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Characterization of mechanisms of resistance in *Spodoptera frugiperda* to synthetic insecticides and insecticidal proteins

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Ao meu avô Gabriel Boaventura

“Todas as vitórias ocultam uma abdicação” (Simone de Beauvoir)

Abstract

Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), is a major lepidopteran pest of maize in the American continent but has recently invaded Africa, Asia, and Australia, and is now present in 107 countries worldwide. The control of FAW has relied mainly on the use of synthetic insecticides and transgenic crops expressing *Bacillus thuringiensis* (Bt) insecticidal proteins. However, the effective control of this pest is challenging, as resistance to 41 different active substances has been reported worldwide, putting at risk the yield of important staple crops.

Diamides act on insect ryanodine receptors (RyR) and are the most modern insecticide class intensively used to control lepidopteran pests. Resistance to this chemical class is found at low levels in the field in Brazil, but a highly resistant population has been selected in the laboratory. Here (Chapter 2) inhibition studies with different synergists (PBO, DEM, and DEF) were performed and suggested the absence of metabolic resistance to diamide insecticides. Sequencing of the C-terminal end (domains II to VI) of the RyR revealed the presence of a conserved point mutation (I4790M) linked to diamide resistance. Diagnostic assays were designed based on gDNA and different FAW populations collected in Brazil were tested for the presence of the RyR target-site mutation. Only the susceptible allele (I4790) was detected in field-collected strains. The diagnostic assays showed robust results, allowing the introduction of these tools across a broad geographic range.

The first case of resistance to Bt crops in Brazil was reported in 2014 for maize expressing the Cry1F protein. Recently, many more cases of field failure have been described, confirming cross-resistance to other Bt proteins expressed in maize, cotton, and soybean.

In Chapter 3 the molecular mechanism conferring Cry1F resistance in Brazilian FAW was investigated and characterized. Different mutations were observed in exon 14 of the Bt receptor ATP-Binding Cassette subfamily C2 (ABCC2) transporter. However, the deletion of glycine and tyrosine (GY deletion) was found in higher frequency in field-collected strains of FAW highly resistant to Cry1F. The mechanism of resistance was investigated by *in vitro* cytotoxicity assays and genetic linkage studies, confirming the role of the GY deletion in Cry1F resistance in Brazil.

Failures of FAW control with Bt crops and consequently high infestation pressure in the field require additional insecticide applications. Therefore, the toxicological profile of the Cry1F-resistant strain (Sf_Des) described in Chapter 3 was also investigated to different commercial insecticides and presented in Chapter 4. Laboratory bioassays with 15 active substances of nine mode of action classes revealed that Sf_Des has a medium level of resistance to deltamethrin and chlorpyrifos in comparison to the Cry1F-susceptible strain (Sf_Bra). Very high

cross-resistance was observed among Cry1 toxins, but high susceptibility against Vip3A. RNA-Seq data support a major role of P450 enzymes in the detoxification of insecticides and RT-qPCR analysis confirmed that *CYP9A*-like and *CYP6B39* are significantly up-regulated (>200-fold) in Sf_Des in comparison to Sf_Bra strain. Moreover, the activity of enzymes involved in insecticide detoxification (P450 monooxygenases, glutathione S-transferase, and carboxylesterase) confirmed the major role of cytochrome P450 enzymes in the toxicological profile observed.

Target-site mutations are among the main mechanisms of resistance and monitoring their frequency is of great value for insecticide resistance management. Pyrosequencing and PCR-based allelic discrimination assays were developed and used to genotype target-site resistance alleles in 34 FAW populations from different continents (Chapter 5). The diagnostic methods revealed a high frequency of mutations in acetylcholinesterase, conferring resistance to organophosphates and carbamates. In voltage-gated sodium channels targeted by pyrethroids, only one population from Indonesia showed a mutation. No mutations were detected in the ryanodine receptor, suggesting susceptibility to diamides. Indels in the ABCC2 associated with Bt-resistance were observed in samples collected in Puerto Rico and Brazil. Additionally, we analyzed all samples for the presence of markers associated with two sympatric FAW host plant strains. The molecular methods established show robust results in FAW samples collected across a broad geographical range and can be used to support decisions for sustainable FAW control and applied resistance management.

The data presented here characterized novel molecular mechanisms conferring resistance to different insecticides/Bt toxins which remained elusive yet. Those findings not only support further research on new insecticides compounds overcoming such resistance mechanisms, but also provide practical guidance for the regional implementation of efficient resistance management strategies.

Zusammenfassung

Spodoptera frugiperda (J.E. Smith), im Englischen als Fall Armyworm („Herbst-Heerwurm“) bezeichnet, aus der Ordnung der Lepidoptera ist einer der wichtigsten Mais-Schädlinge auf dem amerikanischen Kontinent und ist nach Invasion in Afrika, Asien und Australien inzwischen in 107 verschiedenen Ländern weltweit verbreitet. Die Bekämpfung von *S. frugiperda* stützt sich weitestgehend auf synthetische Insektizide, sowie transgene Kulturpflanzen, die insektizide Proteine aus *Bacillus thuringiensis* (Bt) exprimieren. Die effiziente Kontrolle ist jedoch eine große Herausforderung, nicht zuletzt, da weltweit Resistenzen gegenüber 41 verschiedenen Wirkstoffen beschrieben wurden, was ein Risiko für die Ernteerträge wichtiger Nutzpflanzen darstellt.

Diamide sind eine der neuesten, insektiziden Wirkstoffklassen und werden weltweit vor allem zur Kontrolle von Schädlingen aus der Ordnung der Lepidoptera eingesetzt. Während in Brasilien im Feld Resistenzen gegenüber dieser Wirkstoffgruppe bisher nur auf niedrigem Niveau gefunden wurden, ist es gelungen einen hochresistenten Stamm von *S. frugiperda* im Labor zu selektieren. Nach Studien mit verschiedenen Enzym-Inhibitoren (PBO, DEM, DEF) wurde eine essenzielle Beteiligung metabolischer Resistenzmechanismen verworfen (Kapitel 2). Die Sequenzierung der C-terminalen Domänen II bis VI des Ryanodin-Rezeptors – dem Target insektizider Diamide - offenbarte eine konservierte Punktmutation (I4790M), die auch in anderen Schädlingsarten mit Diamid-Resistenz in Verbindung gebracht wird. Basierend auf gDNA wurden diagnostische Tests entwickelt und anschließend verschiedene Feld-Populationen aus Brasilien auf diese Mutation untersucht. Nur das Wildtyp-Allel (I4790) wurde gefunden. Die diagnostischen Tests erwiesen sich als robust, was eine weltweite Implementierung zur Untersuchung dieses Resistenzmechanismus ermöglicht.

Der erste Fall von Resistenz von *S. frugiperda* gegenüber transgenen Bt-Kulturpflanzen in Brasilien wurde 2014 bei Mais gemeldet, welcher das Protein Cry1F exprimiert. Seitdem wurden immer mehr Fälle von ungenügender Kontrolle durch transgene Kulturpflanzen basierend auf der Bt-Technologie registriert, was auf eine mögliche Kreuzresistenz zwischen verschiedenen Bt-Proteinen hindeutet. In Kapitel 3 werden die molekularen Mechanismen der Cry1F-Resistenz in Brasilien untersucht und charakterisiert. Verschiedene Mutationen wurden im Exon 14 des Bt-Rezeptors ATP-Binding Cassette subfamily C2 Transporter (ABCC2) gefunden. Die Deletion von Glycin + Tyrosin (GY-Deletion) wurde dabei am häufigsten in hochresistenten Populationen nachgewiesen. Mithilfe von *in vitro* Cytotoxizitätstests und Genkopplungsstudien wurde der Resistenzmechanismus weiter charakterisiert und seine Bedeutung für die Cry1F-Resistenz in *S. frugiperda* bestätigt.

Durch die mangelnde, vollständige Kontrolle mittels Bt-Technologie ist oftmals die zusätzliche Nutzung von Insektiziden notwendig, um den Befallsdruck von *S. frugiperda* ausreichend zu minimieren. Daher wurde das toxikologische Profil des Cry1F-resistenten Stammes aus Kapitel 3 (Sf_Des) gegenüber verschiedenen, kommerziell erhältlichen Wirkstoffen näher untersucht (Kapitel 4). Biotests mit 15 Wirkstoffen von neun verschiedenen Wirkmechanismen belegen, dass Sf_Des im Vergleich zum Cry1F-sensiblen Referenzstamm (Sf_Bra) erhöhte Resistenz gegenüber Deltamethrin und Chlorpyrifos aufweist. Sehr hohe Kreuzresistenz wurde zwischen verschiedenen Cry1-Toxinen beobachtet, während Vip3A sehr gute Wirksamkeit aufweist. RNA-seq Daten weisen bei der erhöhten Resistenz gegenüber Insektiziden auf eine bedeutende Rolle von P450 Monooxygenasen hin. Eine RT-qPCR Analyse bestätigt eine signifikante Überexpression (>200-fach) von CYP9A-like und CYP6B39 im Sf_Des Stamm verglichen mit Sf_Bra. Die Bedeutung der P450 Monooxygenasen bezüglich des toxikologischen Profils wurde durch Untersuchung der Enzymaktivität verschiedener Enzymfamilien (P450 Monooxygenasen, Glutathion S-Transferasen, Carboxylesterasen) bestätigt.

Target-Site Mutationen gehören zu den wichtigsten Resistenzmechanismen und das Monitoring der Allelfrequenz ist von immenser Bedeutung für erfolgreiches Resistenzmanagement. Diagnostische Methoden basierend auf PCR, sowie Pyrosequenzierung wurden entwickelt und genutzt, um die Allelfrequenz bekannter Mutationen in 34 *S. frugiperda* Populationen aus verschiedenen Regionen zu screenen (Kapitel 5). Hohe Mutationsraten in der Acetylcholinesterase wurden diagnostiziert, welche Resistenz gegenüber Organophosphaten und Carbamaten verleihen. Im spannungsabhängigen Natriumkanal, dem Wirkort der Pyrethroide, wurde lediglich in einer indonesischen Population eine Mutation nachgewiesen. Keine der bekannten Mutationen wurden im Ryanodin-Rezeptor gefunden - Indikator für eine unverändert gute Wirksamkeit der Diamide. Indels im ABCC2-Transporter, verbunden mit Bt-Resistenz, waren in Proben aus Puerto Rico und Brasilien vorhanden. Zusätzlich wurden alle Populationen hinsichtlich genetischer Marker analysiert, die eine Unterscheidung zwischen zwei sympatrischen Unterarten ermöglichen. Die entwickelten molekularen Methoden erwiesen sich als robuste Resistenzdiagnose-Verfahren für *S. frugiperda* Populationen aus verschiedensten geographischen Regionen und können in eine ganzheitliche Strategie zur nachhaltigen Kontrolle des Herbst-Heerwurmes, sowie des angewandten Resistenzmanagements, implementiert werden.

Die hier präsentierten Daten charakterisieren Resistenzmechanismen teils erstmalig gegenüber verschiedene insektizide Wirkstoffe. Die Ergebnisse helfen nicht nur bei der Erforschung neuer, resistenzbrechender insektizider Wirkstoffe, sondern haben auch eine

praktische Relevanz für die Implementierung einer effizienten, regionalen Resistenzmanagement-Strategie.

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List of abbreviations

Å	Angstom
a.i.	Active ingredient
AA	Amino acid
ABC	ATP-binding cassette
AChE	Acetylcholinesterase
ACT	Actin
AFLP	Amplified fragment length polymorphism
AK	Arginine kinase
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
APN	Aminopeptidase-N
ATP	Adenosine-triphosphate
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
BFC	7-benzyloxy-4-trifluoromethyl coumarin
BOMFC	7-benzyloxymethoxy-4-trifluoromethyl coumarin
BOMR	7-benzyloxymethoxy resorufin
bp	Base pair(s)
BPU	Benzoylphenyl urea
BSA	Bovine serum albumin
Bt	*Bacillus thuringiensis*
CAD	Cadherin
Cas9	CRISPR associated protein 9
cDNA	Complementary deoxyribonucleic acid
CDNB	1-chloro-2,4-dinitrobenzene
CE	Carboxylesterase
CHS1	Chitin synthase 1
CI 95%	Confidence interval 95%
CL	Confidence limits
cm	Centimeter
CncC	Cap ‘n’ collar C
COI	Cytochrome oxidase subunit I

CPR	Cytochrome P450 reductase
Cq	Quantification cycle
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cry	Crystalline
Ctrl	Control
CYP	Cytochrome P450-dependent monooxygenase
Cyt	Cytosolic
D	Degrees of dominance
DCJW	Active metabolite from indoxacarb
DDT	Dichlorodiphenyltrichloroethane
DEF	S,S,S-tributyl-phosphorotrithioate
DEG	Differentially expressed genes
DEM	Diethyl maleate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
e.g.	Exempli gratia
EC	7-ethoxy coumarin
EC	Emulsion concentrate
ECL	Extracellular loop
EDTA	Ethylenediaminetetraacetic acid
EF2	Elongation factor 2
EST	Esterase
ET	Economic threshold
EU	European Union
F1	First filial generation
FAO	Food and Agriculture Organization of the United Nations
FAW	Fall armyworm
FBS	Fetal bovine serum
Fig	Figure
FOXA	Forkhead box protein A
g	Gram
g	Relative centrifugation force (g-force)
GABA	Gamma-aminobutyric acid

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
GluCl	Glutamate-gated chloride
GM	Genetically modified
GO	Gene Ontology
GSH	Glutathione
GST	Glutathione S-transferase
GY	Glycine and Tyrosine
h	Hour
ha	Hectare
Hz	Hertz
i. e.	id est (that is)
IPM	Integrated Pest Management
IRAC	Insecticide Resistance Action Committee
IRM	Insecticide Resistance Management
kdr	Knock-down resistance
Kg	Kilogram
km	Kilometers
K_m	Michaelis constant
km	Kilometer
L	Liter
L10	Ribosomal Protein L10
L17	Ribosomal Protein L17
L18	Ribosomal Protein L18
LC	Lethal concentration
LDH	Lactate dehydrogenase
µg	Microgram
µL	Microliter
M	Molar
Maf	Muscle aponeurosis fibromatosis
mALP	Membrane-bound alkaline phosphatase
MAPK	Mitogen-activated protein kinase
MCB	Monochlorobimane

MFO	Mixed-function oxidases
µg	Microgram
µL	Microliter
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
MoA	Mode of action
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance protein
n	Number of replicates
NA	1-and 2-naphthyl acetate
nAChR	Nicotinic acetylcholine receptor
NADPH	Nicotinamide adenine dinucleotide hydrogen phosphate
NBD	Nucleotide-binding domain
NCBI	National Center for Biotechnology Information
ND	Not determined
ng	Nanogram
nm	Nanometer
nM	Nanomolar
nt	Nucleotide
OD	Oil dispersion
OD	Optical density
OP	Organophosphate
ORF	Open reading frame
P450(s)	Cytochrome P450-dependent monooxygenase(s)
PBO	Piperonyl butoxide
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
PVDF	Polyvinylidene fluoride
RFU	Relative fluorescence units

RH	Relative humidity
RNA	Ribonucleic acid
RNA-Seq	RNA-sequencing
rpm	Revolutions per minute
RPS3A	Ribosomal protein S3A
RR	Resistance ratio
RT	Room temperature
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
RyR	Ryanodine receptor
s	Seconds
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SEM	Standard error of the mean
SF	Synergistic factor
skdr	Super knockdown resistance
SNP(s)	Single nucleotide polymorphism(s)
SR	Synergistic ratio
Ta	annealing temperature
TMD	Transmembrane domains
Tpi	Triosephosphate isomerase
UCCR	Ubiquinol-cytochrome C-reductase
UGT	Uridine diphosphate-glucuronosyltransferase
VGSC	Voltage-gated sodium channel
Vip	Vegetative insecticidal protein
vs	Versus
λem	Emission wavelength
λex	Excitation wavelength

Chapter 1

Introduction

Today, modern agriculture faces an enormous challenge – ensuring that enough high-quality food is available to meet the needs of an ever-growing population. The United Nations prognosis suggests an increase to as many as 9.7 billion people by the year 2050 (United Nations, 2019). Beyond that, losses of agricultural land, climate change, and shift in the dietary patterns will require significant enhancements to be made in agricultural productivity (Godfray et al., 2010). Considering that approximately 30 % of yield losses are projected to be attributable to crop protection, the use of crop protection products can contribute to a major rise in food production. Currently, such losses are in the range of 14 % attributable to competition by weeds, 13 % due to fungal pathogens, and 15 % by insect damage (Chrispeels and Sadava, 2003; Jeschke et al., 2018; Oerke, 2006).

Synthetic pesticides against crop pests, pathogens, and weeds allowed for a great improvement in this regard. Moreover, a recent revolution in agriculture was the introduction of genetically modified (GM) crops tolerant to drought and resistant to insects and herbicides. The various benefits brought by those new technologies have driven rapid adoption of GM crops, reaching in 2018 191.7 million ha planted worldwide, an increase of ~113-fold since the first GM crop commercialization in 1996 (“ISAAA,” 2018). Genes from the bacterium *Bacillus thuringiensis* (Bt) encoding proteins with insecticidal activity, were inserted in plants’ genome guaranteeing the protection against key insect pests during the vegetative growth phase (Qaim, 2016). GM maize, for example, brought an economic benefit to farmers of U$ 98.2 billion, an increase in 195 million tons of productivity, and a reduction of 45.2 % (50 million kg) of insecticide applications between 1996 and 2011 (Brookes and Barfoot, 2013).

Together rice, wheat, and maize account for at least 30 % of the food calories of more than 4.5 billion people in developing countries, and the demand for maize is projected to double by 2050. However, though consumption is expected to increase, yields are expected to decline (“CGIAR,” 2020; Rosegrant et al., 2009). Maize production is affected by biotic and abiotic constraints in (sub)tropical regions. Savary et al. (2019) estimate a yield loss of 22.6 % in maize worldwide due to pests and pathogens. Among many defoliator pests, the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is considered the major maize pest in Brazil (Ashley et al., 1989). The yield losses can reach up to 34 %, estimated at U$ 400 million annually, if no control measure is taken (Figueiredo et al., 2005; Lima et al., 2010). More recently, FAW has invaded the Eastern hemisphere, threatening not only maize but also other important staple food, like rice in Asian countries. Moreover, FAW appeared to

be at the top rank of emerging pests causing significant losses in maize yield worldwide (Savary et al., 2019).

Taken the example of a pest of high economic importance such as FAW, research and development of crop protection compounds and insect-resistant traits remain the most effective method for combating losses in agricultural yields (Jeschke et al., 2018). However, the production of new compounds targeting new receptors or displaying novel modes of action in modern agriculture is very demanding, taking an average of 11.3 years and costing about 286 million dollars (Sparks and Lorsbach, 2017). Therefore, strategic life-cycle management of compounds already present in the market is essential, including insecticide resistance management (IRM) strategies. Best agricultural practices to control FAW may be established regionally as supported by international organizations (Insecticide Resistance Action Committee (IRAC) and Food and Organization of The United Nations (FAO)), considering economic and social aspects. The value of integrated pest management (IPM) is also part of future demands and involves chemical, biological, physical, and more recent biotechnological tactics. Rather than the intensive use of synthetic insecticides or the high adoption of Bt crops, which can lead to high selection pressure and development of resistance (Heckel, 2012). A better understanding of mechanisms underpinning resistance to insecticides and Bt traits described in the following chapters can currently provide practical support for more sustainable and efficient control of a global pest such as FAW.

1.1 *Spodoptera frugiperda*, fall armyworm (FAW)

Spodoptera (Lepidoptera: Noctuidae) is a genus composed of 30 species, of which half has the pest status (Pogue, 2002). Among those species, *S. frugiperda* (J.E. Smith), commonly known as fall armyworm (FAW), has gained recently more attention worldwide. The FAW originates from (sub)tropic regions of the American continent and is a major pest of important crops such as maize, soybean, rice, and cotton (Luginbill, 1928; Pogue, 2002). Lately, *S. frugiperda* distribution has been globally broadened. In January 2016, FAW was first reported in São Tomé, Bénin, Togo, and Nigeria (Goergen et al., 2016) and after two years it was recorded in almost all African countries (“FAO,” 2018). In January 2018, this pest has reached Southeast Asia (Shylesha et al., 2018) and early 2020 Australia (“IPPC,” 2020), totalizing its presence in 107 countries worldwide (“EPPO,” 2020). The late identification has been pointed as the main factor of delayed control and fast spread of this species in African countries, as some native *Spodoptera* spp and other noctuid defoliators could easily lead to misidentification (Prasanna et al., 2018). *S. frugiperda* can be morphologically identified from other armyworms by the presence of a typical inverted “Y” in the head, four quadrangular spots on the

penultimate dorsal segment, and typical stripes on the lateral part of the body in the larval stage (Figure 1A) (Prasanna et al., 2018).

The wide and successful establishment of this pest can be attributed to a combination of some biological components (Barros et al., 2010). This pest is highly polyphagous, most recently reported to feed on at least 186 plant species, belonging to 76 families in Brazil (Montezano et al., 2018). Nevertheless, *S. frugiperda* can be genetically divided into two different strains: the rice- and corn-strain according to its host preference (Pashley et al., 1987). Recently, populations collected from corn were composed primarily of corn-strain individuals, with a varied low percentage of rice-strain individuals (Machado et al., 2008; Nagoshi et al., 2007; Silva-Brandão et al., 2018). This event is more likely to happen, in an agronomic system as in Brazil, where natural biomes and agricultural land are connected for extended areas forming what is so-called “green bridges” for connection of insect pests such as *S. frugiperda* (Favetti et al., 2017).

The larvae are feeding mainly on leaves or sometimes fruits and roots (Pogue, 2002) (Figure 1D), potentially causing yield losses of 40 % to 72 % in maize in the American continent (Mura et al., 2006; Wyckhuys and O’Neil, 2006). It is estimated that in just 12 African countries, the yield loss of maize due to FAW damage can reach 8.3 to 20.6 million tons per year if no control measure is taken (Day et al., 2017). In Brazil alone, the cost to control FAW on maize is more than 600 million dollars per year (Ferreira Filho et al., 2010).

The adult moths feed on nutritious liquids, such as nectar and honeydew (Pogue, 2002). Males (Figure 1B) morphologically differ from females (Figure 1C) by the mosaic color of the scales and also by their sexual apparatus. FAW is not able to enter diapause and does not survive at low temperatures, therefore adults are migrating from colder regions to tropics every year in the USA (Barfield et al., 1980). The adults can fly long distances, reaching up to 100 km in one night (Luginbill, 1928). The females are laying the egg masses (100-200 eggs) normally in the upper part of the leaf (Nalim, 1991). The first instar larvae will hatch after two to three days and they have a typical behavior called ballooning. The neonates produce a silk line that can facilitate their dispersion by the wind from one plant to another. In total, the larvae will pass through six instars, but from the third instar on, the larvae enter the inner part of the plant and cover its body with feces (Figure 1D), creating a physical barrier for natural predators and chemical control (Luginbill, 1928). The larvae stage can vary from 15 to 25 days, depending on the environmental conditions. Once the last instar is reached, the larvae migrate to the soil where the pupation occurs and, after five to eight days, the adult will emerge to complete the metamorphosis (Luginbill, 1928). In tropical regions, FAW can have from eight to eleven generations per year (Busato et al., 2005).

