHLWSNEPGL
419                 YDKCVMAIRE KFKDDMAAVT STIFSHGQVA KKNMLVTMLI DHLWSNEPGL
520                 YDKCVMAIRE KFKDDMAAVT STIFSHGQVA KKNMLVTMLI DHLWSNEPGL
619                 YDKCVMAIRE KFKDDMAAVT STIFSHGQVA KKNMLVTMLI DHLWSNEPGL

                    1051                                               1100
BEMITA_QJQ31013     TDELATTLNE LTSLNRSEHS RVALRARQVL IAAHQPAYEL RHNQMESIFL
MYZUPE_XP_022181497 TDELAATLNE LTSLNRSEHS RVALRARQVL IAAHQPAYEL RHNQMESIFL
TRIAVA_ACC          TDELATTLNE LTSLNRSEHS RVALRARQVL IAAHQPAYEL RHNQMESIFL
220O                TDELATTLNE LTSLNRSEHS RVALRSRQVL IAAHQPAYEL RHNQMESIFL
319                 TDELATTLNE LTSLNRSEHS RVALRSRQVL IAAHQPAYEL RHNQMESIFL
419                 TDELATTLNE LTSLNRSEHS RVALRSRQVL IAAHQPAYEL RHNQMESIFL
520                 TDELATTLNE LTSLNRSEHS RVALRSRQVL IAAHQPAYEL RHNQMESIFL
619                 TDELATTLNE LTSLNRSEHS RVALRSRQVL IAAHQPAYEL RHNQMESIFL

                    1101                                               1150
BEMITA_QJQ31013     SAVDMYGHDF HPENLQKLIQ SETSIFDILH DFFYHTNRAV CNAALEVYVR
MYZUPE_XP_022181497 SAVDMYGHDF HPENLQKLIQ SETSIFDILH DFFYHSNRAV CNAALEVYVR
TRIAVA_ACC          SAVDMYGHDF HPENLQKLIQ SETSIFDILH DFFYHTNRAV CNAALEVYVR
220O                SAVDMYGHDF HPENLQKLIQ SETSIFDILH DFFYHTNRAV CNAALEVYVR
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319                 SAVDMYGHDF HPENLQKLIQ SETSIFDILH DFFYHTNRAV CNAALEVYVR
419                 SAVDMYGHDF HPENLQKLIQ SETSIFDILH DFFYHTNRAV CNAALEVYVR
520                 SAVDMYGHDF HPENLQKLIQ SETSIFDILH DFFYHTNRAV CNAALEVYVR
619                 SAVDMYGHDF HPENLQKLIQ SETSIFDILH DFFYHTNRAV CNAALEVYVR

                    1151                                                1200
BEMITA_QJQ31013     RVYISYELTC LKHLELTEEV PLVQFQFLLP SSHPNRQRVT DSASPGRDAP
MYZUPE_XP_022181497 RVYISYDLTC LQHLELSGEI PLVHFQFLLP SSHPNRQQNK INSGAN...G
TRIAVA_ACC          RVYISYDLTC LQHLELSGEI PLVQFQFLLP SSHPNRQKVT DSSPIKADTP
220O                RVYISYELTC LQHLELSGEV PLVQFQFLLP SSHPNRQKVT DPSSPIIAD.
319                 RVYISYELTC LQHLELSGEV PLVQFQFLLP SSHPNRQKVT DPSSPIIAD.
419                 RVYISYELTC LQHLELSGEV PLVQFQFLLP SSHPNRQKVT DPSSPIIAD.
520                 RVYISYELTC LQHLELSGEV PLVQFQFLLP SSHPNRQKVT DPSSPIIAD.
619                 RVYISYELTC LQHLELSGEV PLVQFQFLLP SSHPNRQKVT DPSSPIIAD.