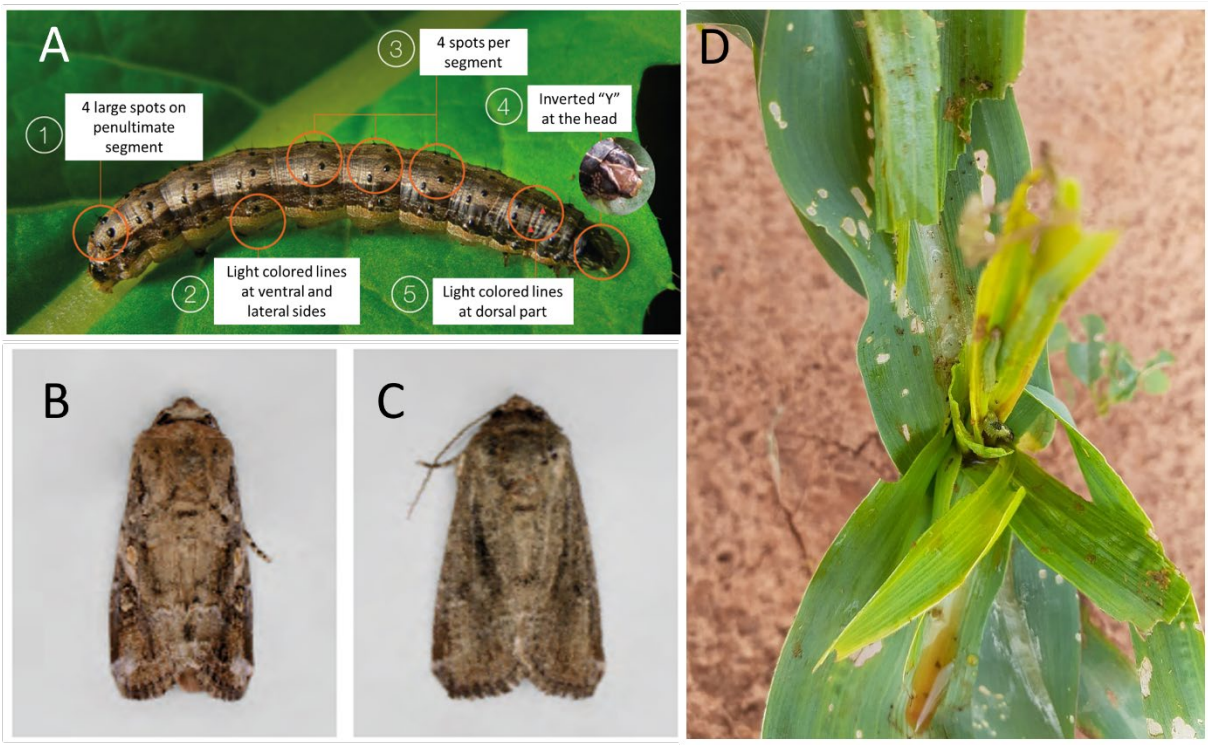

Figure 1 Morphological features used to identify (A) larvae, (B) male, and (C) female of fall armyworm, *Spodoptera frugiperda*. Photos adapted from "Bayer" (2017). (D) Damage caused by *S. frugiperda* feeding in maize plants (Source: personal image).

1.2 FAW control focused on chemical and biotechnological measures

The use of synthetic insecticides in agriculture has contributed to a major rise in food production and the global insecticide sales market was estimated at 19 million dollars in 2018 (Sparks et al., 2020). Among the insect pests, Lepidoptera is economically the most important insect order worldwide (Peters, 1988).

The FAW is not a new species to science; it has been a herbivorous pest for a century and historically its damage has been mitigated by using broad-spectrum synthetic insecticides (Gordy et al., 2015). Early synthesized insecticides have a much broader range to different insect orders, whereas the more recent chemical groups tend to have a narrower and more specific effect, improved ecotoxicological profile towards non-target organisms, decreased environmental persistence, requiring lower use-rates (higher activity), and also reduced human toxicity (Timothy T. Iyaniwura, 1991).

Currently, in Brazil 185 chemical compounds are registered to control *S. frugiperda* ("Agrofit," 2020). However, about 92 are neuroactive insecticides, pyrethroids, and organophosphates, targeting the voltage-gated sodium channel (VGSC) and acetylcholinesterase (AChE), respectively. Some other chemical classes are also available targeting the nicotinic

acetylcholine receptor (nAChR), the GABA-gated chloride channel, and ryanodine receptors (RyR) (Table 1).

Additionally, varieties of maize, cotton, and soybean are commercially available in Brazil since 2008 expressing different single pore-forming proteins or pyramid products (those containing more than one Bt protein) can provide some extent of protection to FAW damages (Table 2) (Fatoretto et al., 2017).

Table 1 Major insecticide modes of action and chemical classes commercialized globally for *S. frugiperda* control (registrations and availability of individual modes of action or chemical classes may differ regionally). Information adapted from "IRAC," (2020).

Chemical class	**Example**	**Mode of Action (MoA)**	**IRAC classification**
Carbamate	thiodicarb	AChE[1] inhibitor	1A
Organophosphate	chlorpyrifos	AChE[1] inhibitor	1B
Pyrethroids	deltamethrin	Sodium channel modulator	3A
Spinosyns	spinosad	nAChR[2] allosteric modulator	5
Avermectins	emamectin benzoate	GluCl[3] allosteric modulator	6
Bacillus thuringiensis	Cry1F	Microbial disruptor of insect gut membrane	11A
Pyrroles	chlorfenapyr	Uncoupler of oxidative phosphorylation via disruption of proton gradient	13
Benzoylureas	triflumuron	Inhibitors of the chitin synthase type O	15
Oxadiazines	indoxacarb	VGSC[4] blocker	22
Diamides	flubendiamide	RyR[5] modulators	28

[1]AChE: acetylcholinesterase; [2] nAChR: nicotinic acetylcholine receptor; [3]GluCl: glutamate-gated chloride, [4]VGSC: voltage gated-sodium channel; [5]RyR: ryanodine receptor

Table 2 Presence (in gray color) of toxins from *B. thuringiensis* expressed in different crops (maize, cotton, and soybean) in Brazil. Table modified from Fatoretto et al. (2017).

Trait	Maize	Cotton	Soybean
Cry1Ac			
Cry1Ab			
Cry1F			
Cry1Ab+Cry2Ae			
Cry1A.105+Cry2Ab2			
Cry1Ab + Cry1F			
Cry1A.105+Cry2Ab2 +Cry1F			
Cry1Ac+Cry2Ab2			
Cry1Ac+Cry1F			
Cry1Ab+Cry2Ae			
Vip3Aa19			
Vip3Aa20			

1.2.1 Pyrethroids and the oxadiazine indoxacarb

Synthetic pyrethroid insecticides are structurally derived from natural pyrethrin isolated from the flower of *Pyrethrum* (*Chrysanthemum*) genus. Improvement of natural pyrethroids resulted in photostability, high effectiveness, and residual activity, allowing effective use under field conditions (Casida, 1980; Elliott et al., 1978). As the mechanism of action, pyrethroids disrupt nerve function by altering the rapid kinetic transitions between conducting (open) and nonconducting (closed or inactivated) states of VGSC, which trigger the generation of nerve action potentials (Soderlund, 2012) (Figure 2). The pyrethroids are belonging to group 3A, according to the IRAC classification scheme. They are classified in type I (Figure 3A), for compounds lacking the *alpha*-cyano-substituent (e.g. pyrethrin I, resmethrin, and permethrin), and type II (Figure 3B) compounds, which contain the *alpha*-cyano-substituent (e.g. deltamethrin and *lambda*-cyhalothrin) (Casida, 1980; Soderlund, 2012; Sparks and Nauen, 2015).

Pyrethroids are broad-spectrum insecticides and extremely lipophilic, therefore present excellent contact and no systemic activity (Elliott et al., 1978). Although present in the market for more than 40 years, pyrethroids sales still represent 15 % of the market share (Sparks et al., 2020). Pyrethroids are acting in different developmental stages of lepidopteran pests (adult, larvae, and egg) (Elliott et al., 1978) and they are acting quite fast on insects, known as “knock-down” symptoms.

Indoxacarb belongs to the chemical class of oxadiazines (IRAC Group 22) with high activity against a number of lepidopterans (including *S. frugiperda*), as well as certain homopteran, and coleopteran pests (Wing et al., 2000). Indoxacarb is a pro-insecticide, which is rapidly

bioactivated by an amidase or carboxylesterase by removing a carbomethoxy group from the amide nitrogen to form a more active metabolite, called DCJW (Figure 3C) (Wing et al., 2000, 1998).

The derivate DCJW is acting on the inactivated state of the sodium channel by shifting the voltage dependence of inactivation to more hyperpolarized potentials (slow inactivation or a combination of slow and fast inactivation) (Song et al., 2006) (Figure 2), causing cessation of feeding, poor coordination, paralysis, and death (Wing et al., 2000).

The intrinsic activity of indoxacarb is determined by the bioactivation rate, which is 2.5-fold higher when administered orally in comparison to topical contact in *S. frugiperda* (Wing et al., 1998). Resistance to pyrethroid insecticides has been reported to FAW in Brazil, Mexico, the USA, and Puerto Rico (Carvalho et al., 2013a; Gutiérrez-Moreno et al., 2019; Leon-Garcia et al., 2012; Yu, 1992). Since indoxacarb acts on a binding site different from pyrethroids, no cross-resistance between these classes has been found. VGSC blockers share 2 % of the insecticide market, corresponding to 277 million dollars (Sparks and Nauen, 2015).

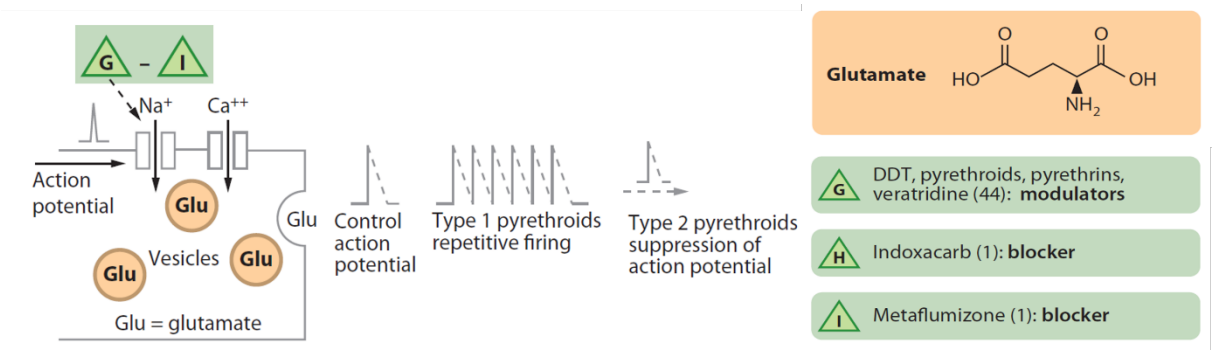

Figure 2 Target site of insecticides acting on the voltage-gated sodium channel (presynaptic nerve terminal) and neuroactive action of pyrethroids. Adapted from Casida and Durkin, (2013).

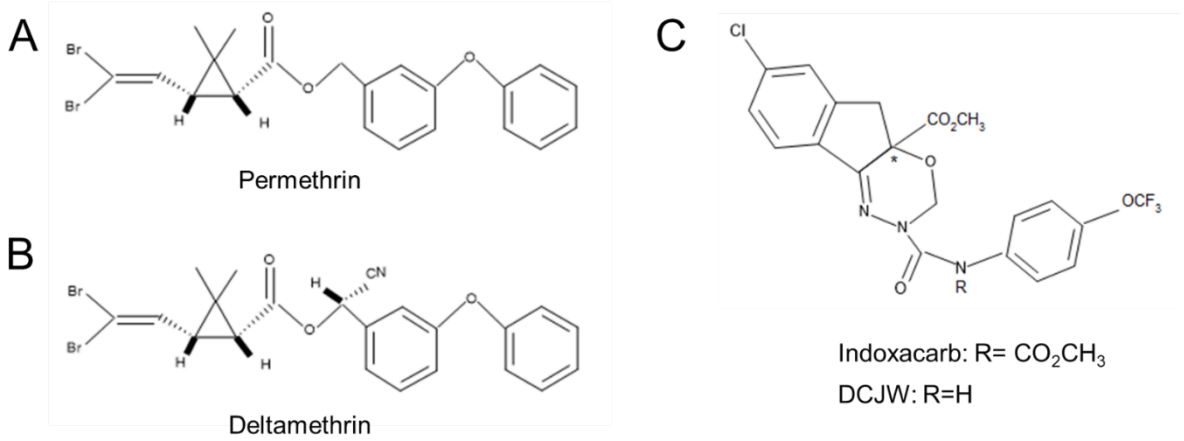

Figure 3 Insecticides targeting the voltage-gated sodium channel. (A) Type I pyrethroid permethrin; (B) Type II pyrethroid deltamethrin; (C) oxadiazine indoxacarb and its active form.

1.2.2 Organophosphates and carbamates

Organophosphates (OPs) and carbamates were introduced to the market over 60 years ago and have a broad spectrum of activity against different insect orders, which make them also toxic to non-target organisms (Nauen and Bretschneider, 2002). Both chemical compounds are classified in Group 1 according to IRAC, acting on the insect nervous system by irreversibly inhibiting acetylcholinesterase (AChE) which hydrolyses the neurotransmitter acetylcholine (Fournier and Mutero, 1994) (Figure 4).

Inhibition of the AChE leads to an accumulation of acetylcholine in the synaptic cleft and consequently a hyperexcitation of the post-synaptic acetylcholine receptors leading to tremors, paralysis, exhaustion, and death (Gunning and Moores, 2001). Among the 165 OPs available in the global market (Sparks et al., 2020), chlorpyrifos is an example of a phosphorothioate ester pro-insecticide that is bioactivated to a respective P=O derivate (Fukuto, 1990) (Figure 5). Carbamate insecticides are currently represented by 43 active ingredients and thiodicarb is an example used in soybean seed treatment to control FAW (Figure 6) (Sparks et al., 2020; Triboni et al., 2019). Organophosphates and carbamates represent together 11 % of the insecticide market (Sparks et al., 2020)

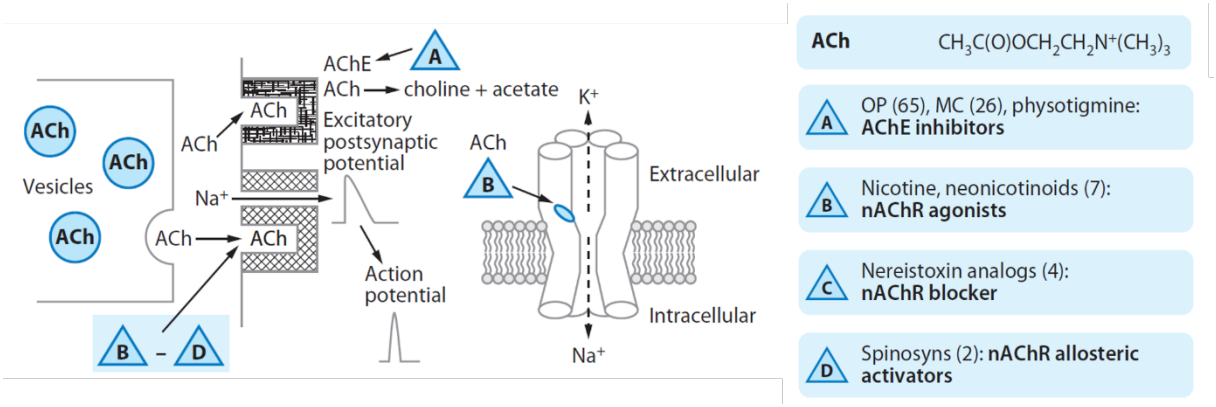

Figure 4 Neuroactive action of acetylcholinesterase inhibitors and nicotinic acetylcholine receptor agonists. Figure adapted from Casida and Durkin (2013).

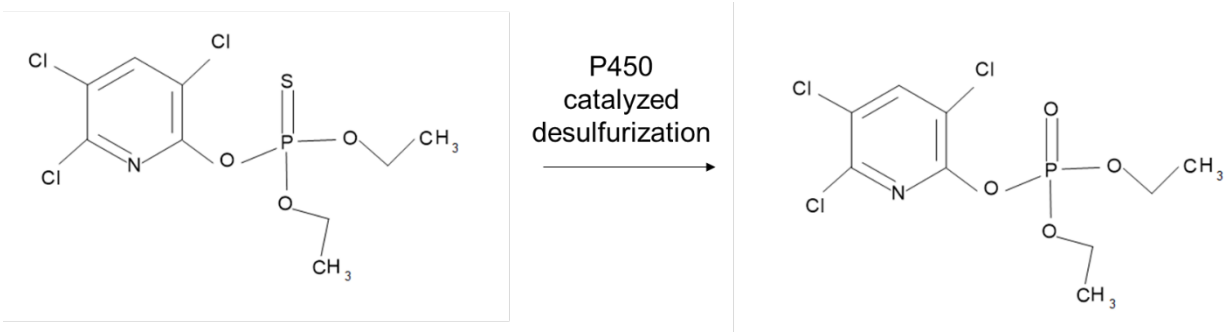

Figure 5 Chemical structure of chlorpyrifos and chlorpyrifos oxon (active molecule) after bioactivation by P450 enzymes.

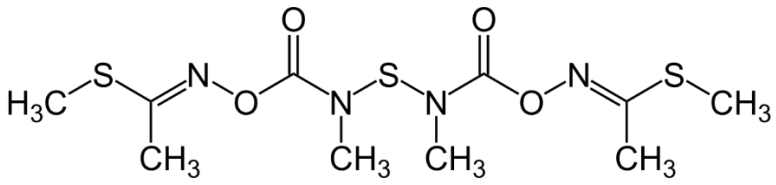

Figure 6 Chemical structure of carbamate thiodicarb.

1.2.3 Benzoylureas

The benzoylureas (BPU) were discovered after the fusion of two herbicides, which resulted in a compound with high insecticide activity in the early 1970s (Sun et al., 2015). The BPUs are classified in IRAC Group 15 as inhibitors of chitin synthase 1, by inhibiting the incorporation of N-acetyl-glucosamine into insect chitin (Merzendorfer, 2013, 2006). This chemistry is widely used in IPM due to its favorable environmental properties and low acute toxicology to mammals and non-target insects (Sun et al., 2015). BPUs have good larval activity with the most recent

compounds acting on a broad spectrum of insects (Lepidoptera, Homoptera, Diptera, and Hemiptera pests). They are really active insecticides but as insect growth regulators, some days after insecticide contact are required until the first symptoms appear (Matsumura, 2010). The use of BPU has increased significantly in the past years in the control of FAW, although resistance to lufenuron (Figure 7) has been reported (Nascimento et al., 2016).

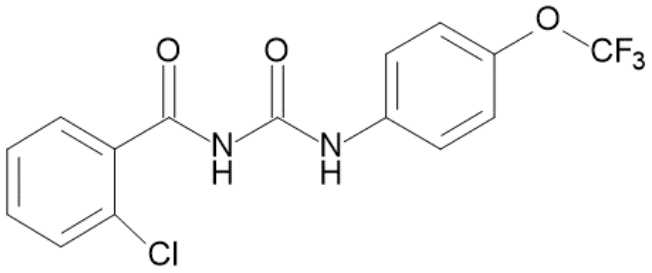

Figure 7 Chemical structure of the benzoylurea triflumuron.

1.2.4 Avermectins

Avermectins are natural products (macrocyclic lactones) produced by the soil actinomycete *Streptomyces avermitilis* with excellent acaricidal and less insecticidal properties (Argentine et al., 2002; Nauen and Bretschneider, 2002). Macrocyclic lactones are produced by large-scale fermentation of the bacterial strains synthesizing these compounds naturally, resulting in a high price product (Jansson et al., 1997). The replacement by a methylamino group in the hydroxy-group in the terminal sugar ring of avermectin originates the derivate emamectin (Figure 8) which has an excellent lepidopteran activity (Argentine et al., 2002; Nauen and Bretschneider, 2002). Emamectin (benzoate), belongs to Group 6 of the IRAC MoA classification scheme, acting on the insect nervous system as an agonist of GABA and glutamate-gated chloride channels. The binding results in strong chloride ion influx into the cells followed by disruption of nerve impulses, paralysis, and finally death (Nauen and Bretschneider, 2002) (Figure 8). Avermectins represent currently 8 % of the global insecticide market share (Sparks et al., 2020).

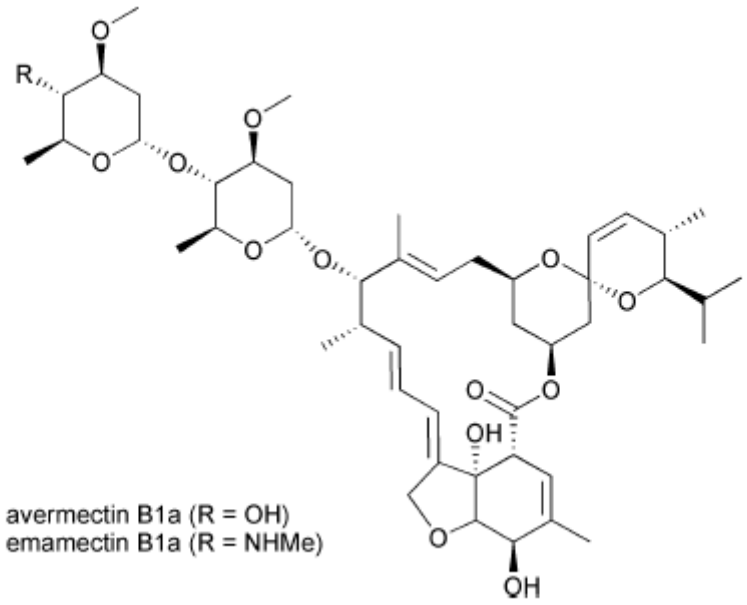

Figure 8 Structure of avermectin and emamectin. Adapted from Nauen and Bretschneider (2002).

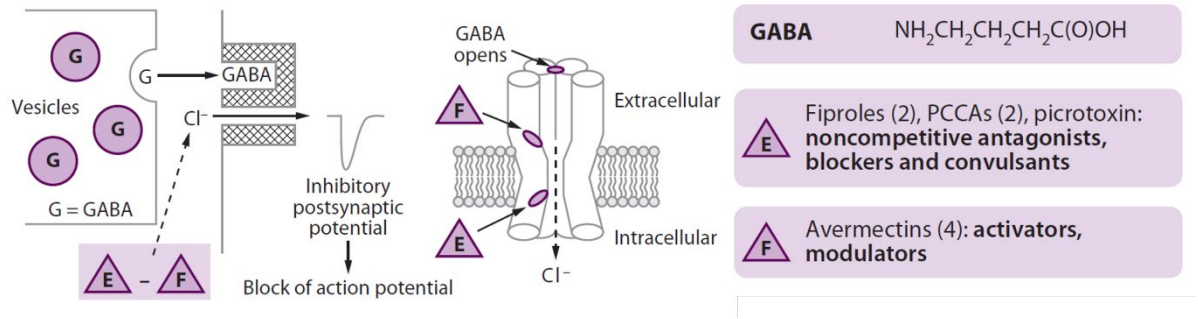

Figure 9 GABA-gated chloride channel and insecticide activity. Adapted from Casida and Durkin (2013).

1.2.5 Spinosyns

Spinosad is composed of a mixture of two macrocyclic lactones, spinosyn A (85 %) and spinosyn D (15 %), derived from the actinobacteria *Saccharopolyspora spinosa*. This mixture is particularly effective against pests in the lepidopteran family Noctuidae (Nauen and Bretschneider, 2002). Spinosyns (IRAC, Group 5) are acting in the insect nervous system by allosterically modulating nicotinic acetylcholine receptors (nAChRs) and prolongation of acetylcholine responses (Thompson and Hutchins, 1999; Nauen and Bretschneider, 2002)

(Figure 4). Spinosyns act on a different site than neonicotinoids on the nAChR. Spinosad and spinetoram (Figure 10) are currently used in the control of *S. frugiperda*, however, resistance has been reported for both compounds (Lira et al., 2020; Okuma et al., 2017).

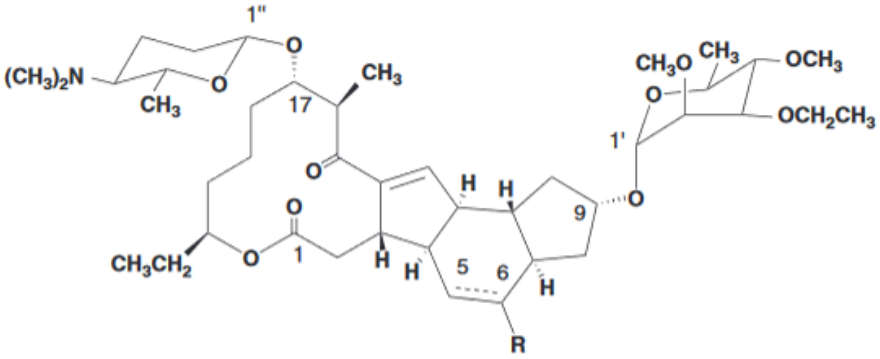

Figure 10 Structure of spinetoram. Adapted from Kirst (2010).

1.2.6 Pyrrole - Chlorfenapyr

Chlorfenapyr (IRAC Group 13) is structurally derived from dioxapyrrolomycin, a natural product isolated from the actinobacteria *Streptomyces fumanus* (Treacy et al., 1994). Chlorfenapyr is a pro-insecticide which is activated metabolically by *N*-dealkylation i.e. oxidative removal of the *N*-ethoxymethyl group (Figure 11). Once converted to its active form, chlorfenapyr uncouples the oxidative phosphorylation and the pyrrole disrupts the proton gradient across the mitochondrial membrane. Thus, the vital energy-production process that converts ADP to ATP is inhibited and finally leading to cell and organism death (Nauen and Bretschneider, 2002; Treacy et al., 1994). Chlorfenapyr is active against larvae and adults of a broad range of pest species and mites (Lepidoptera, Coleoptera, Thysanoptera, Isoptera, Orthoptera, Hymenoptera, and Acarina) (Hunt and Treacy, 1998; N'Guessan et al., 2007).

The uptake of chlorfenapyr is mainly by ingestion and, secondarily, by contact. Owing to its unique mode of action, chlorfenapyr can control pests that are resistant to other insecticide chemical classes, and no instances of target site cross-resistance have been observed (Kanno et al., 2019; N'Guessan et al., 2007). Unfortunately, although chlorfenapyr exhibits a good translaminar movement in plants, it has a very limited systemic and/or ovicidal activity (N'Guessan et al., 2007).

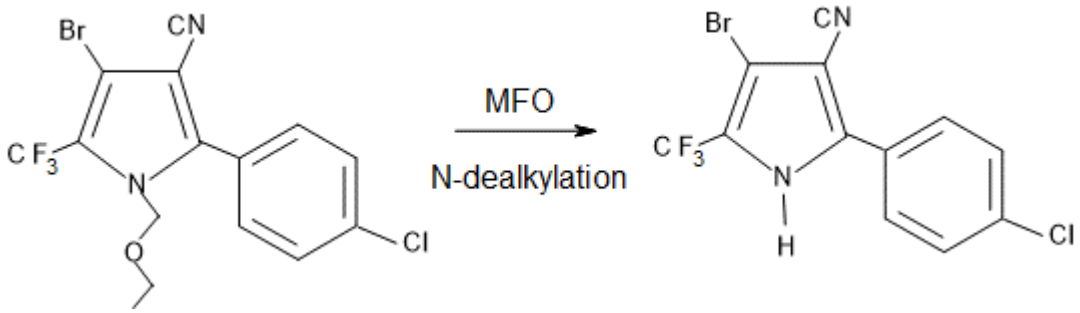

Figure 11 Chemical structure of chlorfenapyr (left) and its activation by mixed-function oxidases.

1.2.7 Diamides

For many decades the plant-derived alkaloid ryanodine is known for acting on the ryanodine receptor (RyR) and exhibits insecticidal activity, however with low activity under field conditions (Jefferies et al., 1997). Diamides (IRAC, Group 28) are the newest major class of insecticides and are divided as phthalic acid diamides such as flubendiamide (Figure 12A) (Ebbinghaus-Kintscher et al., 2007, 2006) and anthranilic diamides such as chlorantraniliprole (Figure 12B) (Cordova et al., 2006; Kambrekar et al., 2017; Lahm et al., 2007, 2005). Diamides are acting on the RyR, which is a large (homo)tetrameric calcium channel located in the sarco- and endoplasmic reticulum in neuromuscular tissues (Cordova et al., 2006; Ebbinghaus-Kintscher et al., 2006; Sattelle et al., 2008). By binding at the RyR, diamides cause calcium release and the depletion of internal calcium stores which leads to uncontrolled muscle contraction, paralysis, and eventually death (Cordova et al., 2006; Ebbinghaus-Kintscher et al., 2006; Tohnishi et al., 2005) (Figure 13).

Currently, diamides represent approximately 12 % of the insecticide market, with a global turnover of > 2.3 billion dollars (Sparks et al., 2020). Diamides, in general, are primarily acting on lepidopteran pests, however, chlorantraniliprole has also activity against coleopteran and cyantraniliprole against sucking pests like aphids and whiteflies (Foster et al., 2012; Grávalos et al., 2015). This chemical class in general has a good toxicological profile to mammals and beneficial insects in many crop settings (Nauen and Steinbach, 2016).

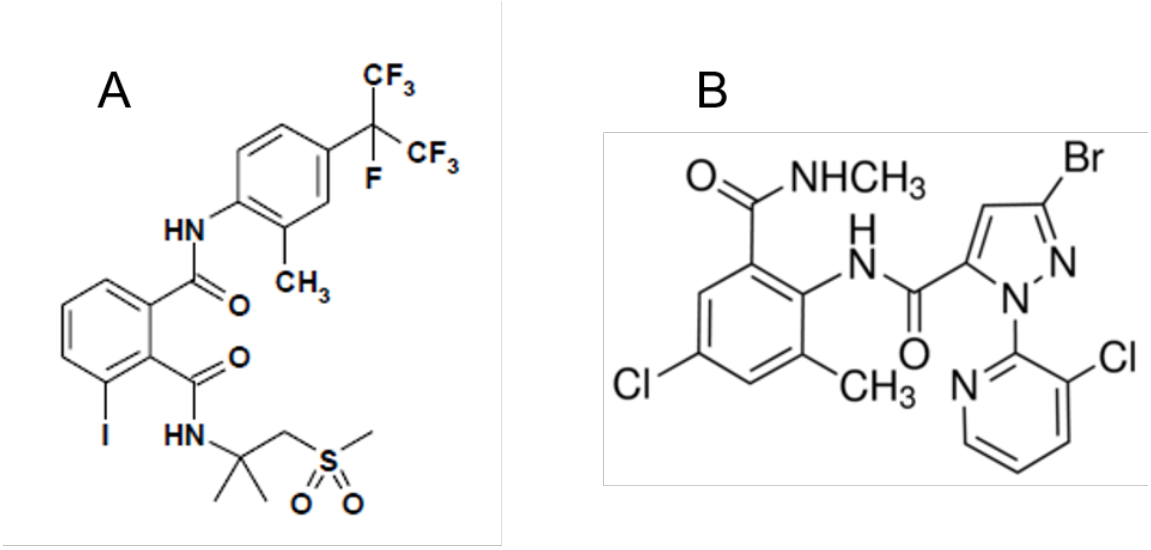

Figure 12 Chemical structures of the diamide (A) flubendiamide, adapted from Lahm et al. (2005), and (B) chlorantraniliprole.

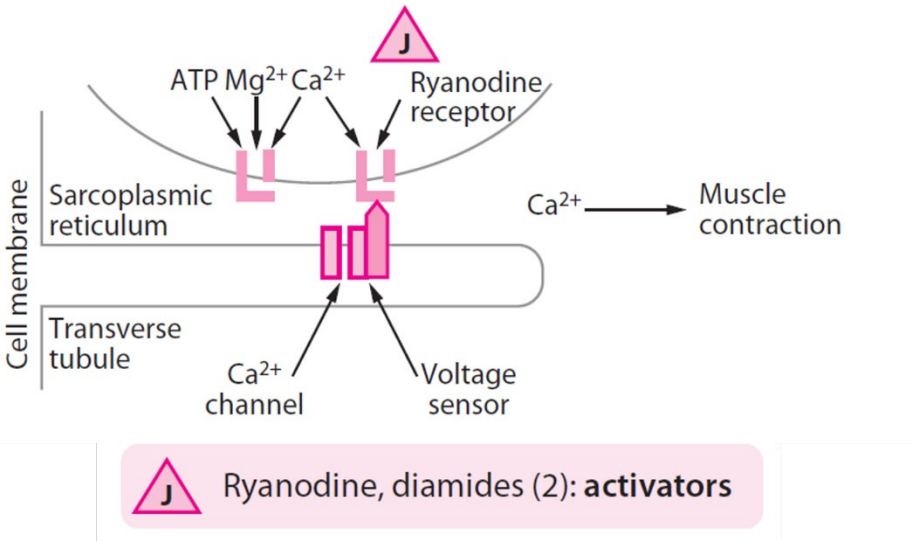

Figure 13 Diamide action on ryanodine receptors present in endo/sarcoplasmic reticulum of nerve and muscle cells. Adapted from Casida and Durkin (2013).

1.2.8 Insecticidal proteins from *Bacillus thuringiensis* (Bt)

The discovery of soil bacteria *B. thuringiensis* Berliner (Bt) proteins with insecticidal activity has led to the development of one of the oldest sprayable biological insecticides, and currently, it is still used as the newest application in insect-resistant GM plants (Bravo et al., 2011). Bt insecticidal proteins have many important properties that make them highly attractive for insect

control, such as low environmental impact, high specificity, and safe to humans (Mendelsohn et al., 2003).

During sporulation, *B. thuringiensis* produces inclusion bodies containing crystalline (Cry) and cytolytic (Cyt) proteins (Estruch et al., 1996). Today over 700 Cry proteins have been identified (Crickmore et al., 2020) and classified into 74 Cry classes based on protein sequence homology (Palma et al., 2014). Although there is no simple correlation between sequence and insecticidal spectrum, Cry1, and Cry9 proteins are active on lepidopteran larvae, whereas Cry3, Cry7, and Cry8 are active on coleopteran larvae (Palma et al., 2014). At the vegetative stage, proteins known as Vip are secreted into the nutrient growth medium. Vip1 and Vip2 proteins are specific for Coleoptera, whereas Vip3 proteins are specific for lepidopteran insects (Estruch et al., 1996).

The specificity of Bt pore-forming proteins remains one of the most intriguing aspects, as any step in the mode of action can influence their intrinsic activity, from proteolytic protoxin activation to interactions with gut receptors (de Maagd et al., 2001; Haider et al., 1986).

Different models have been proposed for the Bt mode of action. Nevertheless, all these models have in common that the toxin interacts with membrane-bound receptors (Vachon et al., 2012). The “classical model” described by Bravo et al. (2007) (Figure 14) is the oldest to explain the Cry toxin mode of action, yet the least resolved. As currently understood, there are several steps involved in the mode of action of Cry pore-forming proteins after ingestion by insect larvae. The crystalline protein is solubilized in the insect midgut, releasing a protoxin (Cry1 toxins, around 135 kDa). The protoxin is activated by the alkaline pH and digestive proteases to a protease-resistant core (~65 kDa) (de Maagd et al., 2001). The toxin binds to membrane-bound proteins on the surface of the midgut epithelial cells. Eventually monomers of the toxin form oligomers, either in solution or after having inserted into the lipid bilayer. Membrane-spanning *alpha*-helix hairpins of the oligomers create a small pore (0.5–1.0 nm) in the membrane. These pores enable cations and water to flow into the cell, possibly through aquaporins, causing the cells to swell and lyse. This is the so-called “colloid-osmotic lysis” mechanism (Knowles and Ellar, 1987). Minor damage might be cured by the insect, but major damage destroys the midgut epithelium, resulting in rapid cessation of feeding and eventual death after a few days (Broderick et al., 2006).

The discovery of specific “receptors” to which Cry toxins bind in insect midgut membranes was a major advance. Fitting this hypothesis into the “sequential binding” model (Bravo et al., 2011), the binding steps are presumed to occur in a specific order. Toxin monomers bind to aminopeptidases-N (APNs) (Garczynski and Adang, 1995; Zhang et al., 2009), alkaline phosphatases (ALP) (Guo et al., 2015; Perera et al., 2009), polycalins (Hossain et al., 2004), glycoconjugates (Valaitis et al., 2001), and other proteins, either to amino acid residues or glycosyl groups (Jurat-Fuentes and Adang, 2006). This reversible binding increases the toxin

concentration at the membrane surface. Then toxin monomers bind sequentially to the cadherin (CAD), accelerating the cleavage of the N-terminal *alpha*1-helix which enables oligomer “pre-pore” formation in solution (Gómez et al., 2002; Xie et al., 2005). Finally, oligomeric “pre-pore” structures will be inserted into the membrane through an irreversible binding to ATP-binding cassette (ABC) transporters (Heckel, 2012).

An alternative model has been proposed in which interaction of monomeric Cry toxin with a cadherin receptor, activates an Mg^{2+}-dependent protein kinase A signaling pathway that leads to cell death (Ibrahim et al., 2010; Zhang et al., 2006, 2005).

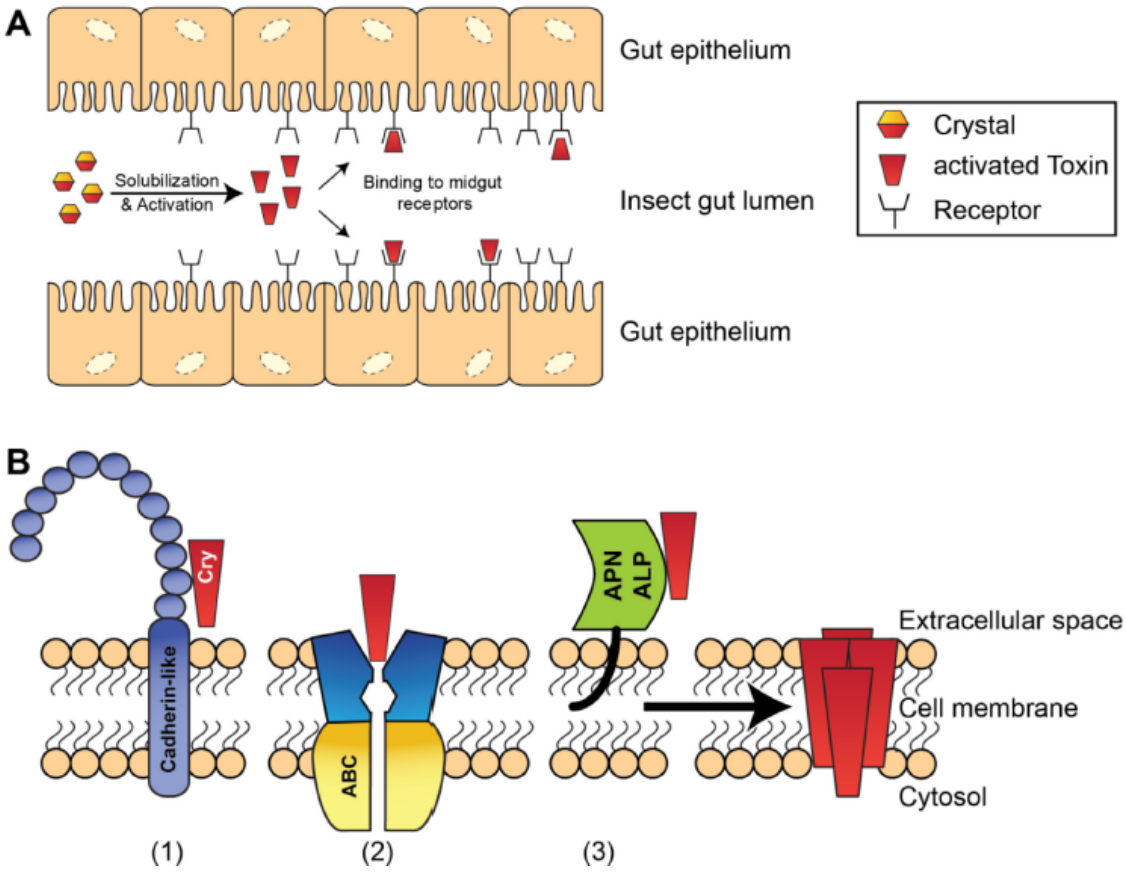

Figure 14 (A) Scheme of *Bacillus thuringiensis* Cry1A mode of action and (B) binding to gut receptors as (1) cadherin, (2) ATP-binding cassette (ABC), (3) alkaline phosphatase (ALP) and aminopeptidase-N (APN). (Source: Bretschneider (2016)).

1.3 Resistance to synthetic insecticides

Resistance is defined as the inherited ability of some organism to survive doses of a toxicant that would kill the majority of individuals in a normal population of the same species (WHO, 1957). Resistance development is usually a consequence of natural selection; an insecticide

prevents susceptible individual insects from reproducing leaving only those individuals carrying genes that confer insecticide resistance (“IRAC,” 2016).

The speed by which resistance develops depends on a combination of factors such as type of crop protection; product and its target specificity; application frequency and rate. Important factors related to pest biology also play an important role, such as the rate of reproduction, time of development, and migration ability (Georghiou and Taylor, 1977; Russell, 2001). A good example is FAW, which is currently among the top 15 resistant insect species (Sparks et al., 2020).

Furthermore, the inheritance of resistance can determine the level and speed of resistance evolution. Resistance can be monogenic, when resistance is conferred by a single allele or poly/multigenic when more than one gene is involved. The dominance of resistance is also a major parameter, i.e. if resistance is functionally recessive, the resistance is less likely to evolve rapidly (Bourguet et al., 2000; ffrench-Constant, 2013; Tabashnik and Carrière, 2017). Moreover, resistance alleles can confer fitness cost (i.e. delayed developmental time, change in sex ratio, fecundity rate) or advantage (high resistance ratio in comparison to susceptible strain, conferring higher survivorship). If so, it will determine rather the resistance allele will fix in the population under both natural and insecticide selection. Having access to such data and the overall understanding of mechanisms conferring resistance provide relevant information for building up prediction models for the spread of resistance (Richardson et al., 2020).

Insecticide resistance occurs worldwide in at least 603 insect species and it is a major element considered by the IRAC (Nauen et al., 2019; Sparks et al., 2020) (Figure 15). In Brazil, before the introduction of Bt crops, insecticides were applied during the growing season up to ten times (Cruz 1998) and even more often (up to 28 times) in maize seed production regions, as Puerto Rico (Blanco et al., 2016). As a result of the frequent use of synthetic insecticides and the adoption of biotech crops, no less than 144 cases of insecticide resistance are reported for FAW. Among the 41 different active substances reported, 45 % of the cases correspond to Bt proteins, 26 % are insecticides targeting the VGSC, and 19 % targeting AChE (“APRD,” 2020; “ISAAA,” 2018) (Figure 16).

Insects can develop resistance through different ways, generally classified into four main mechanisms: behavioral changes, reduced penetration or absorption of the toxicant, biochemical detoxification mediated by metabolic enzymes, and finally a reduction in the sensitivity of the target receptors by mutations (Feyereisen, 1995).

Penetration resistance is determined by a slower absorption of the toxicant through the body in resistant insects in comparison to susceptible ones. This resistance mechanism has been frequently associated with changes in the cuticle structure by increasing the expression of cuticular proteins and consequently cuticular thickness (Ahmad and McCaffery, 1999; Balabanidou et al., 2016; Puinean et al., 2010). The behavioral resistance is, for example, the

prevention of contact to a toxicant, as the avoidance of cockroaches *Blatella germanica* to baits (Wada-Katsumata et al., 2013).

Most resistance mechanisms described are based on metabolic resistance due to higher activity of detoxification enzymes and target-site mutations conferring structural changes in the insecticide receptor (Hawkins et al., 2019; Hemingway, 2000; Li et al., 2007). More than one mechanism of resistance can be found within a single individual, which is called multiple resistance, or one mechanism of resistance can confer resistance to a range of compounds, leading to cross-resistance (Brattsten, 1989). A good example is the peach potato aphid (*Myzus persicae*) which has seven different mechanisms of resistance evolved (Bass et al., 2014).

Changes at the genetic level are diverse and include single nucleotide polymorphisms (SNPs), gene amplification, alternative and/or mis-splicing, gene up-regulation, and many more. The changes at the DNA/RNA level will result in physiological changes which will be described in more detail below.

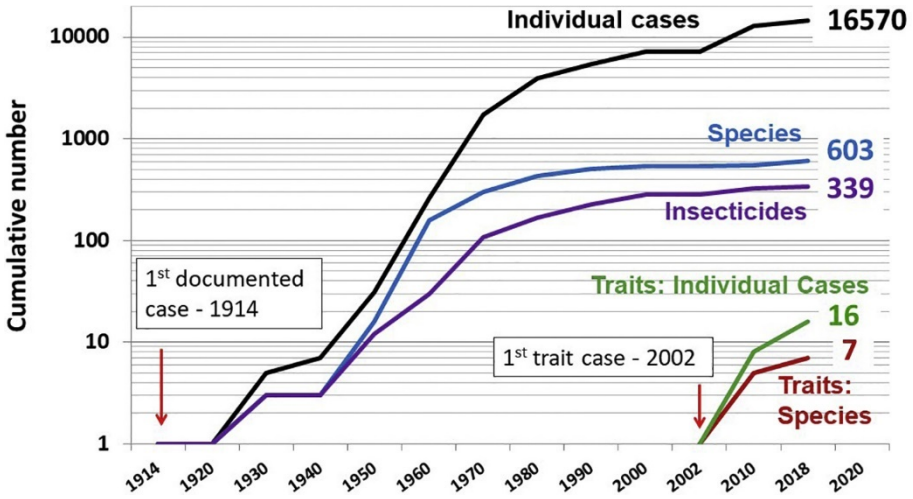

Figure 15 Cumulative number of insecticide resistance shown by individual cases, one or more cases in one species, cases per insecticides registered, and resistance to genetically modified (GM) crops. Adapted from Sparks et al. (2020).

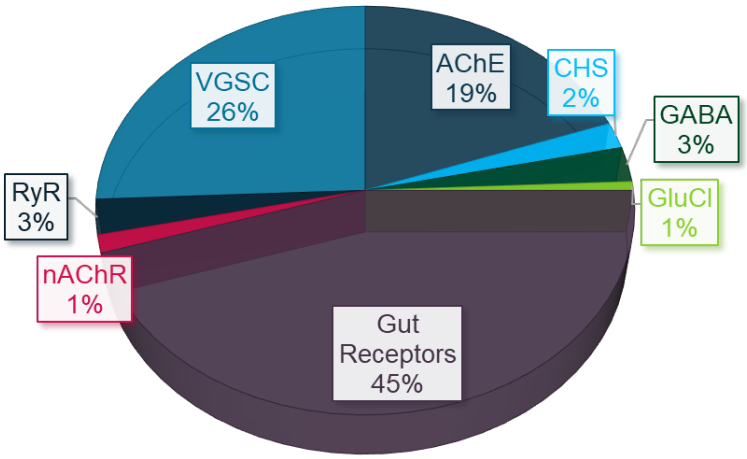

Figure 16 Cases of insecticide resistance in *Spodoptera frugiperda* worldwide according to target-sites of the respective insecticides (Total of 144 cases) (Source: APRD, 2019). VGSC: voltage-gated sodium channel; AChE: acetylcholinesterase; nAChR: nicotinic acetylcholine receptor; GluCl: glutamate-gated chloride channel and RyR: ryanodine receptor.

1.3.1 Metabolism and excretion of xenobiotics

The metabolic resistance is the most common mechanism described and it results in the transformation of toxic compounds to less-toxic, more hydrophilic metabolites to be excreted more readily. The metabolism is mainly based on four enzyme families: microsomal cytochrome P450-dependent monooxygenases (P450s), carboxylesterases (CE), glutathione S-transferases (GSTs), and uridine diphosphate-glucuronosyltransferase (UGTs) (Brattsten, 1989; Li et al., 2018, 2007).