                    1201                                                1250
BEMITA_QJQ31013     ..ETDMTASA APTVIHSYQR TGCMAAFESF DQFESYYDEI LDLLDEL.SP
MYZUPE_XP_022181497 SENLESPTKT PLPYIPTYQR TGCMAAFESF TQFEQYFDEI LDIMEDLSSP
TRIAVA_ACC          PPEIEAGQSS APTIIHSYQR TGCMAAFESF DQFESYYDEI LDLLDDLMSP
220O                SPETEEPPSS TPTIIHSYQR TGCMAAFESF DQFESYYDEI LDLLDEL.SP
319                 SPETEEPPSS TPTIIHSYQR TGCMAAFESF DQFESYYDEI LDLLDEL.SP
419                 SPETEEPPSS TPTIIHSYQR TGCMAAFESF DQFESYYDEI LDLLDEL.SP
520                 SPETEEPPSS TPTIIHSYQR TGCMAAFESF DQFESYYDEI LDLLDEL.SP
619                 SPETEEPPSS TPTIIHSYQR TGCMAAFESF DQFESYYDEI LDLLDEL.SP

                    1251                                                1300
BEMITA_QJQ31013     STVSPRIMEA LESGSESRMS TSINVSLSVD .TQRPAGGEE GLQVEPIHIL
MYZUPE_XP_022181497 VYVSPRIIDA LESGSESRLS SSLNVSLSLG DQRPPD..QE NVEIEPCHIL
TRIAVA_ACC          STVSPRIMEA LESGSESRMS TSINVSLSVS DNPRPQGLEE GLQVEPIHIL
220O                STISPRIMEA LESGSESRMS TSINVSLSVG DNPRPPGLED GLQVEPIHIL
319                 STISPRIMEA LESGSESRMS TSINVSLSVG DNPRPPGLED GLQVEPIHIL
419                 STISPRIMEA LESGSESRMS TSINVSLSVG DNPRPPGLED GLQVEPIHIL
520                 STISPRIMEA LESGSESRMS TSINVSLSVG DNPRPPGLED GLQVEPIHIL
619                 STISPRIMEA LESGSESRMS TSINVSLSVG DNPRPPGLED GLQVEPIHIL

                    1301                                                1350
BEMITA_QJQ31013     CIAVKDNGDL EDDKLSKMFG DFCAKNRDEL KEKSIRRITF LALNRRQFPK
MYZUPE_XP_022181497 CIAMKDTGNM EDDKLGKMYE EFCQQRREEL KKRSIRRITF LALNKRQFPK
TRIAVA_ACC          CIAVKDNGDL EDDKLSKLLG DFCARHRDEL KEKSIRRITF LALNRRQFPK
220O                CIAVKDNGDL EDEKLSKLLG DFCAKHREEL KEKSIRRITF LALNRRQFPK
319                 CIAVKDNGDL EDEKLSKLLG DFCAKHREEL KEKSIRRITF LALNRRQFPK
419                 CIAVKDNGDL EDEKLSKLLG DFCAKHREEL KEKSIRRITF LALNRRQFPK
520                 CIAVKDNGDL EDEKLSKLLG DFCAKHREEL KEKSIRRITF LALNRRQFPK
619                 CIAVKDNGDL EDEKLSKLLG DFCAKHREEL KEKSIRRITF LALNRRQFPK

                    1351                                                1400
BEMITA_QJQ31013     LFTFRNCDNF VEDRIYRHLE PGMAFQLELN RMKTYHLEAL PTSNRKMYLY
MYZUPE_XP_022181497 LFTYRNYEDF AEDRIYRHLE PGMAFQLELN RMRTYELEAL PTSNRKMYLY
TRIAVA_ACC          LFTFRNCDNF VEDRIYRHLE PGMAFQLELN RMKTYHLEAL PTSNRKMYLY
220O                LFTFRNCDNF VEDRIYRHLE PGMAFQLELN RMKTYHLEAL PTSNRKMYLY
319                 LFTFRNCDNF VEDRIYRHLE PGMAFQLELN RMKTYHLEAL PTSNRKMYLY
419                 LFTFRNCDNF VEDRIYRHLE PGMAFQLELN RMKTYHLEAL PTSNRKMYLY
520                 LFTFRNCDNF VEDRIYRHLE PGMAFQLELN RMKTYHLEAL PTSNRKMYLY
619                 LFTFRNCDNF VEDRIYRHLE PGMAFQLELN RMKTYHLEAL PTSNRKMYLY