The metabolism of xenobiotics compounds is classified into three phases (I-III). In phase I, P450 and CE enzymes are playing major roles. During this phase, chemical compounds will be modified by introducing hydrophilic functional groups into lipophilic substrates by oxidation and/or hydrolysis of the parent compound, respectively. The metabolites from phase I will be directly excreted or further modified in phase II. Metabolites from phase I or parent compounds will be conjugated to naturally occurring compounds such as sugars, sugar acids, amino acids, or glutathione to produce polar compounds to facilitate excretion. The conjugated metabolites will be actively eliminated in phase III by ABC transporters or other membrane transporters (Yu, 2008).

Phase I metabolism enzymes are also contributing to the activation of pro-insecticides (i.e indoxacarb and chlorfenapyr) (Wing et al., 2000).

To detect possible involvement of metabolic resistance mechanisms, synergism studies with synergists such as the P450 inhibitor piperonyl butoxide (PBO), the esterase-inhibitor S,S,S- tributyl phosphorotrithioate (DEF), and the glutathione depleter diethyl maleate (DEM)

can be performed. However, limitations of the enzyme specificity for synergists might be taken into account (Feyereisen, 2015; Khot et al., 2008). Particularly metabolic resistance is known to cause cross-resistance among insecticide classes, i.e. one enzyme group can have a wide range of substrate specificity (Brattsten, 1989). Metabolic cross-resistance has been described for a number of P450´s, e.g. CYP6CM1 responsible for the detoxification of neonicotinoids and pymetrozine in the whitefly, *Bemisia tabaci* (Nauen et al., 2015, 2013). The increase of enzyme activity due to over-expression limits the availability of the xenobiotic at its target site. The mechanisms underlying this change in expression level are varied, with gene duplication/amplification the most frequently reported (Bass et al., 2013; Feyereisen, 1995; Zimmer et al., 2018). Another mechanism is mediated by regulatory cis- and trans- elements that influence levels of gene expression (Feyereisen, 1995; Grant and Hammock, 1992; Kalsi and Palli, 2015; Yang et al., 2020). Other than the enhanced expression of an enzyme, there can also be mutations present in the enzymes coding sequence that result in increased metabolism of an insecticide (Newcomb et al., 1997). An overview of the main detoxification enzyme families (P450, CE, and GST) and the transport of xenobiotics by ABC transporters, and their respective role in the resistance of synthetic insecticides is given below.

1.3.1.1 Cytochrome P450-dependent monooxygenases

Cytochrome P450 (encoded by *CYP* genes) is one of the largest and most important superfamily of enzymes found in aerobic organisms (Feyereisen, 1999; Werck-Reichhart and Feyereisen, 2000). P450 are heme-containing proteins, named for the absorption maximum at 450 nm of their reduced carbon-monoxide-bound form (Werck-Reichhart and Feyereisen, 2000). Monooxygenases catalyze a variety of oxidative reactions with a diverse range of endogenous (e.g. steroids, hormones, and fatty acids) and exogenous substrates such as pesticides, plant allelochemicals, and drugs (Hodgson, 1983; Scott, 1999). The complex function of P450s is reflected by the high number of P450 genes which in insect genomes is usually around 100 (Nelson, 2013). Giraudo et al. (2015) have identified 42 full P450 coding sequences in *S. frugiperda* and more recently 200 P450 genes were reported in a FAW genome assembly (Liu et al., 2019). This relatively high number can be due to the polyphagous behavior of *S. frugiperda* encountering a wide range of secondary plant metabolites in its diet (Giraudo et al., 2015). Moreover, to support this co-evolution between host plants and detoxification genes, considerable differences in the CYPome were found comparing FAW corn and rice strains (Gouin et al., 2017).

The CYP genes within each insect order are separated into four distinct clades: CYP2, CYP3, CYP4, and the mitochondrial (Feyereisen, 2006). In insects, the genes belonging to the CYP4,

CYP6, CYP9, and CYP12 families are often associated with detoxification of xenobiotics (Scott, 1999).

Insect monooxygenases are found in many tissues, mainly in the fat body, Malpighian tubules, and midgut (Scott, 1999). The insect subcellular distribution of P450 systems particularly revealed activity in microsomes (endoplasmic reticulum-bound), but it has also been described in mitochondria (Feyereisen, 1999; Hodgson, 1983).

As previously mentioned in section 1.3.1, P450s act in phase I of metabolism, by catalyzing chemical reactions through hydroxylation, epoxidation, O-, N- and S-dealkylation, N- and S-oxidations, and others (Feyereisen, 1999).

For the onset of these reactions, the P450s need to receive two electrons, provided by cytochrome P450 reductase (CPR) or/and cytochrome b5 reductase and NADPH as a co-factor for reduction of the P450-substrate complex. Normally, the oxidative step is followed by other alterations (phase II) in which newly formed hydroxyl, carboxyl, or amino groups are conjugated with an endogenous compound already present in the cell. This renders the xenobiotic more water-soluble and thus more easily excretable (Li et al., 2007).

Monooxygenases often confer resistance to different classes of insecticides, including organophosphates, carbamates, pyrethroids, and chitin biosynthesis inhibitors (Bergé et al., 1998; Feyereisen, 1999; Scott, 1999). Changes in the transcriptional level, caused by cis-acting elements such as cap ‘n’ collar C (CncC) and muscle aponeurosis fibromatosis (Maf) transcriptional factors were reported to cause the up-regulation of CYP6BQ genes in a pyrethroid-resistant strain of *Tribolium castaneaum* (Kalsi and Palli, 2015). More recently trans-acting factors were associated with mitogen-activated protein kinase (MAPK) and the overexpression of CYP6CM1 in *B. tabaci* resistant to neonicotinoid insecticides (Yang et al., 2020).

P450-mediated resistance is well described for many lepidopteran pests and it is mostly related to the constitutive overexpression of a particular enzyme, such as the CYP9A12 and CYP9A14 conferring pyrethroid resistance in *Helicoverpa armigera* (Yang et al., 2008).

In *S. frugiperda* several P450s of the 6B, 321A, and 9A subfamilies were induced after feeding on plant allelochemicals while only a few genes, belonging principally to the CYP9A family, responded to insecticides (Giraudo et al., 2015). CYP9A59, for example, was effectively induced and could be involved in the detoxification of methoxyfenozide (Giraudo et al., 2015). Another example is the overexpression of *CYP9* and *CYP6* genes in lufenuron (benzoylurea) resistant FAW strain (Nascimento et al., 2015). However, induction does not necessarily mean resistance to an insecticide or the ability to metabolize it. Therefore, the expression of a particular P450 in heterologous systems such as yeast, bacteria, or cell lines is required to demonstrate the ability of a specific P450 to metabolize a given insecticide (Giraudo et al., 2015).

Many model substrates from the coumarin and resorufin group are widely used for the biochemical detection of monooxygenase activity in insects (e.g. methoxy- and 7-ethoxycoumarin, 7-benzyloxymethoxy resorufin) by measuring fluorometrically monooxygenase activity via O-dearylation or O-deethylation (Bergé et al., 1998).

1.3.1.2 Carboxylesterases

Carboxylesterases (CE) are important hydrolases for a broad spectrum of endogenous and exogenous substances, such as the metabolism of xenobiotics, development regulation, degradation of pheromones, and neurogenesis. Their function varies according to the species, body region, and developmental stage (Durand et al., 2010; Wheelock et al., 2005).

This large family of enzymes can be characterized based on their amino acid sequence identity and substrate specificities (Ishaaya, 2001). Insect CEs have been classified into 33 major clades (Teese et al., 2010) and three major classes (Claudianos et al., 2006). Recent insect genome analysis has revealed 30 genes in the fruit fly *Drosophila melanogaster;* up to 76 in the silkmoth *Bombyx mori* (Yu et al., 2009), and 84 in *S. frugiperda* (Liu et al., 2019). Juvenile hormone esterase is an example of CEs involved in important biological functions (Kamita et al., 2003).

Nevertheless, insect CEs play an important role in the biotransformation and detoxification of exogenous structures like insecticides that have ester, amide, and phosphate bonds, such as pyrethroids, organophosphates, carbamates, and benzoylureas, by hydrolyzation and/or sequestration (Montella et al., 2012). In many insect species, a correlation of higher enzyme activity and resistance to insecticides has been reported (Pasteur and Georghiou, 1989).

In *S. frugiperda*, CEs are known for mediating resistance to pyrethroid, carbamates, and mainly organophosphates by enhanced activity in resistant individuals (Carvalho et al., 2013a; McCord and Yu, 1987; Yu et al., 2003).

A comprehensive study of the Australian sheep blowfly, *Lucilia cuprina*, revealed a mutation (G137D) in the carboxylesterase E3 which gained the ability to hydrolyze the organophosphate diazinon (Newcomb et al., 1997). The total amount of CE produced by an insect can be substantial. In an insecticide-resistant clone of the green peach aphid, *Myzus persicae*, up to 3 % of total protein correspond to carboxylesterase enzyme (E4), which can have a detrimental fitness cost in the absence of insecticide (Bass et al., 2014).

CE activity is often detected using 1-naphthyl acetate as an artificial substrate in a colorimetric biochemical assay (Yu, 1991).

1.3.1.3 Glutathione S-transferases (GST)

GSTs are a diverse family present in most aerobic organisms (Armstrong, 1997). GSTs play an important role in the detoxification of endogenous and xenobiotic compounds and are involved in intracellular transport, biosynthesis of hormones, and protection against oxidative stress (Habig et al., 1974). GSTs are classified in insects according to their location within the cell (microsomal and cytosolic) and mainly the cytosolic GSTs metabolize insecticides (Enayati et al., 2005). Cytosolic GSTs are hetero- or homo-dimeric proteins (200–250 amino acids) and are encoded by a multigene family while microsomal (150 amino acids) are active as trimmers and constituted by a single gene (Enayati et al., 2005; Ranson et al., 2001; Shi et al., 2012). Recently, 60 putative genes encoding GSTs were identified in *S. frugiperda* and 50 in *D. melanogaster* (Liu et al., 2019).

GSTs primarily catalyze the conjugation of electrophilic compounds with the thiol group of reduced glutathione (GSH), forming products that are more water-soluble and excretable (Li et al., 2007). Many insecticides such as organophosphorus insecticides (Sun et al., 2001), abamectin (Argentine and Clark, 1990; Stumpf and Nauen, 2001), and DDT (Ranson et al., 2001) form with GSH conjugates (phase II metabolism), and so facilitate the detoxification/sequestration and subsequent excretion of the substances from the organism (Hemingway, 2000; Sun et al., 2001). Like P450s, GST-based resistance to insecticides is described to be caused by the increase in the level of expression of one or more GST genes, but the molecular genetic mechanisms responsible for this up-regulation of activity have not yet been resolved in detail (Feyereisen, 1995; Hemingway, 2000). In FAW, the pre-exposure to phenolic plant allelochemical compounds, inhibit the activity of GST and consequently increases the susceptibility towards sequential insecticide exposure (Yu and Abo-Elghar, 2000; Zhu et al., 2015). The involvement of GST in cross-resistance between organophosphate and Bt toxins (Cry1F and Cry1Ac) has been also proposed, but a further functional investigation is necessary (Zhu et al., 2015). Yu (1999) reported FAW larvae has six cytosolic GST isozymes, whereas the fat body contained three cytosolic GST isozymes. GST total activity can be measured using artificial substrates such as 1-chloro-2,4-dinitrobenzene (CDNB) measuring changes in absorbance by 2,4-dinitrophenyl-glutathione formation (Habig et al., 1974) or by the conversion of monochlorobimane (MCB) to its fluorescent bimane–glutathione adduct (Nauen and Stumpf, 2002).

1.3.1.4 ATP-binding cassette (ABC) transporters

ABC transporters belong to phase III of metabolism and are responsible for ATP-dependent translocation of substances across membranes, therefore playing an important role in the transport of xenobiotics (Linton, 2007). They represent one of the largest gene superfamilies

of transporters widely present in all living organisms. ABC transporters cover a broad spectrum of substrates such as ions, amino acids, sugars, peptides, hormones, polysaccharides, lipids, and xenobiotics (Linton, 2007).

A functional eukaryote ABC transporter is composed of four core domains: two transmembrane domains (TMDs), each built up from six membrane-spanning *alpha*-helices, alternating with two cytosolic nucleotide-binding domains (NBDs), characterized by the presence of a P-type traffic ATPase (Dean, 2001; Rees et al., 2009) (Figure 17). The four domains may be fused into a single polypeptide, forming a full transporter (2TMDs-2NBDs), or as a half transporter (1TMD+1NBD) (Linton, 2007; Schneider and Hunke, 1998). The NBDs comprise highly conserved regions, such as the Walker A and B motifs, the A-, Q-, D- and H-loop, and the ABC signature sequence (LSGGQ motif) (Ambudkar et al., 2006; Higgins, 1995; Rees et al., 2009).

ABC transporters have a common mechanism for exporting substrates across the membrane by hydrolyzing ATP as a pump, and the ATP-switch's model provides a scheme for the transport mechanism in which repeated communication between NBDs and TMDs occurs in both directions and involves only non-covalent conformational changes (Higgins and Linton, 2004). A substrate binds into a cavity between the TMs, which causes a conformational change, bringing the NBDs in proximity. This facilitates the binding of two adenosine triphosphate (ATP) molecules. Subsequently, the substrate is released into the extracellular space. Finally, the ATP is hydrolyzed, releasing ADP and Pi into the cytosol and destabilizing the closed dimer conformation to restore its open dimer configuration for another new cycle (Higgins and Linton, 2004) (Figure 17).

ABC transporter genes are highly conserved in many insects and they can be divided into eight subfamilies (ABCA to ABCH) (Bariami et al., 2012). The number of identified ABC genes differs widely among species, 105 were found in the genome of the spider mite, *Tetranychus urticae*, (Dermauw et al., 2013) and most recently, a draft genome from *S. frugiperda* showed the presence of 66 ABC transporter genes (Liu et al., 2019). Members of the subfamilies ABCB and ABCC are associated with the phenomenon of multidrug resistance (Buss and Callaghan, 2008) and in insects, they are highest expressed in Malpighian tubules and midgut tissue (Labbé et al., 2011).

A recent review report cases of insecticide resistance-associated with ABC transporters in insect pests (Dermauw and Van Leeuwen, 2014). The upregulation of some *ABC* transporter genes is associated with resistance to several chemical insecticides such as pyrethroids in mosquitoes, *Aedes aegypti* (Aurade et al., 2010); bed bug, *Cimex lectularius* (Mamidala et al., 2012); and the cotton bollworm, *H. armigera* (Aurade et al., 2010; Srinivas et al., 2004).

Insect ABC transporters are also involved in Bt toxin perforation and will be explained in more detail in section 1.4.1.

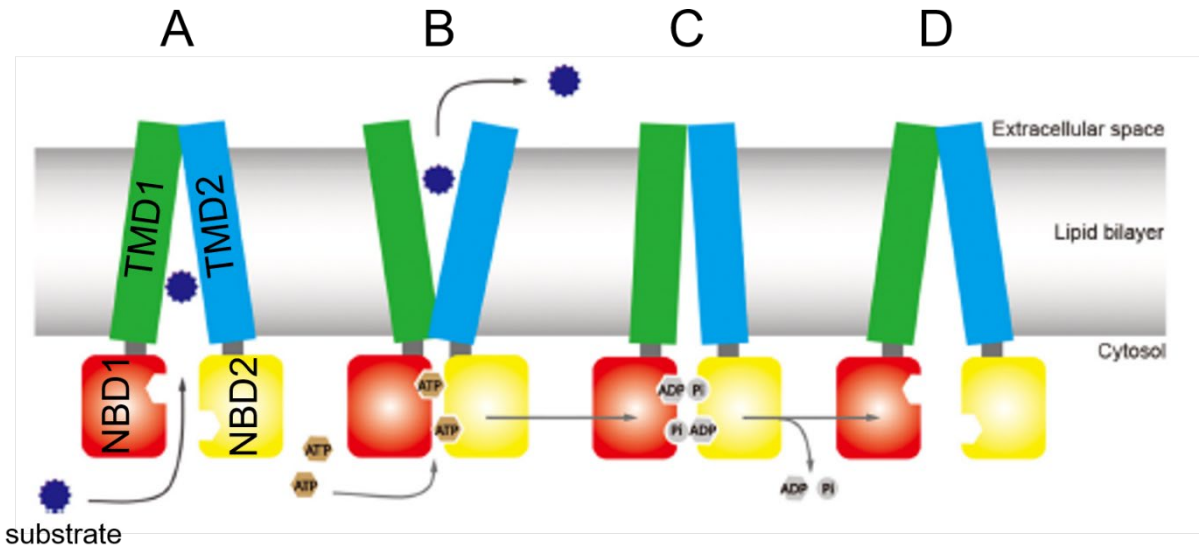

Figure 17 The ATP-switch transport cycle of a full ATP-binding cassette (ABC) transporter composed of two transmembrane domains (TMD1-2) and two nucleotide-binding domains (NBD1-2). (A) Substrate bounds to the closed configuration. (B) ATP binding, conformational change, and expulsion of the substrate. (C) ATP hydrolysis and (D) Return to the closed configuration (Source: Wu et al. (2019)).

1.3.2 Target-site resistance

The second most common resistance mechanism is target-site resistance. Non-synonymous SNPs lead to a change in the amino acid sequence and when it occurs to be within the insecticide binding region of the target protein, it might lead to high levels of resistance (Somers et al., 2018). Target-site resistance is described for many insecticide classes, including pyrethroids (Carvalho et al., 2013a), BPUs (Douris et al., 2016), OPs (Russell et al., 2004), neonicotinoids (Liu et al., 2006), and diamides (Boaventura et al., 2020a; Troczka et al., 2012).

1.3.2.1 Kdr and skdr in the VGSC – resistance to pyrethroids

The *para*-like gene encodes the VGSC in insects which constitutes of a pore-forming *alpha*-subunit with four homologous transmembrane domains (I-IV), each domain contains six transmembrane helices (S1-S6) (Figure 18) and four smaller *beta*-subunits. Many variants of the *para*-like gene can be encoded by alternative splicing events and RNA editing modifications (Rinkevich et al., 2013). O'Reilly et al. (2006) have predicted a unique putative binding site of pyrethroids based on the VGSC structure from *M. domestica.* The binding site is located within the domain IIS4-S5 linker and domain IIIS6 (O'Reilly et al., 2006). The knockdown resistance (kdr) at the VGSC has been described in several pest species (Rinkevich et al., 2013). It

consists of leucine to phenylalanine substitution at position 1014 in *M. domestica*. Different variants of the kdr mutation (L1014F/H/S) were subsequently discovered (Burton et al., 2011). Many more SNPs in the predicted pore region of the VGSC have been reported (Williamson et al., 1996) and kdr can occur together with another mutation, M918T (methionine substitution to threonine) known as super-kdr (skdr). The M918T mutation is located in the domain II S4-S5 linker. The presence of both kdr and skdr can confer up to 10.000-fold lower sensitivity to pyrethroids (Vais et al., 2001; Williamson et al., 1996).

Pyrethroid insecticides have been intensively used in the control of *S. frugiperda*, which largely contributed to the development of resistance to this chemical class of insecticides in early 2000 (Carvalho et al., 2013). The kdr mutation together with two further mutations (T929I and L932F) in the VGSC has been recently described in pyrethroid-resistant *S. frugiperda* from Brazil (Carvalho et al., 2013).

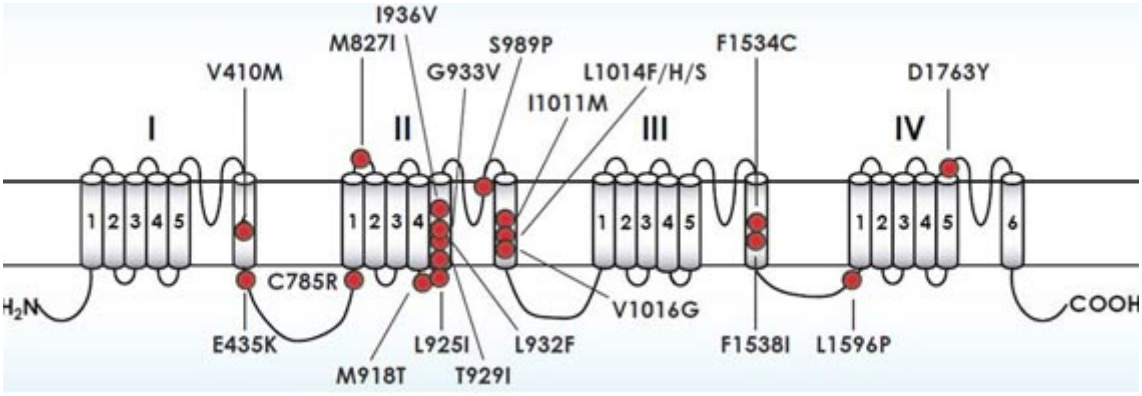

Figure 18 Pore-forming *alpha*-subunit structure composed of four internally homologous domains (I–IV), each having six transmembrane helices (S1–S6). The domains assemble to form a central aqueous pore, lined by the S5, S6, and S5-S6 linkers (P-loops). Numbering shown is according to the voltage-gated sodium channel sequence of the house fly. Adapted from “Prof. Ke Dong's laboratory website”.

1.3.2.2 Target-site mutations in AChE – resistance to carbamates and OP´s

OP´s and carbamates are targeting AChE and mutations linked to resistance are present in the *ace-1* gene, near the active site of the enzyme. Amino acid substitutions may affect the entrance of the insecticide into the AChE active site (Harel et al., 2000).

An *in vitro* study with AChE from *D. melanogaster* harboring different target-site mutations provided functional evidence that all mutations (I161V, G265A, F330Y, and G368A) affect the deacetylation of the enzyme, decreasing or increasing its catalytic efficiency. Moreover, the combination of mutations results in an additive effect in resistance, and the alteration on AChE activity results in fitness cost (Menozzi et al., 2004). The magnitude of the alterations was

related to the allelic frequency in *Drosophila* populations suggesting that the fitness cost is the main driving force for the maintenance of many resistant alleles in insecticide-free conditions (Shi et al., 2004).

Resistance to OPs and/or carbamates were described in many lepidopteran pests such as *P. xylostella* (Konno and Shishido, 1994), *H. virescens* (Brown et al., 1996), *H. armigera* (Gunning et al., 1996), and *S. frugiperda* (Carvalho et al., 2013; Yu, 1992, 1991). However, the effects of altered AChE on acetylcholine hydrolysis seems to vary among lepidopteran species and compounds (Gunning and Moores, 2001). In *H. armigera* resistant to OP´s, AChE activity decreased (Gunning et al., 1996), while in a carbamate-resistant strain of *H. armigera* and *H. virescens* (Brown and Bryson, 1992) the AChE activity increased, and no correlation of AChE activity and cross-resistance patterns was found (Gunning and Moores, 2001). In Lepidoptera, information on the genetics of insensitive AChE is limited, but data for *H. virescens* (Brown et al., 1996) and *Helicoverpa* spp (Gunning et al., 1996) also suggest a single, incompletely dominant gene that may effectively dominant under exposure to insecticides. Heterozygotes are more susceptible to high doses of insecticide than homozygotes (Gunning and Moores, 2001).

Reduced fitness costs have been associated with insensitive AChE, at least in heterozygous Lepidopteran pests. Point mutations linked with OP resistance have been described for *Cydia pomonella* (F399V), *Chilo suppressalis* (A314S), and *P. xylostella* (D131G, A201S, G227A, and A441G) (Baek et al., 2005; Cassanelli et al., 2006; Haddi et al., 2017; Jiang et al., 2015; Lee et al., 2007). Recently, point mutations A201S, G227A, and F290V were found in chlorpyrifos resistant FAW from Brazil (Carvalho et al., 2013) and A201S and F290V in FAW collected in China (Zhang et al., 2019).

1.3.2.3 RyR mutations – resistance to diamides

Diamide insecticide resistance in lepidopteran pests at levels compromising the field efficacy of recommended label rates was first reported for *P. xylostella* (Troczka et al., 2012), followed by *Tuta absoluta* (Roditakis et al., 2015), *Chilo suppressalis* (Yao et al., 2017), *S. exigua* (Cho et al., 2018), and very recently *S. frugiperda* (Bolzan et al., 2019). The most important mechanism of resistance in *P. xylostella* has been functionally linked to target-site mutations in the RyR transmembrane domain, such as the amino acid substitutions G4946E/V and I4790M (Guo et al., 2014; Qin et al., 2018; Steinbach et al., 2015; Tao et al., 2013). The functional relevance of these RyR mutations was confirmed by radioligand binding studies using flight muscle microsomal preparations (Roditakis et al., 2015; Steinbach et al., 2015), by recombinant expression of mutant RyR variants in insect cell lines (Troczka et al., 2015), and by CRISPR/Cas9 genome-edited transgenic *D. melanogaster* and *S. exigua* carrying RyR

M4790I and G4946E mutations, respectively (Douris et al., 2017; Zuo et al., 2017). More recently, novel substitutions (Y4701C/D) were detected in diamide resistant strains of *C. suppressalis* in China (Sun et al., 2018)

Cross-resistance between diamides has been reported in a laboratory selected strain of *S. frugiperda* collected in Brazil, showing 237-fold resistance to chlorantraniliprole and > 42,000-fold resistance to flubendiamide (Bolzan et al., 2019). The mechanism conferring such high levels of resistance has been recently described as a point mutation in the C- terminal transmembrane domain at position 4734 (corresponding to I4790 in *P. xylostella* RyR) (Boaventura et al., 2020a - Described in Chapter 2). RyR homology modeling shows that although G4946E and I4790M mutations are closely located to each other (Richardson et al., 2020; Steinbach et al., 2015) (Figure 19), the presence of I4790M mutation seems to have a different impact on diamide binding according to the diamide chemotype, the methionine residue at this particular position causes higher resistance to flubendiamide (Steinbach et al., 2015), while G4649E strongly affects chlorantraniliprole (Richardson et al., 2020; Steinbach et al., 2015).

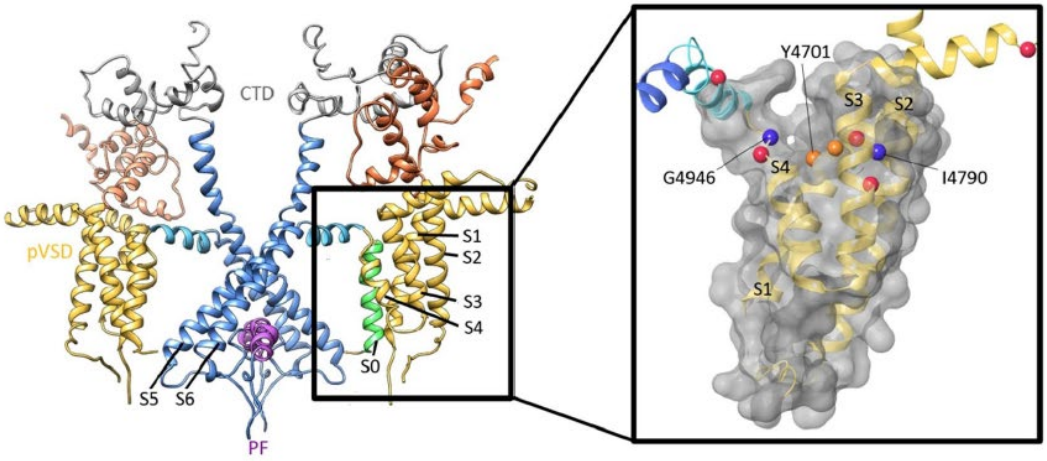

Figure 19 *Plutella xylostella* ryanodine receptor homology model highlighting target-site mutations associated with diamide resistance. Adapted from Richardson et al. (2020).

1.3.2.4 Other target-site mutations conferring resistance to BPUs, indoxacarb, avermectins, and spinosyns

Resistance to BPUs has been often correlated with insects acquiring an increased ability to metabolize and eliminate the compound (Perng et al., 1988). However, a conservative target-site mutation from isoleucine to methionine or leucine (I4290M according to *P. xylostella* numbering) in chitin synthase 1 (CHS1) has been described in insects and mites to confer a high level of resistance to BPUs (Douris et al., 2016; Grigoraki et al., 2017; Guz et al., 2020).

Resistance to lufenuron has been recently described in FAW collected in Brazil, however, the mechanism of resistance was described as polygenic and is still elusive (Nascimento et al., 2015).

The oxadiazine insecticide indoxacarb acts on the VGSC, preferentially blocking open and inactivated states of the sodium channel and have a lower affinity to channels in the resting state (Fozzard et al., 2005; Hille, 2001). The mutations F1845Y and V1848I, in the transmembrane segment 6 of domain IV (IVS6), were identified to be associated with indoxacarb resistance in the diamondback moth, *P. xylostella* (X.-L. Wang et al., 2016), and their functional implications confirmed by CRISPR/Cas9 genome editing in *Drosophila* (Samantsidis et al., 2019).

Avermectins are classified as macrolide neurotoxins, which act by directly activating or potentiating GluCls channels, and to a lesser extent, *γ*-aminobutyric acid (GABA)-gated chloride channels (Nauen and Bretschneider, 2002). To date, mutations occurring in the transmembrane domain of arthropod GluCls were associated with target-site resistance to abamectin: A309V and G315E in *P. xylostella* (X. Wang et al., 2016) and G323D in *T. urticae* (Dermauw et al., 2012; Kwon et al., 2010). Furthermore, recent functional analysis using *Xenopus* oocytes expressing *P. xylostella* GluCl wild and mutant types showed that the A309V and G315E mutations reduced the sensitivity to abamectin by 4.8- and 493-fold, respectively (Wang et al., 2017). The first report of a structural change in the GABA-gated chloride channel, linked to a high level of cyclodiene resistance was a substitution of alanine to a serine (A301S) in *D. melanogaster* (ffrench-Constant et al., 1993). Variations of the A301S/G mutation are found in several insect species (Anthony et al., 1998; Thompson et al., 1993).

Resistance to spinosyns is frequently conferred by target-site mutations such as G275E described in the tomato leafminer *Tuta absoluta* in the *alpha*6 subunit of nAChR (Silva et al., 2016) or truncated disruption of nAChR in *P. xylostella* (Baxter et al., 2010; Rinkevich et al., 2010; Wang et al., 2019). Recent evidence by reverse genetics with CRISPR/Cas9 genome-editing technology confirmed that a truncated *alpha*6 subunit conferred very high levels of resistance to spinetoram and spinosad in *P. xylostella* (Wang et al., 2019) and *S. exigua* (Zuo et al., 2020).

1.4. Mechanism of resistance to Bt pore-forming proteins

The first case of resistance to Bt toxins in lepidopteran pests was reported in 1985 in *Plodia interpunctella* under laboratory conditions (Mcgaughey, 1985), and one year later under field conditions in *P. xylostella*, however in both cases, resistance evolved to a sprayable Bt-based product (Tabashnik et al., 1990).

In the past two decades, the adoption of Bt crops has increased unlike any other agricultural crop protection technology ("ISAAA," 2018). However, the evolution of resistance to different Bt crops is really challenging and it has increased from three cases in 2005 to 21 cases by 2016 (Tabashnik and Carrière, 2019, 2017). Field-evolved resistance to Bt crops in lepidopterans has been reported to different species and Cry toxins around the world, for example, *P. xylostella*, *Trichoplusia ni*, *Busseola fusca*, *Diatraea saccharalis*, *S. frugiperda*, and *Pectinophora gossypiella* (Baxter et al., 2005; Dhurua and Gujar, 2011; Farias et al., 2014; Gassmann et al., 2011; Huang et al., 2012; Janmaat and Myers, 2003; Storer et al., 2010; Tabashnik et al., 2013, 1990; van Rensburg, 2007).

It is evident that the evolution of resistance in many pests is a major threat to the sustainable use of Bt-crops (Tabashnik et al., 2013). Therefore, the elucidation of the mechanisms conferring resistance is crucial for developing sustainable measures of IRM. Several mechanisms have been observed in Cry-resistant insect strains, such as altered binding to midgut receptors, inefficient protoxin activation (Gong et al., 2020), toxin degradation, more efficient repair of the damaged midgut cells, CE sequestration (Gunning et al., 2005), and elevated immune status (Ferré and Van Rie, 2002a; Ma et al., 2005) (Figure 20). Although virtually all steps in the Bt mode of action have potential to evolve resistance, the most reported mechanism of field-evolved resistance seems to be altered binding of Cry toxins to gut receptors (Ferré and Van Rie, 2002a), either due to differences in the expression level or point mutations (Guo et al., 2015; Jakka et al., 2016; Jurat-Fuentes et al., 2011). In some cases, one mechanism of resistance has been found in different pest species or can confer resistance to different toxins in the same insect. The major mechanisms of resistance and their practical implication will be described below.

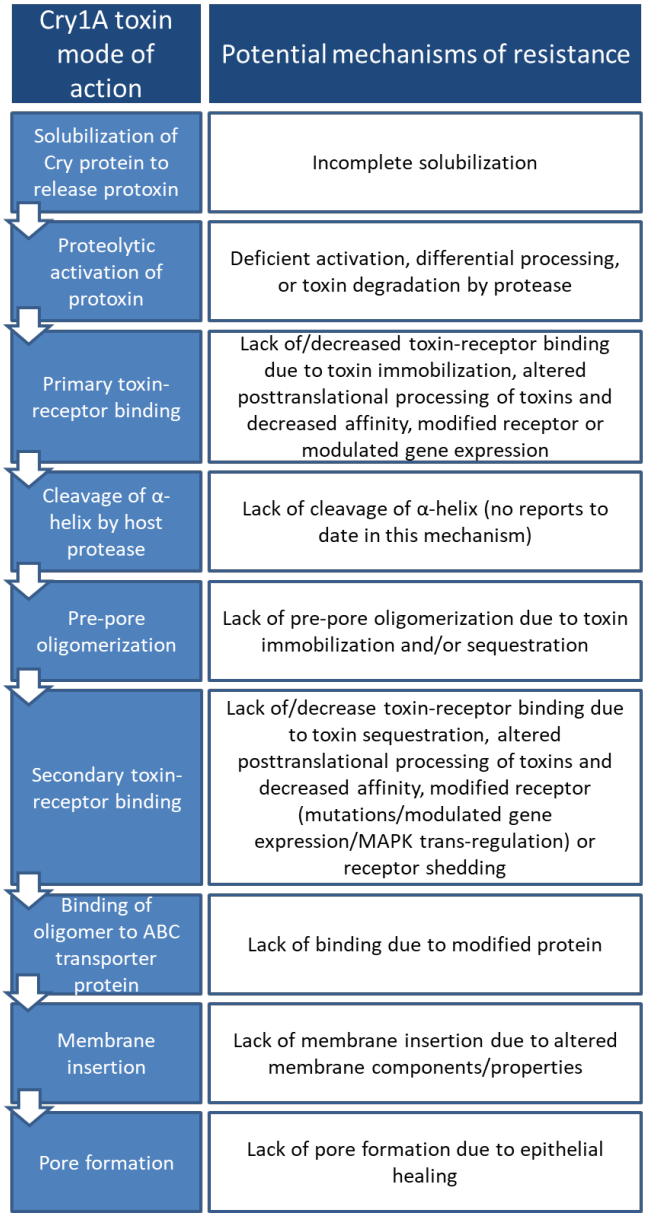

Figure 20 Summary of potential mechanisms of resistance reported for lepidopteran pests under laboratory and field conditions (white blocks) for each step in the Cry1A toxin mode of action (gray blocks) (Source: adapted from Peterson et al. (2017)).

1.4.1 Altered expression level of receptors/enzymes and target-site mutations

Binding studies comparing susceptible and resistant strains have supported the multistep mode of action of Cry toxins and the identification of important Bt-receptors in the midgut, such as cadherin (CAD), alkaline phosphatase (ALP), aminopeptidase N (APN), and ABC transporters.

CAD was the first Cry1A receptor identified in the tobacco budworm, *H. virescens* (Martinezramirez et al., 1994; Vadlamudi et al., 1993) and later on a retro-transposon mediated disruption of CAD was genetically linked to Cry1Ac resistance (Gahan, 2001).

Different mutations/insertions resulting in an incomplete CAD and alternative splicing, coding two or more different transcript isoforms were also observed in Cry1Ac-resistant strains of *P. gossypiella* (Fabrick et al., 2014; Morin et al., 2003; Tabashnik et al., 2005, 2004). Furthermore, several resistant alleles carrying CAD mutations were found in strains of *H. armigera* (J. Wang et al., 2016; Xu et al., 2005; Yang et al., 2007, 2006; Zhang et al., 2013).

Besides target modifications, the down-regulation of *CAD* gene expression was also linked to Cry1Ab resistance in *D. saccharalis* (Yang et al., 2011), Cry1Ac in *H. virescens* (Jurat-Fuentes et al., 2011), *P. xylostella* (Yang et al., 2012) and Cry2Ab in *P. gossypiella* (Fabrick et al., 2020).

Many APN (1-6) isoforms have been identified in lepidopteran pests and supposed to play an important role in the binding specificity of Cry toxins (Van Rie and Ferré, 2000). A mutation in APN1 has been linked to Cry1Ac resistance in *H. armigera* (Zhang et al., 2009), while APN1 and APN6 were reported as receptors of Cry1Ac in *T. ni*. Nevertheless, only the down-regulation of *APN1* was correlated with resistance (Tiewsiri and Wang, 2011). In the case of a Cry1Ca-resistant strain of *S. exigua*, a total lack of *APN1* expression was observed. However, the linkage between resistance and lack of expression has not been determined yet (Herrero et al., 2005).

Interestingly, reduced *ALP* transcript levels were reported in a Cry1Ac-resistant strain of *H. zea* but increased ALP enzyme activity (Caccia et al., 2012). ALP in *B. mori* could be solubilized by enzymes present in the midgut epithelium, and it has been suggested that differences of intracellular ALP levels might be involved in Cry toxin-mediated signaling (Jurat-Fuentes and Adang, 2006).

Earlier studies revealed a significant down-regulation of membrane-bound ALP isoenzyme 2 (*mALP2*) in gut tissue in Cry1 resistant lepidopteran pests such as *H. virescens*, *H. armigera*, and *S. frugiperda* (Jurat-Fuentes et al., 2011). However, more recently, Banerjee et al. (2017) did not find a co-segregation of down-regulation of *mALP2* and Cry1F resistance in FAW collected in Puerto Rico.

During the last years, ABC transporters played an increasing role as Bt receptors. Some ABC transporters are functional receptors for more than one Cry1 toxin and they might mediate the insertion of toxin into the lipid membrane, consequently leading to pore formation in the insect midgut (Heckel, 2012). Heterologous expression in *D. melanogaster* has been used to validate the involvement of ABCC2 together with CAD in Cry1Ac toxin binding and has reinforced the major role of ABCC2 in Bt toxicity (Stevens et al., 2017).

ABCC2 in *H. virescens* was the first ABC transporter linked with Cry1Ac resistance, and later on, the finding supported by the description of a 22 base pair (bp) deletion in exon 2 in *ABCC2* (Gahan, 2001; Gahan et al., 2010).

Changes of the protein conformation and activity, such as mis-splicing of ABCC2 or point mutations, reduce the binding of Cry1Ac toxin and has conferred high levels of Bt resistance in different target pests (Atsumi et al., 2012; Gahan et al., 2010; Xiao et al., 2015).

In a *P. xylostella* strain resistant to Cry1Ac, 30 bp were deleted from exon 20 of *ABCC2*, causing the removal of carboxyl-terminal of TM12, harboring then ATP-binding loop and resulting in a dysfunctional transporter (Baxter et al., 2011).

Moreover, ABCC transporters are also receptors of Cry1Ca and Cry1Fa toxins (Coates and Siegfried, 2015; Park et al., 2014). A GC insertion in the *ABCC2* of *S. frugiperda* leading to a premature stop codon was linked to Cry1F resistance in populations from Puerto Rico (Banerjee et al., 2017; Flagel et al., 2018). More recently a two amino acid deletion (glycine and tyrosine) (GY deletion) at the extracellular loop 4 (ECL4) was linked to Cry1F-resistance in FAW populations from Brazil, together with more rare mutations at the same exon 14 of *ABCC2* (Boaventura et al., 2020b, described more in details in Chapter 3).

The heterologous expression of ABC transporters and cytotoxicity assays showed that the binding affinity of ABC transporters to Cry toxin is largely linked to amino acids at ECL4 in *B. mori* by determining the specificity of ABCC to Cry toxins (Endo et al., 2018).

Another ABC transporter, ABCC3, was suggested to be a receptor for Cry1Ca in *S. exigua* larvae (Park et al., 2014) and mutations at the ABCA2 in *H. armigera* and *H. punctigera* were linked to Cry2Ab resistance (Tay et al. 2015). In the case of three Cry1Ac resistant strains of *P. xylostella,* resistance was not caused by a mutation in an *ABC* gene, but rather down-regulation of an *ABCG* (Guo et al. 2015a).

Also, insect ABCA2 has been reported to bind to Cry2Ab (Tabashnik, 2015; Tay et al., 2015), and deletions have been described to confer resistance to Cry2Ab in *H. armigera* and *H. punctigera* strains (Tay et al., 2015). Moreover, the complete loss of *ABCA2* exon 6 caused by alternative splicing has been described in resistant larvae of *P. gossypiella* (Mathew et al., 2018).

Bt resistance mediated by ABC transporters has not been only associated with target-site mutations but also the regulation of gene expression. A study of Bt resistance in *B. mori* revealed a trans-regulatory mechanism involved in the expression of ABCC2 in response to Cry1Ab toxin, which may play an important role in insect Bt resistance (Chen et al., 2014). The pore formation caused by Cry1Ac toxin binding to ABCC transporters has been reported to be regulated by the MAPK signaling pathway (Guo et al., 2015). Also, Forkhead box protein A (FOXA) upregulates the expression of *ABCC2* and *ABCC3* genes in Sf9 cells (Li et al., 2017). MicroRNA, miR-998-3p was proved to be able to regulate the expression of *ABCC2*, and thus to mediate the Cry1Ac resistance in three representative lepidopteran pests (*H. armigera*, *S. exigua,* and *P. xylostella*) (Zhu et al., 2020).

1.5 Integrated pest and insecticide resistance management

Bioecological considerations such as life cycle, fecundity, migration capacity, feeding behavior, and also the ability to evolve resistance to different chemical compounds must be taken into account when planning FAW control strategies. IPM and IRM approaches need to be taken locally and according to the control measures and insecticides available.

The FAW management in Latin America and the USA is based mainly on Bt technology and additional insecticide applications when necessary. One of the IRM strategies recommended on Bt-crops is the refuge (i.e., non-Bt crops planted with Bt-crops). This strategy is most effective when the initial frequency of resistance alleles is low ($< 1 \times 10^{-3}$, according to Roush (1997)); resistance is functionally recessive; and the Bt crop produces a “high-dose” (25-fold the dose needed to kill all homozygous susceptible larvae) of the Bt protein against the target insect population (Georghiou and Taylor, 1977; Tabashnik and Croft, 1982). Moreover, to obtain an effective delay in resistance, refuge areas should be sufficiently large (20 % for maize and 10 % for soybean in Brazil) and near (800 m) to the Bt-crop (Comins, 1977; Shelton et al., 2000).

The refuge should generate a great number of susceptible insects that will mate with potential homozygous resistant survivors in the Bt area. If resistance is recessive, the offspring generated would be heterozygous and controlled by the Bt crop (Tabashnik et al., 2009).

The monitoring of pest pressure in the field is trivial for deciding on when to intervene in the field. The monitoring of early migration events can be done through pheromone traps (at least one per ha) placed in different areas in the field and by assessing the foliar damage at five sites (scout 10-20 plants in each site) (Figure 21A). In Brazil, IRAC recommends making no more than two foliar applications (from different MoA) until V6 (sixth leaves fully expanded) maize growing stage when 20 % of the plants reach a damage score of three according to Davis Scale (Figure 21B) (Davis et al., 1992) in the refuge area (IRAC-BR, 2016). In the areas where Bt crops are cultivated, chemical intervention is required when 10 % of plants screened reach scale three. The use of seed treatment is recommended in the field with high infestation history. In case more than one insecticide application is required for sufficient pest control, the rotation of MoA is essential to delay resistance. Moreover, the applications of compounds with a high hazard profile should be avoided, especially in the late vegetative or reproductive maize growth phases (Figure 22) (IRAC-BR, 2016).

The choice of a crop variety is also essential in determining the durability and effectiveness of the Bt technology in case field-evolved resistance is known. The continuous expression of *cry* genes in transgenic plants exerts a strong selection for resistance in the target pests (Mcgaughey, 1985). Therefore, the industry has moved toward the pyramiding or stacking of multi genes in Bt crops to increase the durability of traits compared to single events (Storer et al., 2012). Cross-resistance of Cry1F-resistant FAW has been described to Cry1Ab, Cry1Ac,

and Cry1A.105, but not to Vip3Aa20 and Cry2Ab (Bernardi et al., 2015; Murúa et al., 2019; Santos-Amaya et al., 2016; Vélez et al., 2013).

The rapid evolution of resistance to Bt and synthetic insecticides in the Americas challenges FAW control. Therefore, it is important to establish a baseline susceptibility in the newly invaded countries to monitor the shift in sensitivity for insecticides and take efficient decisions on what mode of action/chemical to use.

In sub- Saharan Africa, most of the maize grown areas are composed of small farmers (<2 ha), and the final products are mainly produced for family consumption (Hruska, 2019). Asia produced about 32 % of the maize globally, China is the second-largest maize producer in the world, growing maize on over 40 million ha ("ISAAA," 2018), but most maize is grown in small scale farms as well, and produced for animal feed (Hruska, 2019).

The use of intercropping systems, push-and-pull strategy, entomopathogenic fungi, parasitoids, baculoviruses, and botanical compounds are among a diverse range of tactics implemented in the newly invaded countries in Africa and India to control FAW and other endogenous lepidopteran pests (Baudron et al., 2019; Feldmann et al., 2019; Gebreziher, 2020; Hruska, 2019; Kumela et al., 2019; Sharanabasappa et al., 2019; Sisay et al., 2019). Some of the control measures mentioned above have shown significant FAW control in small farms and at rather low infestation levels. Synthetic insecticides have been used to manage FAW outbreaks in countries with large agricultural input support programs (Hruska, 2019; Kumela et al., 2019; Sisay et al., 2019). However, in some countries, farmers have claimed for the low efficacy of insecticides used, such as OP´s and pyrethroids (Baudron et al., 2019; Kumela et al., 2019).

Research on mechanisms conferring insecticide resistance is essential for the understanding of the potential risk for resistance evolution against new and old chemical compounds, and therefore improves resistance management strategies in different regions worldwide.