                    1401                                                1450
BEMITA_QJQ31013     LGKAKVAKGQ EVTDYRFFIR SIIRHSDLIT KEASFEYLQN EGERVLLEAM
MYZUPE_XP_022181497 LGKAKVPRGQ VVTDYRFFIR SIIRHQDLIT KEASFEYLQN EGERVLLEAM
TRIAVA_ACC          LGKAKVAKGQ EVTDYRFFIR SIIRHSDLIT KEASFEYLQN EGERVLLEAM
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220O                LGRAKVAKGQ EVTDFRFFIR SIIRHSDLIT KEASFEYLQN EGERVLLEAM
319                 LGRAKVAKGQ EVTDFRFFIR SIIRHSDLIT KEASFEYLQN EGERVLLEAM
419                 LGRAKVAKGQ EVTDFRFFIR SIIRHSDLIT KEASFEYLQN EGERVLLEAM
520                 LGRAKVAKGQ EVTDFRFFIR SIIRHSDLIT KEASFEYLQN EGERVLLEAM
619                 LGRAKVAKGQ EVTDFRFFIR SIIRHSDLIT KEASFEYLQN EGERVLLEAM

                    1451                                                1500
BEMITA_QJQ31013     DELEVAFSHP LAKRTDCNHI FLNFVPTVIM DSAKIEESVT NMVMRYGPRL
MYZUPE_XP_022181497 DELEVAFSHP HARRTDCNHI FLNFVPTVIM DPAKIKESVT NMVMRYGPRL
TRIAVA_ACC          DELEVAFSHP LAKRTDCNHI FLNFVPTVIM DSAKIEESVT NMVMRYGPRL
220O                DELEVAFSHP LARRTDCNHI FLNFVPTVIM DSAKIEESVT NMVMRYGARL
319                 DELEVAFSHP LARRTDCNHI FLNFVPTVIM DSAKIEESVT NMVMRYGARL
419                 DELEVAFSHP LARRTDCNHI FLNFVPTVIM DSAKIEESVT NMVMRYGARL
520                 DELEVAFSHP LARRTDCNHI FLNFVPTVIM DSAKIEESVT NMVMRYGARL
619                 DELEVAFSHP LARRTDCNHI FLNFVPTVIM DSAKIEESVT NMVMRYGARL

                    1501                                                1550
BEMITA_QJQ31013     WKLRVLQAEL RMTIRASPNA KTTNVRLCLA NDSGYYLDIC LYKEVVDPKT
MYZUPE_XP_022181497 WKLRVLQAEL RMTIRPSPTS KTSNVRLSLA NGSGYHLDIC LYKEITDSKL
TRIAVA_ACC          WKLRVLQAEL RMTIRASPNS KTTNVRLCLA NDSGYYLDIC LYKEVVDPKT
220O                WKLRVLQAEL RMTIRTSPNS KTTNVRLCLA NDSGYYLDIC LYKEVVDPKT
319                 WKLRVLQAEL RMTIRTSPNS KTTNVRLCLA NDSGYYLDIC LYKEVVDPKT
419                 WKLRVLQAEL RMTIRTSPNS KTTNVRLCLA NDSGYYLDIC LYKEVVDPKT
520                 WKLRVLQAEL RMTIRTSPNS KTTNVRLCLA NDSGYYLDIC LYKEVVDPKT
619                 WKLRVLQAEL RMTIRTSPNS KTTNVRLCLA NDSGYYLDIC LYKEVVDPKT

                    1551                                                1600
BEMITA_QJQ31013     GIIKLESYGS KQGPLHGLPV ATPYVTKDYL QQKRFQAQSA GTTYCHDIPD
MYZUPE_XP_022181497 GMIKFESYES KQGPLHGLPV STPYVTKDFL QQKRFQAQSA GTTYVYDIPD
TRIAVA_ACC          GIIKLESYGS KQGPVHGLPV SLPYVTKDYL QQKRFQAQSA GTTYCHDIPD
220O                GIIKLESYGS KQGPLHGLPT AIPYVTKDYL QQKRFQAQSA GTTYCHDIPD
319                 GIIKLESYGS KQGPLHGLPT AIPYVTKDYL QQKRFQAQSA GTTYCHDIPD
419                 GIIKLESYGS KQGPLHGLPT AIPYVTKDYL QQKRFQAQSA GTTYCHDIPD
520                 GIIKLESYGS KQGPLHGLPT AIPYVTKDYL QQKRFQAQSA GTTYCHDIPD
619                 GIIKLESYGS KQGPLHGLPT AIPYVTKDYL QQKRFQAQSA GTTYCHDIPD