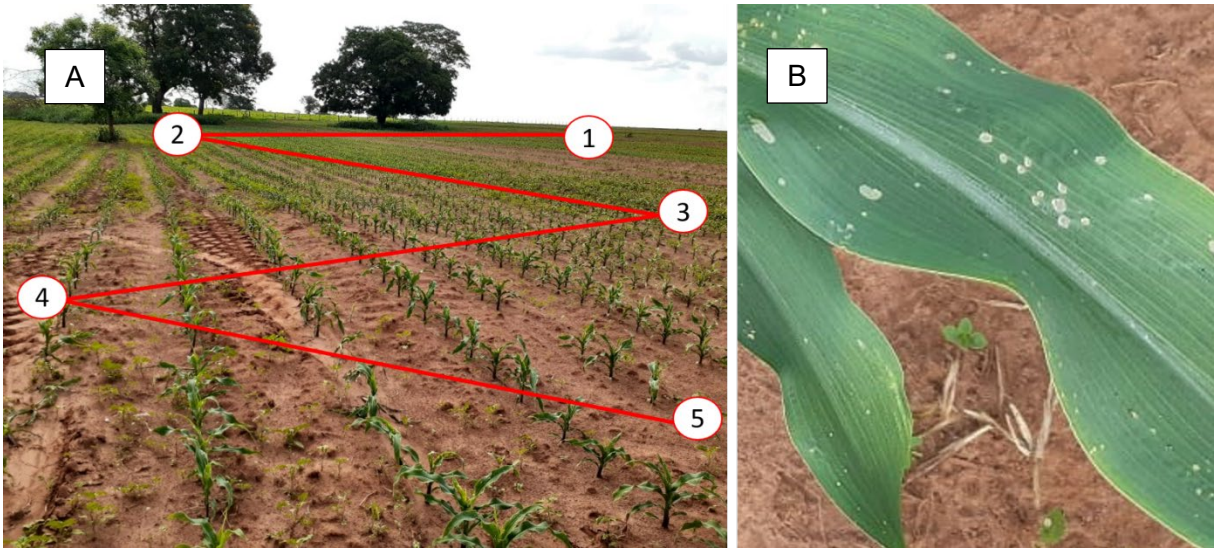

Figure 21 Scouting scheme for inspection of five consecutive plants at five sites dispersed across the maize field (zig-zag pattern) (left side) and visual illustration of Davis scale 3 damage by fall armyworm in maize (right side), as the timepoint recommended for chemical control (Source: personal images).

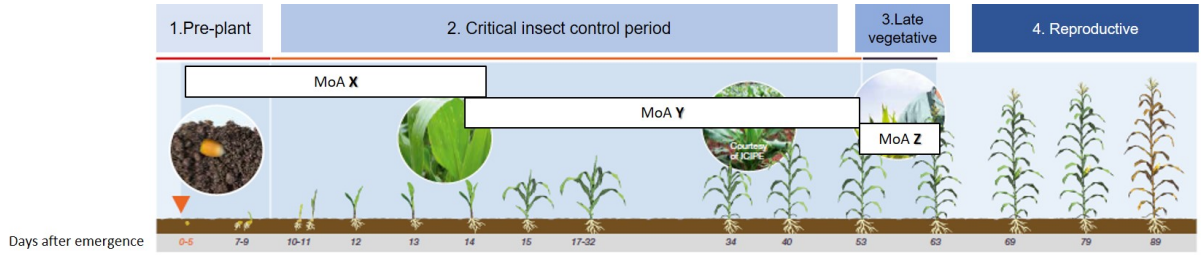

Figure 22 Model for IRM strategy against fall armyworm based on windows-treatments and rotation of insecticide mode of action. A window corresponds to one complete life cycle, one generation. Adapted from Bayer (2017).

1.6 Objectives

The development of resistance to many chemical classes of insecticides and more recently Bt toxins in FAW highlights the importance of the implementation of resistance management strategies. Additionally, the new invasion of this pest in the African and Asian continent reinforced the need for sustainable pest management according to a broad geographical extent. Therefore, it is of utmost importance to understand the molecular mechanisms of resistance involved, and to assess alternative chemical control options not yet affected by cross-resistance issues.

Diamide insecticides are the most modern class of insecticides used to control lepidopteran pests worldwide, including FAW. The aim of Chapter 2 was to describe the mechanism underlying high levels of diamide resistance obtained in a laboratory-selected FAW population from Brazil and to provide robust and simple genotyping diagnostic tools that could be used in resistance monitoring programs to follow the spread of the identified resistance alleles in field populations.

Chapter 3 aimed to characterize the mechanisms involved in Cry1F resistance in FAW collected in Brazil. Genetic inheritance studies, molecular and cellular *in vitro* assays were performed in order to functionally validate and link resistant alleles to the Cry1F-resistant phenotype. Moreover, a genotyping tool was developed and used to provide an overview of the geographical distribution of the resistance alleles.

Chapter 4 objective was to investigate the toxicological profile of a Cry1F-resistant strain to different chemistries of diverse insecticide MoA classes and Bt toxins by bioassays. Next-generation sequencing was used to identify genes encoding insecticide target sites and detoxification enzymes. Gene expression profiling together with biochemical assays was used to identify major enzyme families involved in the detoxification of insecticides and to clarify their contribution to cross-resistance patterns observed in bioassays.

Chapter 5 aimed to provide broad monitoring of target-site mutations conferring resistance to pyrethroids, organophosphates, diamides, and Cry1F in FAW populations collected on three different continents (Brazil, Puerto Rico, Kenya, and Indonesia).

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Chapter 2

Detection of a ryanodine receptor target-site mutation in diamide insecticide resistant fall armyworm, *Spodoptera frugiperda*

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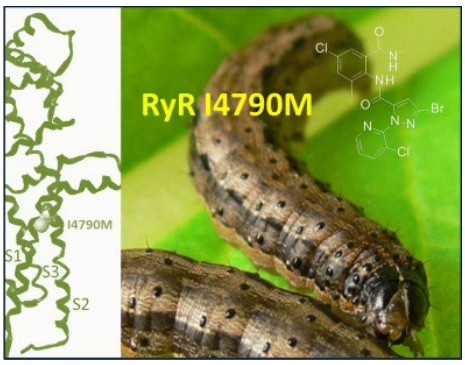

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Detection of a ryanodine receptor target-site mutation in diamide insecticide resistant fall armyworm, *Spodoptera frugiperda*

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Abstract

BACKGROUND: Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), a major lepidopteran pest in Latin and North America, has very recently invaded the continents of Africa and Asia. FAW has evolved resistance to different insecticides and transgenic corn expressing *Bacillus thuringiensis* (Bt) toxins. Here, we investigated the extent and mechanisms of resistance to diamide insecticides in a Brazilian field-collected FAW strain selected using chlorantraniliprole.

RESULTS: Continuous laboratory selection of a field-collected FAW strain with chlorantraniliprole resulted in resistance ratios of 225-fold and > 5400-fold against chlorantraniliprole and flubendiamide, respectively, when compared with a susceptible strain. Pre-exposure to different synergists known to inhibit detoxification enzymes did not result in significantly increased larval toxicity, suggesting a minor role for metabolic resistance. Sequencing of the FAW ryanodine receptor (RyR) C-terminal domains II to VI revealed a single nucleotide polymorphism, resulting in a I4734M mutation recently said to confer target-site resistance to diamides in lepidopteran pests. Genotyping by pyrosequencing of field-collected FAW larvae sampled in the 2018 crop season suggests a low resistance allele frequency. Furthermore, we developed a fluorescent polymerase chain reaction (PCR)-based allelic discrimination assay for rapid genotyping of field-collected FAW samples, because diamides are increasingly used in Bt−/non-Bt corn.

CONCLUSIONS: Recently, the identified RyR mutation has been shown to confer field resistance in other lepidopteran pests such as diamondback moth, tomato leafminer and striped rice stem borer. The developed PCR-based allelic discrimination assay will help to monitor the frequency and future spread of diamide resistance allele in FAW field populations and help to implement appropriate resistance management measures.

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

Keywords: fall armyworm; flubendiamide; chlorantraniliprole; resistance; Brazil; ryanodine receptor

1 INTRODUCTION

Fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith, 1797) (Lepidoptera: Noctuidae), is a species native to the (sub)tropical regions of North and South America and is a major pest of corn, particularly in Brazil.[1,2] This pest species has recently invaded the African continent,[3] and was first described as being present in Asia in 2018.[4] There are two corn cropping seasons in Brazil, covering more than 16 million hectares in the 2017/2018 crop season.[5] Prior to the introduction in 2008 of transgenic corn, expressing *Bacillus thuringiensis* (Bt) protein toxic to FAW larvae, FAW was kept below economic damage thresholds by frequent applications of insecticides.[6,7] FAW control by synthetic contact insecticides has limitations as larvae tend to be protected from direct foliar insecticide treatments because they stay inside the corn whorl.[8] The introduction of Bt corn in Brazil 10 years ago – covering > 80% of the total corn cropping area in 2016 – resulted in much less insecticide spraying against FAW.[9] However, the first cases of reduced efficacy of Bt corn towards FAW were reported in 2014 in some regions in Brazil,[10] and subsequent studies in Brazilian FAW populations revealed a high frequency of Cry1F resistance alleles compromising the field effectiveness of Bt corn technology.[11–13] FAW resistance to Bt corn expressing Cry1F was also reported

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in 2010 in Puerto Rico, ~6 years after its introduction.[14] Irrespective of the presence of Bt resistance, a dramatic increase in insecticide applications (up to 28) in a single cropping season has been described.[13] Such a treatment frequency is conducive to the development of insecticide resistance in FAW, so it is not surprising that high levels of resistance to several chemical classes of insecticides have been reported recently in Puerto Rico.[15] In Brazilian populations of FAW, resistance to a range of synthetic insecticides, addressing different modes of action, including organophosphates, carbamates, pyrethroids, benzoylureas and spinosyns, has been also described.[16–19]

The most recent chemical class introduced to the insecticide market in 2007 comprised the phthalic and anthranilic acid diamides, targeting insect ryanodine receptors (RyR).[20,21] Diamide insecticides such as flubendiamide and chlorantraniliprole rapidly gained market share exceeding US $1.4 billion in 2013.[22] These are broad spectrum, multiple crop utility insecticides combining high efficacy on numerous pest insects, including lepidopterans, with an excellent safety profile.[21] Diamides affect insect neuromuscular function, acting as selective modulators of insect RyR, as demonstrated by biochemical and physiological mode of action studies.[23,24] RyRs are large homo-tetrameric calcium channels endogenously activated by calcium, and are present in the sarco−/endoplasmic reticulum in nerve and muscle tissue.[25] RyR activation results in Ca^{2+} efflux into the cytosol, triggering a physiological response such as muscle contraction. In those insects investigated to date, RyR protomers are encoded by a single gene with an open reading frame (ORF) of > 15 000 bp.[26] These channels consist of a large N-terminal cytosolic domain and six transmembrane domains at the C terminus comprising the voltage sensor.[27] Diamide insecticides such as flubendiamide and chlorantraniliprole, although different chemotypes, allosterically enhance [^{3}H]ryanodine binding, and address a common binding site in lepidopteran RyRs, as shown by radioligand binding studies with *Heliothis* flight muscle microsomal membrane preparations.[28,29] A crucial role of the RyR transmembrane domain for diamide action was demonstrated by binding studies with RyR deletion mutants using a photoaffinity labelled flubendiamide derivative, as well as with *Drosophila* mutant receptors expressed in insect cell lines.[30,31]

Diamide insecticide resistance in lepidopteran pests, at levels compromising the field efficacy of recommended label rates, was first reported for *Plutella xylostella*,[32,33] followed by *Tuta absoluta*,[34] *Chilo suppressalis*[35] and very recently *Spodoptera exigua*.[36] However, a number of additional studies reviewed by Nauen and Steinbach on lepidopteran pests reported rather low levels of diamide resistance.[37] The most important mechanism of resistance in diamondback moth has been functionally linked to target-site mutations in the RyR transmembrane domain, such as the amino acid substitutions G4946E and I4790M.[31,38,39] The functional relevance of these RyR mutations was not confirmed not only by radioligand binding studies using flight muscle microsomal membrane preparations of diamondback moth and tomato leafminer adults,[38,40] but also by recombinant expression of mutant RyR variants in insect cell lines,[41] and by CRISPR/Cas9 genome-edited transgenic *Drosophila melanogaster* and *S. exigua* carrying RyR M4790I and G4946E mutations, respectively.[42,43] Both amino acid residues have also been shown to be mutated in diamide-resistant tomato leafminer and rice stem borers.[35,40,44]

Despite increasing selection pressure, particularly in Brazil,[45,46] high levels of diamide field resistance in noctuid pests conferred by RyR target-site mutations, as described for some non-noctuid lepidopterans, have not been reported. High levels of FAW resistance to diamides (resistance ratios of 160- and 500-fold against chlorantraniliprole and flubendiamide, respectively) due to frequent applications were recently found in a Puerto Rico population, but the mechanism of diamide resistance was not investigated.[15]

Levels of diamide resistance much higher than those reported for FAW from Puerto Rico, were very recently described for a Brazilian field-collected FAW strain, Chlorant-R, resulting from an F2 screen and selection with chlorantraniliprole.[45] The authors reported resistance ratios of > 230-fold and > 42 000-fold against chlorantraniliprole and flubendiamide, respectively. However, the molecular mechanism of resistance remained unknown, but genetic studies suggested monogenic resistance and an incompletely recessive mode of inheritance.[45]

Here, we investigated the mechanism of diamide resistance in FAW strain Chlorant-R using synergist studies and a molecular approach by sequencing polymerase chain reaction (PCR)-amplified FAW RyR fragments encompassing those sites, reported to be mutated in other diamide-resistant lepidopteran pests. Because of the high level of resistance, we expected to detect a target-site mutation in the RyR transmembrane domain conferring diamide resistance. Furthermore, we wanted to design a simple PCR-based allelic discrimination assay to monitor the extent and spread of the respective resistance allele to support resistance management strategies.

2 MATERIALS AND METHODS

2.1 Insects

All laboratory strains and field-collected populations of *S. frugiperda* used in the study originated from Brazil (Table 1; exception permit SG 46/18-DE-NW for working with *S. frugiperda* was provided by the chamber of Agriculture, NRW, Germany, and a letter of authority issued under directive 2008/61/EC). Diamide resistant (Chlorant-R) and susceptible (Sus) strains of *S. frugiperda* were maintained under controlled conditions (25 ± 1 °C, 70 ± 10% relative humidity (RH) and 14:10 h light/dark photoperiod). Strain Chlorant-R was maintained under continuous selection pressure by chlorantraniliprole as described previously.[45]

2.2 Chemicals

Chlorantraniliprole (Premio® SC 200, DuPont do Brasil S.A., Brazil) and flubendiamide (Belt® SC 480, Bayer S.A., Brazil) were used as commercial formulations. All organic solvents used were of analytical grade. Piperonyl butoxide (PBO) and diethyl maleate (DEM) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and *S,S,S*-tributyl phosphorotrithioate (DEF) was obtained from Chem Service (West Chester, PA, USA).

2.3 Bioassays with synergists

Concentration – response bioassays were performed on third instar larvae of strains Sus and Chlorant-R, using both chlorantraniliprole and flubendiamide. Artificial diet overlay assays were conducted in 24-well plates as described previously.[45] To evaluate the impact of metabolic resistance to diamide insecticides, 1 µL acetonic solutions of PBO, DEM and DEF were applied topically to third instar larvae 2 h prior to insecticide exposure. Synergist solutions were prepared in acetone and applied onto the larval pronotum using a micro applicator (Burkard Manufacturing Co. Ltd, Rickmansworth, UK). Synergists were applied at the following doses: PBO, 0.1 µg

per larva; DEM, 1 µg per larva; and DEF, 0.32 µg per larva. Acetone alone served as a solvent control. The highest non-lethal concentration of each synergist was established in preliminary bioassays. For each insecticide concentration, three to four replicates of ~ 24 larvae were used. After infestation, the larvae were kept under controlled conditions (25 ± 1 °C, 70 ± 10% RH and 14:10 h light/dark photoperiod). Larvae were scored for mortality after 4 days.

2.4 Partial sequencing of *S. frugiperda* RyR

Total RNA was extracted from ten single fourth instar larvae of strains Sus and Chlorant-R previously stored in RNAlater® (Life Technology, Carlsbad, CA, USA). TRIzol® reagent (ThermoFisher Scientific, Waltham, MA, USA) was used for RNA separation followed by RNA purification according to RNeasy® Plus Universal Mini Kit (QIAGEN, Hilden, Germany) recommendations, including a genomic DNA eliminator column step. The RNA was quantified by spectrophotometry (NanoQuant Infinite 200, Tecan, Switzerland) and its integrity assessed by an automated gel electrophoresis system, according to CL-RNA method (QIAxcel RNA QC Kit v2.0, QIAGEN). The RNA was normalized to 200 ng µL^{-1} and 1 µg total RNA was used in 20 µL reactions for cDNA synthesis using SuperScript™ III Reverse Transcriptase and oligo(dT) 20 primer (ThermoFisher Scientific) according to manufacturer's instructions.

Primer pairs were designed by Primer3 v. 2.3.7 based on the cDNA sequence of the RyR of beet armyworm, *Spodoptera exigua* (GenBank KJ573633) covering the transmembrane domains II to VI. Primer pairs Sf. 1-F, Sf. 1-R, Sf. 2-F, Sf. 1-R, Sf. 3-F and Sf. 2-R (Table S1) were used to amplify fragments, which overlapped each other and revealed a 1479 bp FAW RyR fragment. The PCRs contained 100 ng cDNA, 500 nM of each primer, 25 µL of high-fidelity Phusion Flash PCR Master Mix (Thermo Scientific) and nuclease-free water, resulting in a 50-µL reaction mix. The reactions were subjected to cycling conditions of: 10 s at 98 °C followed by 30 cycles at 98 °C for 1 s, 60 °C for 5 s and 72 °C for 15 s, and a final extension step at 72 °C for 1 min in CFX-96 (Bio-Rad, Hercules, CA, USA). The PCR products were verified by an automated gel electrophoresis system, according to OM500 method (QIAxcel DNA Screening Kit v2.0, QIAGEN) purified using innuPREP PCRpure kit (Analytik Jena, Jena, Germany) and directly Sanger-sequenced by Eurofins Genomics (Konstanz, Germany) using the forward and reverse primers described in Table S1. The obtained *S. frugiperda* RyR sequence consisted of 1413 bp (GenBank MK226188) and was aligned with the RyR partial sequence of *Spodoptera exigua* (GenBank KJ573633) and the translated corresponding sequences aligned with *P. xylostella* (GenBank AET09964), *T. absoluta* (GenBank APC65631), *C. suppressalis* (GenBank AFN70719) and *S. exigua* (GenBank AFC36359) sequences using Geneious software v. 10.2.3 (Biomatters Ltd, Auckland, New Zealand).

2.5 Target-site resistance diagnostics by pyrosequencing

Genomic DNA from 10–23 single third/fourth instar larvae from the populations described in Table 1 was extracted using DNeasy Blood & Tissue Kit (QIAGEN) or QuickExtract™ DNA Extraction Solution 1.0 (Epicentre, Madison, WI, USA) according to the suppliers' recommended protocols.

Primer pairs were designed with Assay Design Software (QIAGEN) targeting the mutations G4946E and I4790M (*P. xylostella* RyR numbering) separately, based on cDNA partial sequence *S. frugiperda* RyR described above (GenBank: MK226188) (Table S1). Afterwards, the PCR for pyrosequencing was performed in 30 µL reaction mixture containing 15 µL JumpStart™ Taq ReadyMix™ (Sigma-Aldrich), 500 nM of forward and reverse primer (one biotinylated, see Table S1), ~ 50 ng gDNA and nuclease-free water. The cycling conditions comprised of 95 °C for 3 min, followed by 40 cycles at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. The pyrosequencing reaction was carried out as described previously using the sequencing primers described in Table S1 (i.e. Sf. G4946-F.Seq for G4946E and Sf. I4790-R.Seq for I4790M).[31]

2.6 PCR-based allelic discrimination assay using fluorescent probes

Probes containing different fluorescent dyes were used for allele-selective detection of wild-type and/or mutant gene fragments in a modified real-time PCR assay. Primers and probes (Table S1) were designed using Primer3 v. 2.3.7 software for detection of I4790M (*P. xylostella* RyR numbering) mutation. Individuals with known genotype from strain Sus and Chlorant-R as well as artificial heterozygotes (mixture of gDNA from Sus and Chlorant-R individuals) were tested. To validate the assay, seven field populations collected in Brazil (RO-VI, MT-SZ, MT-PL, MT-TS, MS-CS, SP-IT and PR-PG) were used (Table 1).

The reaction setup consisted of a final volume of 10 µL with 5 µL SsoAdvanced™ Universal Probes Supermix (Bio-Rad), 700 nM of forward and reverse primers, 200 nM of probes (Table S1) and 20–50 ng of genomic DNA, and were run in duplicate. The conditions of PCR amplification was 95 °C for 5 min, and 35 cycles at 95 °C for 15 s and 60.6 °C for 30 s. The real-time PCR was conducted

Table 1. Laboratory strains (Sus and Chlorant-R) and field-collected samples of *Spodoptera frugiperda* used

Strain	State	City	Collection date	Crop season
Sus	Minas Gerais	Sete Lagoas	1996	1996
Chlorant-R	Bahia	Correntina	Dec-2015	2016
PR-PG	Paraná	Ponta Grossa	Feb-2018	2018
SP-IT	São Paulo	Ituverava	Jan-2018	2018
MS-CS	Mato Grosso do Sul	Chapadão do Sul	Nov-2017	2018
MT-SZ	Mato Grosso	Sapezal	Nov-2017	2018
MT-TS	Mato Grosso	Tangará da Serra	Dec-2017	2018
MT-PL	Mato Grosso	Primavera do Leste	Nov-2017	2018
MT-LV	Mato Grosso	Lucas do Rio Verde	Nov-2017	2018
BA-SD	Bahia	São Desiderio	Nov-2017	2018
RO-VI	Rondônia	Vilhena	Nov-2017	2018

in a CFX-384 real-time thermocycler (Bio-Rad) and the end-point fluorescence values, taking cycle 30 as threshold were plotted in scatter-plot using Bio-Rad qPCR analysis software CFX Maestro 1.0.

2.7 Data analyses

The mortality data obtained in dose response experiments were submitted to Probit analysis,[47] using the Polo-Plus program (LeOra Software, Cape Girardeau, MO, USA), to calculate lethal concentration values (LC_{50}) and confidence intervals (95% CI). LC_{50} values were considered different when there was no overlap of the 95% CI. Synergistic ratios (SR) were calculated by dividing the LC_{50} of the control (insecticide without synergist) by the LC_{50} of the insecticide plus synergist treatment. Resistance ratios (RR) were calculated by dividing the LC_{50} value of the resistant strain by the corresponding LC_{50} value of the susceptible strain in each of the insecticide and synergist combination.

3 RESULTS

3.1 Bioassays

Third instar larvae of strain Chlorant-R, continuously maintained under selection pressure with chlorantraniliprole, showed RR values of > 220-fold and > 5400-fold against chlorantraniliprole and flubendiamide, respectively, when compared with strain Sus in diet overlay assays. All tested synergists increased the toxicity of chlorantraniliprole slightly by 1.5–2.2-fold in strain Sus. The highest synergistic ratio was observed with PBO, followed by DEF and DEM; no such synergistic effects were detected in combination with flubendiamide (Table 2). When applied to strain Chlorant-R, none of the tested synergists significantly increased the toxicity of either chlorantraniliprole or flubendiamide, suggesting a lack of metabolic resistance.

3.2 RyR transmembrane domain sequencing

To identify nucleotide polymorphisms leading to non-synonymous mutations in RyR domains previously described to confer diamide resistance in other lepidopteran pests, we PCR amplified and sequenced a partial stretch of the RyR encompassing transmembrane domains II to VI (Fig. S1). Some of the shipped larval material was of lower quality resulting in difficulties in RNA extraction. Therefore, we amplified the selected partial sequence by using three different primer pairs, which amplified smaller overlapping fragments. After sequencing and assembling, a total fragment of 1413 bp (GenBank: MK226188) was obtained successfully. The amplified partial *S. frugiperda* Sus RyR sequence shows high similarity (98,3% pairwise identity at amino acid level) to the respective stretch of the *S. exigua* RyR (GenBank: AFC36359).

Analysis of the obtained sequences from individuals of strains Sus and Chlorant-R revealed only a single non-synonymous single nucleotide polymorphism causing an isoleucine to methionine substitution at position 4734 (corresponding to I4790M in *P. xylostella* RyR) (Fig. 1 and Fig. S1). All individuals sequenced by Sanger sequencing were either homozygote for the wild-type allele (larvae of strain Sus) or for the I4790M allele (larvae of strain Chlorant-R).