                    1601                                                1650
BEMITA_QJQ31013     MFRQMVERQW KEHIDQRPDD GIVKPAQLMD FAELVLEEDH LVEQKRLPGE
MYZUPE_XP_022181497 MFRQMIETLW QEYILEHPND GVLKSSLVFD YVELVVEDNH LIEQKRFSGE
TRIAVA_ACC          MFRQMVERQW KEHIEQRPND GIVKPSQLMD FVELVMEEDH LIEVKRLSGE
220O                MFRQMVERQW KEFIEQRPND GIVKPSQLMD YVELVLEDDH LVEQKRLAGE
319                 MFRQMVERQW KEFIEQRPND GIVKPSQLMD YVELVLEDDH LVEQKRLAGE
419                 MFRQMVERQW KEFIEQRPND GIVKPSQLMD YVELVLEDDH LVEQKRLAGE
520                 MFRQMVERQW KEFIEQRPND GIVKPSQLMD YVELVLEDDH LVEQKRLAGE
619                 MFRQMVERQW KEFIEQRPND GIVKPSQLMD YVELVLEDDH LVEQKRLAGE

                    1651                                                1700
BEMITA_QJQ31013     NNVGMVAWRI TLNTPEYPDG RDIIVIANDI TVRIGSFGPE EDLVFDLASK
MYZUPE_XP_022181497 NTAGMVAWRF TMHTPEYPSG RDIIVIANDL TVNIGSFGPQ EDIVFDLASK
TRIAVA_ACC          NNVGMVAWRI TLNTPEYPDG RDIIVIANDI TVRIGSFGPE EDIVFDLASK
220O                NTIGMVAWRI TLNTPEYPDG REIIVIANDI TVRIGSFGPE EDLVFDLASK
319                 NTIGMVAWRI TLNTPEYPDG REIIVIANDI TVRIGSFGPE EDLVFDLASK
419                 NTIGMVAWRI TLNTPEYPDG REIIVIANDI TVRIGSFGPE EDLVFDLASK
520                 NTIGMVAWRI TLNTPEYPDG REIIVIANDI TVRIGSFGPE EDLVFDLASK
619                 NTIGMVAWRI TLNTPEYPDG REIIVIANDI TVRIGSFGPE EDLVFDLASK

                    1701                                                1750
BEMITA_QJQ31013     IARQRKIPRI YIAANSGARI GLAEEVKSLF RVAWEDPDEP DKGFKYLYLS
MYZUPE_XP_022181497 EARRKKIPRI YISANSGARI GLAEEIKSLF NVAWEDPSDP EKGFKYLYLT
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TRIAVA_ACC              IARARKIPRI YIAANSGARI GLAEEVKSLF RVAWEDPDEP DKGFKYLYLS
220O                    IARQMKVPRI YIAANSGARI GLAEEVKSLF RVAWEDPDEP DKGLKYLYLS
319                     IARQMKVPRI YIAANSGARI GLAEEVKSLF RVAWEDPDEP DKGLKYLYLS
419                     IARQMKVPRI YIAANSGARI GLAEEVKSLF RVAWEDPDEP DKGLKYLYLS
520                     IARQMKVPRI YIAANSGARI GLAEEVKSLF RVAWEDPDEP DKGLKYLYLS
619                     IARQMKVPRI YIAANSGARI GLAEEVKSLF RVAWEDPDEP DKGLKYLYLS

                        1751                                                1800
BEMITA_QJQ31013         TEDFTKISSC NSVHAILIED EGEARYKITD IIGKEDGLGV ENLRYAGMIA
MYZUPE_XP_022181497     PDDYGKLAGQ NSVEAELIED EGEPRYKLTD IIGKDFGYGV ENLKFAGMIA
TRIAVA_ACC              TEDFTKISSL NSVHAILIED EGEARYKITD IIGKEDGLGV ENLRYAGMIA
220O                    TEDFIKISAC NSVRAILIED EGEARYKITD IIGKEDGLGV ENLRYAGMIA
319                     TEDFIKISAC NSVRAILIED EGEARYKITD IIGKEDGLGV ENLRYAGMIA
419                     TEDFIKISAC NSVRAILIED EGEARYKITD IIGKEDGLGV ENLRYAGMIA
520                     TEDFIKISAC NSVRAILIED EGEARYKITD IIGKEDGLGV ENLRYAGMIA
619                     TEDFIKISAC NSVRAILIED EGEARYKITD IIGKEDGLGV ENLRYAGMIA

                        1801                                                1850
BEMITA_QJQ31013         GETSEAYREI VTISMVSCRA IGIGAYLVRL GQRVIQVENS HIILTGYSAL
MYZUPE_XP_022181497     GETSRAYQDI VTISMVTCRA IGIGAYLVRL GQRVIQIENS HIILTGYSAL
TRIAVA_ACC              GETSEAYREI VTISMVSCRA IGIGAYLVRL GQRVIQVENS HIILTGYSAL
220O                    GETSEAYREI VTISMVSCRA IGIGAYLVRL GQRVIQVENS HIILTGYSAL
319                     GETSEAYREI VTISMVSCRA IGIGAYLVRL GQRVIQVENS HIILTGYSAL
419                     GETSEAYREI VTISMVSCRA IGIGAYLVRL GQRVIQVENS HIILTGYSAL
520                     GETSEAYREI VTISMVSCRA IGIGAYLVRL GQRVIQVENS HIILTGYSAL
619                     GETSEAYREI VTISMVSCRA IGIGAYLVRL GQRVIQVENS HIILTGYSAL

                        1851                                                1900
BEMITA_QJQ31013         NKLLGREVYA SNNQLGGIQI MYNNGVSHKT EPRDLDGIYS IVKWLSYIPK
MYZUPE_XP_022181497     NKLLGREVYA SNNQLGGIQI MYNNGVSHKT EARDLDGVYR ILKWLSYIPK
TRIAVA_ACC              NKLLGREVYA SNNQLGGIQI MHNNGVSHKT EPRDLDGIYS IVKWLAFVPK
220O                    NKLLGREVYA SNNQLGGIQI MYNNGISHKT EPRDLDGIYS IVKWLGYIPK
319                     NKLLGREVYA SNNQLGGIQI MYNNGISHKT EPRDLDGIYS IVKWLGYIPK
419                     NKLLGREVYA SNNQLGGIQI MYNNGISHKT EPRDLDGIYS IVKWLGYIPK
520                     NKLLGREVYA SNNQLGGIQI MYNNGISHKT EPRDLDGIYS IVKWLGYIPK
619                     NKLLGREVYA SNNQLGGIQI MYNNGISHKT EPRDLDGIYS IVKWLGYIPK

                        1901                                                1950
BEMITA_QJQ31013         DKLSPVPVIK PADPIDREVG YMPTKTPYDP RWMLAGRYSP .NNSNEWESG
MYZUPE_XP_022181497     TKESPLPVIK SVDSVERDID YVPTKVPYDP RWMIAGKE.. .DTNGHWESG
TRIAVA_ACC              DKMSPVPVLK PADPVDREVG YMPTKTPYDP RWMLAGHFSF LDSSNEWESG
220O                    DKYSPVPVIK PADPVDREVG YMPTKTPYDP RWMIAGRYSP .NNSNEWESG
319                     DKYSPVPVIK PADPVDREVG YMPTKTPYDP RWMIAGRYSP .NNSNEWESG
419                     DKYSPVPVIK PADPVDREVG YMPTKTPYDP RWMIAGRYSP .NNSNEWESG
520                     DKYSPVPVIK PADPVDREVG YMPTKTPYDP RWMIAGRYSP .NNSNEWESG
619                     DKYSPVPVIK PADPVDREVG YMPTKTPYDP RWMIAGRYSP .NNSNEWESG