3.3 Genotyping by pyrosequencing

We designed a pyrosequencing assay allowing us to genotype individual larvae for the presence of the RyR I4790M mutation in nine field populations of *S. frugiperda* collected in six different states in Brazil in crop season 2018 (Table 1). The pyrosequencing assay was validated by genotyping 18 individual larvae of both strain Sus and Chlorant-R being 100% homozygous I4790 (ATA) and M4790 (ATG), respectively (Table 3). Furthermore, we analysed 10–23 field-collected larvae per sampling site, but all of the field-collected samples were homozygous wild-type, i.e. I4790 (Fig. 2). Owing to the low number of field-collected larvae available, we were not able to assess the precise frequency of the RyR I4790M resistance allele in those field samples; however, based on the data shown it is fair to claim that it is rather low (Table S2). We also analysed all field samples for the presence of an amino acid substitution at position G4891 (corresponding to G4946 in *P. xylostella* RyR) shown to confer diamide resistance, but no amino acid substitution was found. All analysed samples, including individuals of strains Sus and Chlorant-R were homozygous SS, i.e. G4946 (Table 3).

3.4 PCR-based allelic discrimination assay for genotyping

Because pyrosequencing is a rather expensive diagnostic tool, we decided to develop a PCR-based allelic discrimination assay using fluorescent probes, which could be easily implemented for large-scale monitoring of the I4790M resistance allele in field-collected samples. The fluorescent PCR assay was validated with genomic DNA samples prepared from individual larvae of

Table 2. Log-dose probit-mortality data for different diamide insecticides tested in combination with synergists against third instar larvae of two different laboratory strains of *Spodoptera frugiperda* in diet overlay assays (96 h)

	Sus (reference strain)				Chlorant-R (selected strain)				
Treatment	LC_{50} µg cm^{-2}	95% CI	Slope	SR[a]	LC_{50} µg cm^{-2}	95% CI	Slope	SR[a]	RR[b]
Chlorantraniliprole	0.020	0.016–0.024	2.1	-	4.5	3.4–5.6	3.4	–	225
+PBO	0.009	0.008–0.010	2.1	2.2	3.1	2.6–3.5	4.0	1.5	344
+DEF	0.013	0.011–0.015	1.8	1.5	2.9	2.2–3.5	2.8	1.6	223
+DEM	0.010	0.008–0.013	2.2	2.0	2.6	2.0–3.5	2.3	1.7	260
Flubendiamide	0.051	0.039–0.065	1.7	-	>280	–	–	–	>5400
+PBO	0.055	0.043–0.070	1.7	0.9	>280	–	–	–	>5000
+DEF	0.045	0.033–0.059	1.6	1.1	>280	–	–	–	>6200
+DEM	0.051	0.040–0.066	1.6	1.0	>280	–	–	–	>5400

[a] Synergistic ratio: LC_{50} of the insecticide alone divided by the LC_{50} of the insecticide + synergist.
[b] Resistance ratio: LC_{50} of strain Chlorant-R divided by LC_{50} of strain Sus.
PBO, piperonyl butoxide; DEF, *S,S,S*-tributyl phosphorotrithioate; DEM, diethyl maleate.

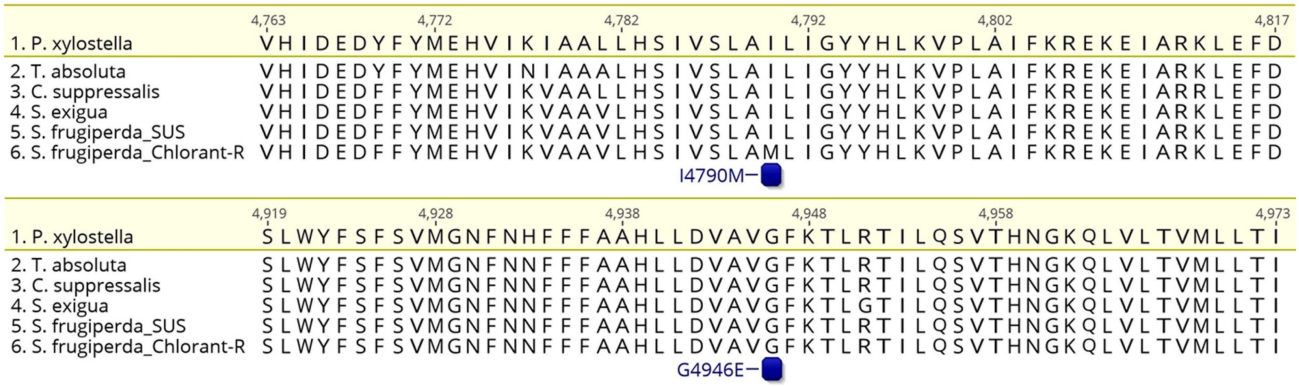

Figure 1. Multiple amino acid sequence alignment of a partial ryanodine receptor transmembrane domain encompassing sites harbouring potential mutation sites G4946E and I4790M (numbering according to *Plutella xylostella* RyR; GenBank AET09964) recently described in different lepidopteran pests. Ryanodine receptor mutation sites known to confer diamide insecticide resistance in *P. xylostella* are indicated by a blue square. GenBank accession numbers of the aligned ryanodine receptor wild-type sequences: *P. xylostella* (AET09964), *Tuta absoluta* (APC65631), *Chilo suppressalis* (AFN70719), *Spodoptera exigua* (AFC36359) and *S. frugiperda* strain Sus (MK226188). The alignment shows the presence of mutation I4790M, but not G4946E in the diamide-resistant *S. frugiperda* strain Chlorant-R.

Table 3. Genotyping by pyrosequencing of fall armyworm larvae of strains Sus (susceptible) and Chlorant-R (selected) for the presence of ryanodine receptor target-site mutations in the C-terminal transmembrane domain at amino acid positions I4790 and G4946 (numbering according to *Plutella xylostella* RyR). The diamide susceptible strain Sus is homozygous wild-type (SS) at the respective positions

Strain	Larvae tested	I4790 ATA	I/M4790 ATA/ATG	M4790 ATG	G4946 GGC		
Genotype	*n*	SS (%)	SR (%)	RR (%)	SS (%)	SR (%)	RR (%)
Sus	18	100	0	0	100	0	0
Chlorant-R	18	0	0	100	100	0	0

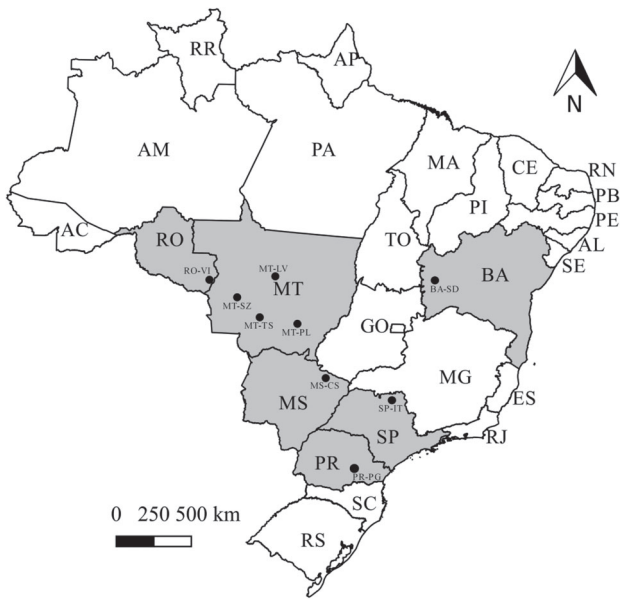

Figure 2. Genotyping for RyR target-site mutation I4790M in fall armyworm larvae collected in corn fields in Brazil during the 2018 crop season in the following states (see also Table 1): Paraná (PR), São Paulo (SP), Mato Grosso do Sul (MS), Mato Grosso (MT), Bahia (BA) and Rondônia (RO). All samples analysed were homozygous wild-type I4790.

strains Sus and Chlorant-R and allowed a clear differentiation of wild-type I4790 and mutated M4790 alleles, respectively (Fig. 3a). Mixing DNA samples of strains Sus and Chlorant-R revealed the capacity of the assay to detect (artificial) heterozygotes. In addition, we analysed all field-collected samples and were able to confirm the obtained pyrosequencing results, i.e. the absence of both RyR mutant I4790M heterozygotes and homozygotes (Fig. 3b).

4 DISCUSSION

Because of the evolution of resistance of FAW to some Bt proteins expressed in corn in Brazil,[10,46,48] the use of chemical insecticides has increased in some corn-producing regions.[9] However, frequent reports of resistance to major chemical insecticides such as spinosad,[19] *lambda*-cyhalothrin,[8,17] chlorpyrifos[8] and lufenuron,[18] challenges the control of this pest. Thus, the number of effective modes of action available to implement resistance management strategies, is shrinking. As an alternative, diamide insecticides have been introduced recently and used intensively for foliar, drench and seed treatment applications in diverse agronomic settings, thus increasing the selection pressure for the evolution of resistance, mainly in lepidopteran pests.[37,49] However, intense and frequent use of diamide insecticides has selected for field-relevant levels of resistance in a few lepidopteran pests such as diamondback moth and tomato leafminer,[31–34] and more recently beet armyworm – a noctuid pest.[36] In diamondback moth, tomato leafminer and rice stem borer, it has been shown that diamide resistance is conferred by RyR target-site mutations leading to amino acid substitutions at two major sites, G4946 and I4790.[31,39,40,44] It has been shown using radioligand binding studies, cell-based studies and CRISPR/Cas9 genome editing that these mutations are functional in conferring diamide resistance.[38,40–43] However, the molecular mechanisms of high levels of diamide resistance (RR > 100 000-fold) in beet armyworm, *S. exigua* remain unknown.

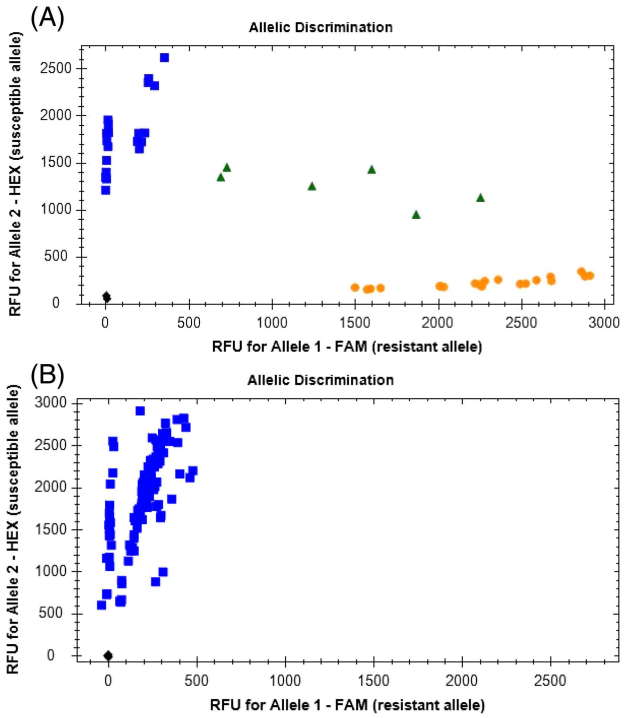

Figure 3. Bivariate plot showing the discrimination of different ryanodine receptor alleles in *Spodoptera frugiperda* samples by an allele-specific real-time PCR fluorescent probe assay. Each dot represents a single insect. (A) Blue squares represent strain Sus wild-type SS homozygotes (I4790; allele 2), orange circles strain Chlorant-R mutant RR homozygotes (M4790; allele 1), and green triangles SR heterozygotes (I4790/M4790) based on mixed RR and SS individuals. (B) Analysis of FAW field samples collected in Brazil (Table 2). All tested individuals were susceptible wild-type SS homozygotes (I4790; allele 2).

A low frequency of resistance to chlorantraniliprole has been reported in Brazilian field populations of FAW collected in 2012, 2013 and 2014.[50] More recently, a F2 screen and subsequent laboratory selection resulted in a FAW strain, Chlorant-R, showing high levels of diamide resistance.[45] The genetics of resistance in this strain was characterized as monogenic, incompletely recessive, and it has been shown that some heterozygotes would survive field-recommended rates of chlorantraniliprole, thus increasing the risk of rapid evolution of resistance.[45] At present, there are no reports of diamide field failure against FAW in Brazil, but this is likely to change, as selection pressure steadily increases, particularly due to the development of Bt toxin resistance, compromising the effectiveness of transgenic crops. Such a scenario is not unlikely, as reported recently from Puerto Rico, where FAW developed high levels of field resistance to chlorantraniliprole and flubendiamide due to increased selection pressure.[15]

Our study aimed to investigate the mechanism of resistance in a field-collected FAW strain, Chlorant-R, resulting from an F2 screen and subsequent laboratory selection with chlorantraniliprole. The strain unambiguously demonstrates the potential risk of the development of diamide resistance in FAW under applied conditions. It is interesting to note that selection with chlorantraniliprole resulted in extremely high levels of cross resistance against flubendiamide in strain Chlorant-R; in fact, rendering it completely inactive, whereas anthranilic diamides still show decent efficacy levels.[45] Synergists inhibiting detoxification enzyme families such as cytochrome P450s, esterases and glutathione *S*-transferases did not increase either chlorantraniliprole or flubendiamide efficacy in strain Chlorant-R, suggesting the absence of metabolic resistance. In almost all serious cases of diamide insecticide resistance in lepidopteran pests, metabolic resistance has been shown to play only a minor role, if any.[37]

Our molecular studies including the PCR amplification and sequencing of a FAW RyR fragment, spanning those C-terminal regions formerly described to contain target-site mutations, revealed the presence of an I4790M mutation (numbering according to *P. xylostella* RyR), described previously in other diamide-resistant lepidopteran pests.[39,40,44] The mutation I4790M is located at TM3, which is important for formation of the calcium channel pore and suggested to be part of the putative diamide binding site in insect RyRs.[38,39] The isoleucine in position 4790 is highly conserved in Lepidoptera but not in other insect orders such as Coleoptera or Diptera.[37] Therefore, it is hypothesized that this amino acid residue may contribute to the observed selectivity of the two chemotypes, phthalic and anthranilic diamides,[29,38] because phthalic diamides are less active against coleopteran pests, which have a methionine at this position in the RyR wild-type.[26,37] Flies that naturally have a methionine at the position corresponding to RyR I4790 in *P. xylostella* have been shown to be naturally ~70-fold more sensitive to clorantraniliprole than to flubendiamide.[42] However, CRISPR/Cas9 genome-modified transgenic flies expressing a RyR M4790I mutant were shown to be significantly more sensitive to both flubendiamide and chlorantraniliprole, although chlorantraniliprole was still 35-fold more toxic than flubendiamide.[42] The difference in toxicity between flubendiamide and chlorantraniliprole in diamide-resistant FAW (> 62-fold) is quite close to that reported in wild-type *Drosophila*, whereas the efficacy in the susceptible FAW strain differs just twofold (Table 2). Thus, suggesting other structural variations between dipteran and lepidopteran RyRs mediating differences in flubendiamide and chlorantraniliprole binding. Studies characterizing the diamide binding sites by radioligand competition assays in different insect species support our view as they revealed differences in anthranilic and phthalic-acid diamide binding affinity in *Musca domestica*, where high-affinity binding sites for flubendiamide are lacking.[28,29] On the other hand, in *Heliothis virescens* (syn. *Chloridea virescens*), similar binding affinities and identical sites for both chemotypes were observed.[29] The recently described G4946E substitution – not detected in Chlorant-R RyR – has been functionally linked to high levels of diamide resistance in at least two lepidopteran pests, *P. xylostella* and *T. absoluta*.[38,40,41] Recently, the G4946E mutation has been introduced *via* CRISPR/Cas9 technology in *S. exigua* and resulted in high levels of diamide resistance in transgenic beet armyworm.[43]

Although present at low frequency, FAW individuals carrying the RyR mutation I4790M conferring target-site resistance to diamides, have a high potential to develop field-relevant resistance levels compromising diamide efficacy under applied conditions. Therefore, we developed a PCR diagnostic test, i.e. a fluorescence-based allelic discrimination assay, based on FAW genomic DNA and differentiating the homozygous genotypes I4790 and M4790 as well as heterozygous M/I4790. The test is robust, cheap and easy to implement, and will help to monitor the extent and spread of the diamide resistance allele in cropping systems at risk and invaded by *S. frugiperda*. The presence of this mutation in Brazilian FAW field populations – albeit at very low levels – highlights the urgent need to implement resistance management strategies as recently suggested for diamide insecticides.[49] Otherwise there is a high risk of losing efficacy of this important chemical class in foliar, soil

and seed applications within the next few years due to the evolution of resistance based on RyR target-site mutations. It is of utmost importance to implement resistance management strategies based on mode of action rotation, biological control and effective trait technology to guarantee sustainable yields.

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SUPPORTING INFORMATION

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

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Chapter 3

Molecular characterization of Cry1F resistance in fall armyworm, *Spodoptera frugiperda* from Brazil

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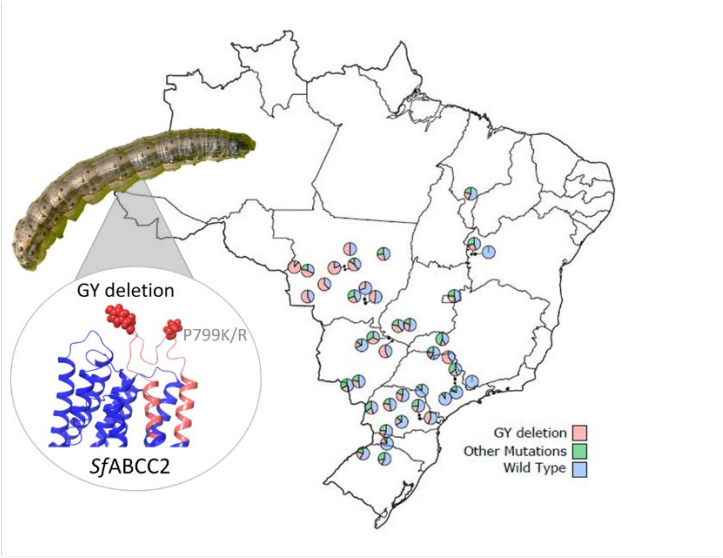

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Molecular characterization of Cry1F resistance in fall armyworm, *Spodoptera frugiperda* from Brazil

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ABSTRACT

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) is a major lepidopteran pest of maize in Brazil and its control particularly relies on the use of genetically engineered crops expressing *Bacillus thuringiensis* (Bt) toxins such as Cry1F. However, control failures compromising the efficacy of this technology have been reported in many regions in Brazil, but the mechanism of Cry1F resistance in Brazilian fall armyworm populations remained elusive. Here we investigated the molecular mechanism of Cry1F resistance in two field-collected strains of *S. frugiperda* from Brazil exhibiting high levels of Cry1F resistance. We first rigorously evaluated several candidate reference genes for normalization of gene expression data across strains, larval instars and gut tissues, and identified ribosomal proteins L10, L17 and RPS3A to be most suitable. We then investigated the expression pattern of ten potential Bt toxin receptors/enzymes in both neonates and 2nd instar gut tissue of Cry1F resistant fall armyworm strains compared to a susceptible strain. Next we sequenced the ATP-dependent Binding Cassette subfamily C2 gene (*ABCC2*) and identified three mutated sites present in ABCC2 of both Cry1F resistant strains: two of them, a GY deletion (positions 788–789) and a P799 K/R amino acid substitution, located in a conserved region of ABCC2 extracellular loop 4 (EC4) and another amino acid substitution, G1088D, but in a less conserved region. We further characterized the role of the novel mutations present in EC4 by functionally expressing both wild type and mutated ABCC2 transporters in insect cell lines, and confirmed a critical role of both sites for Cry1F binding by cell viability assays. Finally, we assessed the frequency of the mutant alleles by pooled population sequencing and pyrosequencing in 40 fall armyworm populations collected from maize fields in different regions in Brazil. We found that the GY deletion being present at high frequency. However we also observed many rare alleles which disrupt residues between sites 783–799, and their diversity and abundance in field collected populations lends further support to the importance of the EC4 domain for Cry1F toxicity.

1. Introduction

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is a polyphagous lepidopteran pest species, causing significant damage in several economically important crops, particularly maize in Brazil (Ivan Cruz, 1995; Barros et al., 2010). The control of this pest has relied mainly on synthetic insecticides, which led to the evolution of resistance to different chemical classes (Diez-RodríGuez and Omoto, 2001; Carvalho et al., 2013; Nascimento et al., 2016; Okuma et al., 2017; Bolzan et al., 2019). Currently, the main measure to control fall armyworm in Brazil involves the use of genetically engineered crops expressing *Bacillus thuringiensis* (Bt) toxins (O. Bernardi et al., 2015).

Since the introduction of transgenic maize expressing Cry1F in 2009, the Bt technology has been adopted in large scale year-round production in Brazil, with limited refuge areas of non-Bt plants (Horikoshi et al., 2016). Subsequently, the presence of the Cry1F protein in both maize and cotton products contributed to the evolution of Cry1F-resistance in fall armyworm, which was first reported in 2014

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(Farias et al., 2014).

The inheritance of Cry1F resistance in Brazil has been described for *S. frugiperda* as (incompletely) recessive, autosomal and monogenic (Farias et al., 2014; Leite et al., 2016; Santos-Amaya et al., 2016a), and many recent studies have shown cross-resistance among Cry1F, Cry1A.105, Cry1Ac and Cry1Ab (Vélez et al., 2013; D. Bernardi et al., 2015; Santos-Amaya et al., 2016b; Burtet et al., 2017). Field-evolved resistance of fall armyworm to Cry1F in Argentina was characterized as autosomal and incompletely recessive (Chandrasena et al., 2018). In order to implement reliable resistance management strategies, it is important to understand the molecular mechanism of Bt resistance. A broadly accepted model of Bt toxicity is that once the crystalline inclusions containing the Cry proteins are ingested by the insect, they act in a sequential manner on different targets in the insect midgut. The Cry proteins have to be solubilized and processed to an active toxin. By crossing the peritrophic matrix, the activated toxins then interact with different enzymes and receptors, resulting in pore formation, osmotic cell lysis and insect death (Bravo et al., 2007; Adang et al., 2014). A number of proteins have been reported as receptors for the Cry toxins, including aminopeptidase N (APN), cadherin (CAD), alkaline phosphatases (ALP) and ATP-binding cassette (ABC) transporters (Bravo et al., 2007). A well-known Bt toxin resistance mechanism is the reduction of Cry toxin binding to their specific midgut receptors, by changes in the expression level and/or mutations (Bravo et al., 2007; Heckel et al., 2007). Many studies have indicated a major role of ABC transporter subfamily C2 (ABCC2) in mediating the insertion of Cry toxins into the midgut membrane of lepidopteran species (Gahan et al., 2010). Mutations in the ABCC2 transporters have been linked to Cry1-type resistance in many lepidopteran pests (Gahan et al., 2010; Atsumi et al., 2012; Park et al., 2014; Xiao et al., 2015), including *S. frugiperda* (Banerjee et al., 2017; Flagel et al., 2018). The resistance to Cry1F in fall armyworm populations from Puerto Rico has been linked to an insertion of two nucleotides in the *ABCC2* gene, which lead to a premature stop codon and consequently a non-functional receptor for the Bt toxin (Banerjee et al., 2017; Flagel et al., 2018).

Nevertheless, until now the mechanism of Cry1F resistance in fall armyworm populations from Brazil is unknown. Therefore, understanding the molecular basis is critical to develop effective resistance management programs and sustain the Bt technology (Tabashnik et al., 2013). In the present study we elucidated the molecular mechanism of Cry1F resistance in *S. frugiperda* from Brazil. For this purpose we first selected stable reference genes with low expression variance among strains, larval stages and gut tissue. We then investigated in one susceptible and two Cry1F-resistant fall armyworm strains the expression pattern of known receptors/enzymes involved in Bt toxin mode of action. Next we screened for mutations in full length sequences of *ABCC2* and characterized the functional role of non-synonomous mutations using cell toxicity assays. Finally, we assessed the frequency of the mutant alleles in fall armyworm populations recently collected from maize fields in different regions in Brazil by different technologies.