                        1951                                                2000
BEMITA_QJQ31013         FFDEGSWEEV MQPWAQTVVV GRARLGGIPM GVIAVETRTV EVKLPADPAN
MYZUPE_XP_022181497     FFDKGSWDEI MQPWAQTVVC GRARLGGIPV GVIAVETRTV EVTLPADPAN
TRIAVA_ACC              FFDEGTWEEV MQPWAQTVVV GRARLGGIPM GVIAVETRTV ELKWPADPAN
220O                    FFDEGSWEEV MQPWAQTVVV GRARLGGIPM GVIAVETRTV EVKLPADPAN
319                     FFDEGSWEEV MQPWAQTVVV GRARLGGIPM GVIAVETRTV EVKLPADPAN
419                     FFDEGSWEEV MQPWAQTVVV GRARLGGIPM GVIAVETRTV EVKLPADPAN
520                     FFDEGSWEEV MQPWAQTVVV GRARLGGIPM GVIAVETRTV EVKLPADPAN
619                     FFDEGSWEEV MQPWAQTVVV GRARLGGIPM GVIAVETRTV EVKLPADPAN

                        2001                                                2050
BEMITA_QJQ31013         LDSEAKTLSQ AGQVWFPDSA YKTAQAIKDF EHEDLPLIIF ANWRGFSGGM
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MYZUPE_XP_022181497    LDSESKTVSQ AGQVWFPDSA YKTSQAIKDF AHEDLPLFIF ANWRGFSGGM
TRIAVA_ACC             LDSEAKTLSQ AGQVWFPDSA YKTAQAIKDF QHEDLPLIIF ANWRGFSGGM
2200                   LDSEAKTLSQ AGQVWFPDSA FKTAQAIKDF EHEDLPLIIF ANWRGFSGGM
319                    LDSEAKTLSQ AGQVWFPDSA FKTAQAIKDF EHEDLPLIIF ANWRGFSGGM
419                    LDSEAKTLSQ AGQVWFPDSA FKTAQAIKDF EHEDLPLIIF ANWRGFSGGM
520                    LDSEAKTLSQ AGQVWFPDSA FKTAQAIKDF EHEDLPLIIF ANWRGFSGGM
619                    LDSEAKTLSQ AGQVWFPDSA FKTAQAIKDF EHEDLPLIIF ANWRGFSGGM

                       2051                                            2100
BEMITA_QJQ31013        KDMYEQVMKF GAYIVDELRV YKQPVIIYIP PNGELRGGAW AVVDPTINPR
MYZUPE_XP_022181497    KDMYEQIMKF GAYIVDELRQ YNQPIITYIP PFGELRGGAW AVVDTTINPR
TRIAVA_ACC             KDMYEQVMKF GAYIVDELRL YKQPVIIYIP PNGELRGGAW AVVDPTINPR
2200                   KDMYEQVMKF GAYIVDELRV YKQPVIIYIP PNGELRGGAW VVVDPTINPR
319                    KDMYEQVMKF GAYIVDELRV YKQPVIIYIP PNGELRGGAW AVVDPTINPR
419                    KDMYEQVMKF GAYIVDELRV YKQPVIIYIP PNGELRGGAW AVVDPTINPR
520                    KDMYEQVMKF GAYIVDELRV YKQPVIIYIP PNGELRGGAW AVVDPTINPR
619                    KDMYEQVMKF GAYIVDELRV YKQPVIIYIP PNGELRGGAW AVVDPTINPR

                       2101                                            2150
BEMITA_QJQ31013        HMEMYADPES RGGVLEPEGI VEIKFREKDI LKTMHRIDQV IVPLKQRLSS
MYZUPE_XP_022181497    HIEMYADPDS RGGVLEPEGI VEIKFREKDI LKSINRIDTN ILSLKAN...
TRIAVA_ACC             YMEMYADPES RGGVLEPEGI VEIKFREKDI LKTMHRIDQI IVPLKQKLAN
2200                   HMEMYADPES RGGVLEPEGI VEIKFREKDI LKTMHRIDQV IVPLKQKLAV
319                    HMEMYADPES RGGVLEPEGI VEIKFREKDI LKTMHRIDQV IVPLKQKLAV
419                    HMEMYADPES RGGVLEPEGI VEIKFREKDI LKTMHRIDQV IVPLKQKLAV
520                    HMEMYADPES RGGVLEPEGI VEIKFREKDI LKTMHRIDQV IVPLKQKLAV
619                    HMEMYADPES RGGVLEPEGI VEIKFREKDI LKTMHRIDQV IVPLKQKLAV

                       2151                                            2200
BEMITA_QJQ31013        TDISPEEKAD VESRIVEREQ YLKPMYHQVA VHFADLHDTP ERMVEKGVIH
MYZUPE_XP_022181497    ASPTPEEAVE IEKNVAERIS VLKPIYHQVA IHFADLHDTP KCMLSKGVIK
TRIAVA_ACC             PDISPEEKAE VENQIVEREQ YLKPMYHQVA VHFADLHDTP ERMMEKGVIH
2200                   PDLSPEEKAE VENQIVEREQ YLKPMYHQVA IHFADLHDTP ERMMEKGVIH
319                    PDLSPEEKAE VENQIVEREQ YLKPMYHQVA IHFADLHDTP ERMMEKGVIH
419                    PDLSPEEKAE VENQIVEREQ YLKPMYHQVA IHFADLHDTP ERMMEKGVIH
520                    PDLSPEEKAE VENQIVEREQ YLKPMYHQVA IHFADLHDTP ERMMEKGVIH
619                    PDLSPEEKAE VENQIVEREQ YLKPMYHQVA IHFADLHDTP ERMMEKGVIH

                       2201                                            2250
BEMITA_QJQ31013        DIVPWRKSRT ILHWRVKRLL LENQIKSNLL KVQPQMDDGQ AQAMLRRWFV
MYZUPE_XP_022181497    DIVQWKKSRN TLYWRLKRRL LQNQIQKVIT KSNDTIQDDV AYEMLRRWFV
TRIAVA_ACC             DIVPWRKSRS IIHWRMKRLL LENQIKSNLI RVQPQLDDGQ AQAMLRRWFV
2200                   NIVPWRKSRT LLHWRVKRLL LENQIKCNLL KVQPQMDNGQ AQAMLRRWFV
319                    NIVPWRKSRT LLHWRVKRLL LENQIKCNLL KVQPQMDNGQ AQAMLRRWFV
419                    NIVPWRKSRT LLHWRVKRLL LENQIKCNLL KVQPQMDNGQ AQAMLRRWFV
520                    NIVPWRKSRT LLHWRVKRLL LENQIKCNLL KVQPQMDNGQ AQAMLRRWFV
619                    NIVPWRKSRT LLHWRVKRLL LENQIKCNLL KVQPQMDNGQ AQAMLRRWFV

                       2251                                            2300
BEMITA_QJQ31013        EDIGTTTAYL WENNESVVSW LMGQLS.PDG SISPNSIVAN NIRCVQRDAL
MYZUPE_XP_022181497    EDKGTTASYL WDNNQAVVQW LTSQLDESDG TIVADSLIGN NIKSVRKDAV
TRIAVA_ACC             EDKGTTTAYL WENNESVVSW LMTQLS.PDG SISPHSIVAN NIRCVQRDAL
2200                   EDKGTTTAYL WENNESVVAW LMSQLS.PDG SISPNSIVAN NIRCVQRDAL
319                    EDKGTTTAYL WENNESVVAW LMSQLS.PDG SISPNSIVAN NIRCVQRDAL
419                    EDKGTTTAYL WENNESVVAW LMSQLS.PDG SISPNSIVAN NIRCVQRDAL
520                    EDKGTTTAYL WENNESVVAW LMSQLS.PDG SISPNSIVAN NIRCVQRDAL
619                    EDKGTTTAYL WENNESVVAW LMSQLS.PDG SISPNSIVAN NIRCVQRDAL

                       2301                                            2350
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BEMITA_QJQ31013         INQIKTSMEE SPDVAQDAVV EMFQTLSASE RSEVLRKLSH LETISKPEPQ
MYZUPE_XP_022181497     INQVKSTIND TPEVTSDVIM GMFQSLSEMQ RLDLIHNLTQ ATSIGNVKLN
TRIAVA_ACC              INQIKTSMEE SPDVAQDAVV EMFQALSSSE RSEVLRKLSH LEMINSSEPQ
220O                    INQIKTSMEE SPDVAQDAVV EMFQTLSANE RSEVLRKLSH LETISKPEPQ
319                     INQIKTSMEE SPDVAQDAVV EMFQTLSANE RSEVLRKLSH LETISKPEPQ
419                     INQIKTSMEE SPDVAQDAVV EMFQTLSANE RSEVLRKLSH LETISKPEPQ
520                     INQIKTSMEE SPDVAQDAVV EMFQTLSANE RSEVLRKLSH LETISKPEPQ
619                     INQIKTSMEE SPDVAQDAVV EMFQTLSANE RSEVLRKLSH LETISKPEPQ

                        2351
BEMITA_QJQ31013         S~
MYZUPE_XP_022181497     S~
TRIAVA_ACC              NS
220O                    S~
319                     S~
419                     S~
520                     S~
619                     S~
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Table S3: Genotyping by pyrosequencing of individuals of *Aleyrodes proletella* (ALEUPR) for the presence of the A2083V mutation in the ACC carboxyltransferase (CT) domain (*Bemisia tabaci* numbering).

			Genotype ACC 2083, %		
	ALEUPR strain	**Year**	**A/A**	**A/V**	**V/V**
Strains reared in laboratory	2/19	2019	80	20	0
	3/19	2019	100	0	0
	4/19	2019	100	0	0
	5/19	2019	70	20	10
	SPI-5/19	2019	0	20	80
	6/19	2019	100	0	0
	1/20	2020	100	0	0
	2/20	2020	10	60	30
	SPI-2/20	2020	10	0	90
	4/20	2020	100	0	0
	5/20	2020	100	0	0
	6/20	2020	29	0	71
	SPI-6/20	2020	0	0	100
Strains preserved in alcohol	NAR2019-131	2019	100	0	0
	NAR2019-132	2019	100	0	0
	NAR2019-148	2019	100	0	0
	NAR2019-149	2019	90	10	0

			Genotype ACC 2083, %		
	ALEUPR strain	**Year**	**A/A**	**A/V**	**V/V**
Strains preserved in alcohol	NAR2019-150	2019	100	0	0
	NAR2019-151	2019	100	0	0
	NAR2019-162	2019	0	0	100
	NAR2019-163	2019	100	0	0
	NAR2019-164	2019	100	0	0
	NAR2019-165	2019	100	0	0
	NAR2019-166	2019	100	0	0
	NAR2019-183	2019	100	0	0
	NAR2020-119	2020	100	0	0
	NAR2020-120	2020	100	0	0
	NAR2020-121	2020	100	0	0
	NAR2020-122	2020	0	0	100
	NAR2020-123	2020	0	33	67
	NAR2020-124	2020	0	50	50
	NAR2021-048	2021	100	0	0
	NAR2021-049	2021	100	0	0
	NAR2021-050	2021	100	0	0
	NAR2021-051	2021	100	0	0
	NAR2021-052	2021	100	0	0
	NAR2021-053	2021	100	0	0
	NAR2021-054	2021	0	100	0
	NAR2021-055	2021	20	80	0
	NAR2021-060	2021	100	0	0
	NAR2021-061	2021	100	0	0
	NAR2021-110	2021	0	10	90
	NAR2021-111	2021	0	0	100
	NAR2021-112	2021	0	10	90
	NAR2021-113	2021	0	10	90
	NAR2021-114	2021	100	0	0
	NAR2021-115	2021	0	10	90
	NAR2021-116	2021	0	0	100
	NAR2021-117	2021	0	0	100

			Genotype ACC 2083, %		
	ALEUPR strain	**Year**	**A/A**	**A/V**	**V/V**
Strains preserved in alcohol	NAR2021-120	2021	100	0	0
	ALTHFL_DEU21_0001	2021	30	40	30
	ALEUPR_DEU21_0002	2021	0	60	40