2. Material and methods

2.1. Fall armyworm strains

Three *S. frugiperda* strains, Sf_Bra (susceptible to Cry1F), Sf_Cor and Sf_Des (field-resistant to Cry1F) were collected in Brazil according to Table S1. The insects were reared under controlled conditions (25 ± 1 °C, 55 ± 5% relative humidity) in the laboratory on standard noctuid artificial diet without exposure to any Bt toxin or synthetic insecticides. For pyrosequencing genotyping larvae were collected at ten sites in Brazilian non-Bt maize fields in 2017–2018, and preserved in alcohol before shipment (Table S1).

An additional 30 populations were collected in 2016 from non-Bt maize fields in Brazil for pooled population sequencing (Table S1; those named FAW_). Field-collected larvae were transferred to the laboratory and reared on artificial diet. The adults (P0) of the different populations were mass-mated and the resulting F1 generation neonate larvae were tested on leaf tissue expressing Cry1F (TC1507 maize) and non-Bt maize using a leaf-disc bioassay. Briefly: Completely expanded leaves were removed from the maize whorl of greenhouse-grown plants at the V4–V6 stage. Leaf discs measuring 2.0 cm in diameter were cut using a metallic cutter and placed on a non-gelled mixture of water and agar at 20 g/L (1 ml/well) in acrylic plates with 12 wells (Costar®, Corning, Tewksbury, MA, USA). Leaf discs were separated from the water-agar layer by a filter paper disc. One neonate larva (< 24 h old) was placed on each maize leaf disc using a fine brush. Plates were sealed with plastic film and appropriate lids and placed in a climatic chamber (temperature: 25 ± 1 °C; relative humidity: 60 ± 10%; photoperiod: 14:10 (L:D)). The experimental design was completely randomized with 10 replicates per treatment, totaling 120 neonates of each *S. frugiperda* collection tested on TC1507 and non-Bt maize. Larval survivorship was recorded at five days after leaf disc infestation (Table S1). A sub-set of the adults (P0) of each of the field populations were flash frozen in liquid nitrogen for molecular analysis.

2.2. Diet overlay assays

Cry1F used throughout the study was produced by a *B. thuringiensis* recombinant strain and kindly provided internally in 50 mM sodium carbonate buffer (pH 10.4). Xentari™ (Neudorff, Germany), a Bt-based insecticide (composed of Cry1Aa, Cry1Ab, Cry1C and Cry1D), is registered in different crop systems for the control of fall armyworm in Brazil (Horikoshi et al., 2019).

The diet overlay assays were performed according to Marçon et al. (1999), with modifications. Briefly: seven different concentrations (66.87–48,750 ng cm^{-2} for the protoxin Cry1F and 22,500–30.86 ng cm^{-2} for Xentari™) diluted in 50 mM sodium carbonate buffer (pH 10.4) and 0.1% (v/v) Triton X-100 were applied (25 µL/well) to the surface of the artificial diet in a 48-well plate (Greiner CELLSTAR®, Merk). A single *S. frugiperda* neonate larvae (< 24 h old) of susceptible (Sf_Bra) and Cry1F-resistant strains (Sf_Cor and Sf_Des) was placed into each and plates were incubated under controlled conditions for five days (25 ± 1 °C, 55 ± 5% relative humidity, 16:8 h (L:D) photoperiod). Twelve larvae (for the two highest concentrations of Xentari™) or 24 larvae (for all Cry1F concentrations and for the five lowest concentrations of Xentari™) in total were used per toxin concentration and control (buffer and 0.1% (v/v) Triton X-100). The bioassay was replicated four times. The mortality was scored after five days and larvae that did not reach the second instar after this time were counted as dead. Control mortality was corrected according to Abbott (1925) and data were fitted by a logistic regression model to calculate LC_{50}/LC_{95} values and 95% confidence intervals (GraphPad Software Inc. v.5, CA). Survivors from Sf_Des and Sf_Cor exposed to the highest concentration of Cry1F (48,750 ng cm^{-2}) were collected and kept at −80 °C, for later genomic DNA extraction.

2.3. Genetics of resistance

Both Cry1F resistant strains, Sf_Des and Sf_Cor, were originally collected in Bahia State within short distance from one another and showed similar resistance levels to Cry1F, therefore the experiments on the genetics of resistance were confined to strain Sf_Des. Neonates from Sf_Des were selected against Cry1F on artificial diet overlaid with Cry1F (48,750 ng cm^{-2}). The survivors of Cry1F exposure were used to generate the F1 progeny. Briefly: Newly emerged virgin females of strain Sf_Des were crossed with males of strain Sf_Bra and *vice versa* (n = 20 couples each). The F1 eggs were collected and neonates were tested against Cry1F as described above. Since there was no significant Cry1F toxicity difference observed between F1 cohorts (based on overlapping 95% confidence intervals) the F1 generation was pooled for further studies, similar to other studies (Storer et al., 2010;

Chandrasena et al., 2018). Subsequently the F1 generation was backcrossed with the parental strain Sf_Des to check for monogenic resistance (single pair mating, n = 14). From each backcross, 30 larvae were tested against a discriminating dose of Cry1F (5,400 ng cm^{-2}) and 12 larvae were tested against 50 mM sodium carbonate as control. Larvae were scored for mortality after five days and survivors on Cry1F treated and untreated diet were stored at −80 °C for later genotyping by pyrosequencing. The proportion of genotypes (RR and RS) obtained in larvae untreated was analyzed by Chi-square test (GraphPad Software Inc. v.8, CA).

To estimate dominance we used two methods, one based on LC_{50}-values as described by Bourguet et al. (1996), and a single-concentration method (Liu and Tabashnik, 1997; Bourguet et al., 2000), based on survival at a discriminating dose of Cry1F of pooled F1 obtained from reciprocal crosses of strains Sf_Bra and Sf_Des. The level of dominance of Cry1F resistance based on LC_{50}-vaues (D_{LC}) obtained in dose-response bioassays was calculated by the following formula: D_{LC} = ($logLC_{50}$RS-$logLC_{50}$SS)/($logLC_{50}$RR-$logLC_{50}$SS). Dominance based on corrected mortality (ML) in single discriminating dose bioassays (D_{ML}) was determined by the following formula: D_{ML} = (ML_{RS}-ML_{SS})/(ML_{RR}-ML_{SS}). Values of D_{LC} and D_{ML} range from 0 (completely recessive) to 1 (completely dominant). Resistance is called codominant when D_{ML} is 0.5 (Liu and Tabashnik, 1997).

2.4. Total RNA extraction and cDNA synthesis

Total RNA was extracted from larvae (pools of 10 larvae per replicate) of three *S. frugiperda* strains (Sf_Bra, Sf_Cor and Sf_Des) with TRIzol® reagent (Invitrogen, CA, USA) and followed by RNA purification according to RNeasy® Plus Universal Mini Kit (QIAGEN, Germany) according to manufacturer's instruction including a genomic DNA eliminator column step. ARCTURUS® PicoPure® RNA Isolation Kit (Applied Biosystems, USA) was used to extract RNA from neonates (pool of 30 larvae per replicate) and gut tissue of second instar larvae (pool of 10 guts per replicate) following the manufacturer's instructions, including a DNA digestion step with DNase I (QIAGEN, Germany). The RNA was quantified by spectrophotometry (NanoQuant Infinite 200, Tecan, Switzerland) and its integrity verified by an automated gel electrophoresis system, according to CM-RNA and CL-RNA methods (QIAxcel RNA QC Kit v2.0, QIAGEN, Germany). One µg total RNA was used in 20 µL reactions for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad, USA) for RT-qPCR analysis and SuperScript™ III Reverse Transcriptase and oligo(dT) 20 primer (Thermo Fisher Scientific, USA) were used for ABCC2 amplification, following the manufacturer's instructions.

2.5. RT-qPCR

RT-qPCR reactions were performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) 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), 400 nM of reverse/forward primers (Table S2), and nuclease-free water. Reactions were run in triplicate using CFX384™ Real-Time system (Bio-Rad, USA) and non-template mixtures as negative controls. The PCR conditions were: 3 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A final melt-curve step was included post-PCR (ramping from 65 °C–95 °C by 0.5 °C every 5 s) to check for non-specific amplification. Amplification efficiencies were determined by a 5-fold dilution series revealing for all primers an efficiency ≥93%.

2.5.1. Expression analysis of putative Cry1F target genes

The expression level of ten genes coding for putative Bt-binding proteins was investigated by quantitative RT-PCR in neonates and gut tissue of second instar larvae, as described above (2.4). Primer pairs used in this study were described in Table S2. The most stable reference genes (*L17*, *L10*, and *RPS3A*) were used for normalization. The normalized expression levels were calculated from four to six biological replicates (30 individuals each for neonates and 10 individuals for the gut tissue). The values obtained for Sf_Cor and Sf_Des were compared to Sf_Bra and statistically tested by an unpaired *t*-test ($P < 0.05$) with qbase + software (v. 3.2; Biogazelle, Belgium).

2.6. Screening for reference genes

Reference gene expression stability was tested in all three *S. frugiperda* strains (Sf_Bra, Sf_Cor and Sf_Des). The samples contained pools of neonates (n = 30 each), guts and whole body from different larval developmental stages (2nd to 6th instar; n = 10 each). For the different sample groups three biological replicates were used.

We selected ten candidate reference genes that have been commonly used as internal controls in qPCR data analysis in the literature (Lu et al., 2013; Zhu et al., 2014; Jakka et al., 2016; Li et al., 2017): *ACT1, ACT2, AK, EF2, GAPDH, L10, L17, L18, RPS3A* and *UCCR* (Table S2). The fall armyworm reference gene sequences were identified in a custom *S. frugiperda* transcriptome generated at Bayer AG through queries using local BLAST searches and deposited in GenBank (for accession numbers see Table S3). The primers were designed using Gene Runner software v. 6.5.48 Beta (http://www.generunner.com) or Geneious software v. 10.2.3 (Biomatters Ltd., New Zealand).

The expression stability of the ten candidate reference genes was evaluated using geNorm qBase Plus v3.1 software (Biogazelle, Belgium) and Normfinder (moma.dk/normfinder-software) by using raw Cq values obtained from CFX Maestro 1.0 v4.0 (Bio-Rad) software. Detailed information on the geNorm qBase Plus application can be found in Vandesompele et al. (2002). The Excel based tool Normfinder was used to estimate the reference gene stability according to Andersen et al. (2004).

2.7. Expression analysis of putative Cry1F target genes

The expression level of ten genes coding for putative Bt-binding proteins was investigated by quantitative RT-PCR in neonates and gut tissue of second instar larvae, as described above (2.4). Primer pairs used in this study were described in Table S2. The most stable reference genes (*L17*, *L10*, and *RPS3A*) were used for normalization. The normalized expression levels were calculated from four to six biological replicates (30 individuals each for neonates and 10 individuals for the gut tissue). The values obtained for Sf_Cor and Sf_Des were compared to Sf_Bra and statistically tested by one-way ANOVA, followed by Tukey-Kramer multiple comparison test with qbase + software (v. 3.2; Biogazelle, Belgium).

2.8. Amplification of SfABCC2 cDNA

The full-length *S. frugiperda ABCC2* (syn. Sf*ABCC2*) (GenBank accession No. KY489760) was amplified from cDNA from six biological replicates of ten guts of 4th instar larvae. Primers and PCR conditions were used as described in Flagel et al. (2018). The PCR products were validated by an automated gel electrophoresis system, according to OM500 method (QIAxcel DNA Screening Kit v2.0, QIAGEN, Germany), purified using innuPREP PCRpure kit (Analytik Jena, Germany) and cloned according to TOPO® TA Cloning® (Thermo Fisher Scientifics, Germany) recommendations. Samples were purified and Sanger-sequenced by Eurofins Genomics (Ebersberg, Germany). The sequencing results were assembled and mapped to the reference Sf*ABCC2* sequence using Geneious software v. 10.2.3 (Biomatters Ltd., New Zealand) in order to identify non-synonymous mutations in the *ABCC2* of Cry1F-resistant fall armyworm strains. The full-length ABCC2 amino acid sequence of strain Sf_Bra (GenBank MN399979) is virtually identical to the ABCC2 reference sequence (*S. frugiperda*; GenBank KY489760; Figure S6). The translated ABCC2 amino acid sequences were then

aligned with *Bombyx mori* (BAK82126); *Helicoverpa armigera* (AHL68986); *Heliothis virescens* (syn. *Chloridea virescens*) (ADH16740); *Plutella xylostella* (AEI27593); *Spodoptera exigua* (AIB06822) and *S. frugiperda* (KY489760) using the Geneious Alignment tool in Geneious software v. 10.2.3 (Biomatters Ltd., New Zealand).

2.9. Modelling SfABCC2

Homology models for the SfABCC2 protein were generated using the Advanced Homology Modeling tool within the software suite Maestro v.2018/2 (Schrödinger, 2018). The cryo-EM structure of bovine (*Bos taurus*) Multidrug Resistance Protein (MRP1) (PDB- identity number: 5UJ9) (Johnson and Chen, 2017) was suggested as the most suitable template after pasting the SfABCC2 sequence as the fold query. For bovine MRP1 two conformational states are known and structurally available: the apo form (3.5 Å resolution) and a substrate-bound state (3.3 Å resolution), complexed with one of its substrates (leukotriene C4); as both states differ considerably from each other, homology models based on each of them were generated stepwise.

A knowledge-based homology model building procedure was chosen to generate the respective 3D-structures, followed by energy minimization calculations, to correct for distortions and unwanted clashes from sidechain replacements and backbone insertions/deletions.

After visual inspection of both models (based on the coordinates of the apo- and substrate-bound conformational state) the mutations potentially linked to Cry1F resistance were substructure-labelled and illustrated by a space-fill representation of the respective residues following the numbering of the bovine MRP1 sequence. A region at the C-terminal end, that was poorly resolved (just backbone atoms, no residues assigned) in the cryo-EM study, was omitted in the presented illustration of the SfABCC2 homology model.

2.10. Recombinant expression of SfABCC2 using a baculovirus expression system

Wild type *SfABCC2* (GenBank KY489760) was purchased by GeneArt (USA) containing 3xFLAG tag in the C-terminal end and inserted into the vector pFastBac™1 (Invitrogen). SfABCC2 mutants were constructed using Q5® Site-Directed Mutagenesis Kit (New England, Biolabs) according to manufacturer's instructions. The primers used for mutagenesis, SfABCC2_GYdel and SfABCC2_P799K were designed by NEBaseChanger™ tool (TableS2). Each mutation was created separately, followed by plasmid purification with Monarch® Plasmid Miniprep Kit (New England, Biolabs), checked by restriction digestion and sequenced by Eurofins Genomics (Ebersberg, Germany).

The expression vector containing one of the four ABCC2 variants (SfABCC2_wt, SfABCC2_GYdel, SfABCC2_P799K and SfABCC2_GYdel + P799K) was transformed in MAX Efficiency® DH10Bac™ according to manufacturer's instructions (Gateway® technology, Invitrogen). Recombinant baculovirus DNA was purified using Qiagen® Large-construct kit (QIAGEN, Germany) and transfected to Sf9 insect cells (Gibco) using the Bac-to-Bac® baculovirus expression system (Thermo Fisher Scientific, USA), according to manufacturer's instructions. Sf9 cells were maintained in suspension culture under serum-free conditions (SF-900™ III SFM, Gibco) at 27 °C, with orbital shaking at 130 rpm and 10 μg mL^{-1} gentamycin (Thermo Fisher, USA) until the first signs of cell infection were observed. The titer of the recombinant viruses was determined according to the protocol described by Kitts and Green (1999). At the density of 2×10^6 cells mL^{-1}, Sf9 cells were infected with a multiplicity of infection (MOI) of 1. The cells were kept as aforementioned, but provided with 0.5% heated-inactivated fetal bovine serum (FBS) (Gibco). After 48 h, the cells were harvested and re-suspended at 2×10^6 cells $mL^{-1}$with either phosphate-buffered saline (PBS) or SF-900™ III SFM media.

2.10.1. Detection of expressed SfABCC2 by Western blotting

Cells expressing different variants of SfABCC2 were re-suspended in ice-cold lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS solution and 1 tablet of cOmplete™ proteinase inhibitor cocktail for 50 mL buffer). Total protein concentration of samples was determined by BCA method (Pierce), using BSA (Bio-rad, USA) as reference. Twenty five μg of protein was loaded onto SDS-PAGE (NuPAGE Novex Bis-Tris Mini Gel 4–12%, Invitrogen) and coomassie stained (Imperial™ Protein Stain, Thermo Fisher Scientific) after separation. Cell lysates were also separated by SDS-PAGE and blotted on PVDF membrane (Thermo Fisher Scientific) according to manufacturer's protocol. The membrane was blocked with 5% BSA in TBST for 1 h at room temperature and incubated overnight at 4 °C with monoclonal ANTI-FLAG M2 (Merck) as primary antibody (1:1,000 dilution in 5% BSA blocking solution). Anti-mouse IgG1 coupled to horseradish peroxidase (Invitrogen) (1:10,000 dilution in 5% BSA blocking solution) was used as secondary antibody and detected using SuperSignalWest Dura kit (Pierce) on an Imaging System (G:Box, Syngene).

2.11. Cytotoxicity test with wild type and mutant SfABCC2

Cry1F and Xentari™ solutions in 50 mM sodium carbonate buffer (pH 10.4) were adjusted to pH 8.5 by adding 1 M Tris buffer. The protoxins were activated with trypsin (Sigma-Aldrich, USA) using a ratio 1:30 (trypsin/protoxin, w/w) for 1 h at 37 °C. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride (PMSF) at 1 mM final concentration. The activated toxins were analyzed by 4–12% SDS gradient PAGE (NuPAGE, Novex Bis-Tris, Thermo Fisher Scientific) and their concentrations determined by spot densitometry by ImageJ analysis (Rasband, 2018) using bovine serum albumin (BSA) (Bio-rad, USA) as standard. Aliquots were kept at −20 °C until use.

The cytotoxic tests were performed after 48 h incubation with baculovirus harboring the different *SfABCC2* variants. Each treatment consisted of three replicates and the experiment was performed using two independent batches of cells expressing the *SfABCC2* variants. The cell number was normalized according to the viable cells assessed with Trypan blue (Merck, Germany) and the automated cell counter LUNA-FL™ (Logo, Biosystems). Cell pellets were collected by centrifugation and used for cytotoxicity tests and Western blotting.

Briefly, initial cytotoxicity tests comprised of cells expressing each SfABCC2wt, SfABCC2_GYdel, SfABCC2_P799K, or SfABCC2_GYdel + P799K mutants in PBS solution seeded on a 96-well plate (Corning®, USA) and treated with approximately 0.2 μg mL^{-1} of Cry1F at 27 °C for 1 h. The release of lactate dehydrogenase (LDH) was measured using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Germany) according to the manufacture's protocol. Percentage viability was calculated in relation to cells treated without toxin (considered as 100% viable) and cells treated with lysis buffer (considered 0% viable).

To back up the results we performed a second cytotoxicity test based on image analysis. Sf9 cells expressing different forms of SfABCC2 were kept under the conditions described above. Medium was removed and new SF-900™ III SFM (Gibco) was added to obtain 2×10^6 cells mL^{-1}. The cells were seeded in 384-well plates (PerkinElmer™) one day before the experiment. Activated Cry1F or Xentari™ (approximately 0.2 μg mL^{-1}) were incubated with the cells for 1 h at 27 °C. After that, the medium was rinsed and the cells were stained by adding 50 μL of SFM III medium containing Calcein AM (Thermo Fisher Scientific) (2 μM) for 20 min. The medium was rinsed again, and wells were filled with 50 μL of SFM III medium with Hoechst33342 (Sigma-Aldrich, USA) (2 μg mL^{-1}) for 10 min. Measurements were taken with ImageXpress Micro XLS (Molecular Devices, USA) using EGFP and DAPI filters and analyzed by MetaView® imaging system (Universal Imaging Co., Westchester, PA). For each well, the average fluorescence intensity was measured in three different replicates per treatment and relative cytotoxic effect was calculated by dividing the average intensity of Cry1F/Xentari™ treated cells by the average intensity of cells treated

with PBS.

The data for both cytotoxicity tests were separately analyzed by one-way ANOVA, followed by Tukey's multiple comparison test (GraphPad Software Inc. v.5, CA).

2.12. Pyrosequencing genotyping assay of fall armyworm field samples

Genomic DNA was extracted from single 3rd instar larvae preserved in RNAlater® (Life technology, USA) (n = 9 to 22 individuals per population, Table S1) from populations collected in the field, as well as from survivors of the diet overlay assay using DNeasy Blood & Tissue Kit (QIAGEN, Germany) or QuickExtract™ DNA Extraction Solution 1.0 (Epicentre, USA) according to the suppliers' recommended protocols.

Primer pairs were designed with Assay Design Software (QIAGEN, Germany) targeting the GY deletion at positions 788–789 and the target site mutation P799K, separately. Next, the PCR for pyrosequencing was performed in 30 μL reaction mixture containing 15 μL JumpStart™ Taq ReadyMix™ (Sigma-Aldrich, USA), 500 nM of forward and reverse primer (one biotinylated, see Table S2), around 50 ng gDNA and nuclease-free water. The cycling conditions comprised 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s and a final elongation step at 72 °C for 5 min. The pyrosequencing reaction was carried out as described elsewhere (Troczka et al., 2012) using the sequencing primers described in Table S2.

2.13. Pooled population sequencing

Approximately 50 individuals from each of 30 populations were collected from the field during the Brazilian summer of 2016–2017 and pooled (Table S1). DNA was bulk extracted from each pool using the E.Z.N.A. Insect DNA isolation kit (Omega Bio-Tek). Illumina sequencing libraries were prepared for each pool using the Kapa HyperPlus PCR-Free kit (Roche) and sequenced on an Illumina NextSeq 500 genome sequencer in 2 × 151 bp paired-end configuration. Each pool was sequenced to approximately 50X genomic coverage.

Following sequencing, reads were quality trimmed using trimmomatic (Bolger et al., 2014), and aligned to the *S. frugiperda* corn variant reference genome (Gouin et al., 2017) (v3.1; downloaded from http://bipaa.genouest.org/data/public/sfrudb/corn_assembly_v3.1_20141112/) using Bowtie2 (Langmead and Salzberg, 2012). In the *S. frugiperda* corn variant reference genome (Gouin et al., 2017), SfABCC2 is annotated as GSSPFG00033000001.2, which occurs on scaffold_7154. We focused on mutations in the 14th exon of this gene (closely corresponds to the extracellular loop 4 (EC4)), which occurs from 98 to 150 bp on scaffold_7154. From every pool we extracted all reads that covered the entire interval listed above and translated each one into its corresponding protein sequence. Furthermore a "full-length gene" indel polymorphism analyses was conducted in *ABCC2* of field-collected populations (Table S1) by mapping reads on GenBank contig MKQC01018747.1, a complete copy of *ABCC2* containing 24 exons from the *S. frugiperda* Sf9 cell genome. Illumina data have been deposited in NCBI under the BioProject accession number PRJNA545483 (Table S4). Reads that only partially covered the interval were discarded.

3. Results

3.1. Bioassays and genetics of resistance

Both Sf_Cor and Sf_Des strains showed high levels of resistance to Cry1F (Fig. 1A), when compared to the susceptible strain, Sf_Bra (Table S5). On the other hand, the bioassay results against Xentari™ showed very low resistance ratios (RR ≤ 5-fold) for the Cry1F-resistant strains most likely not relevant under applied aspects, i.e. almost exhibiting a similar susceptibility to this Bt-based product as the susceptible strain Sf_Bra (Fig. 1B). Reciprocal crosses of strains Sf_Des and Sf_Bra revealed

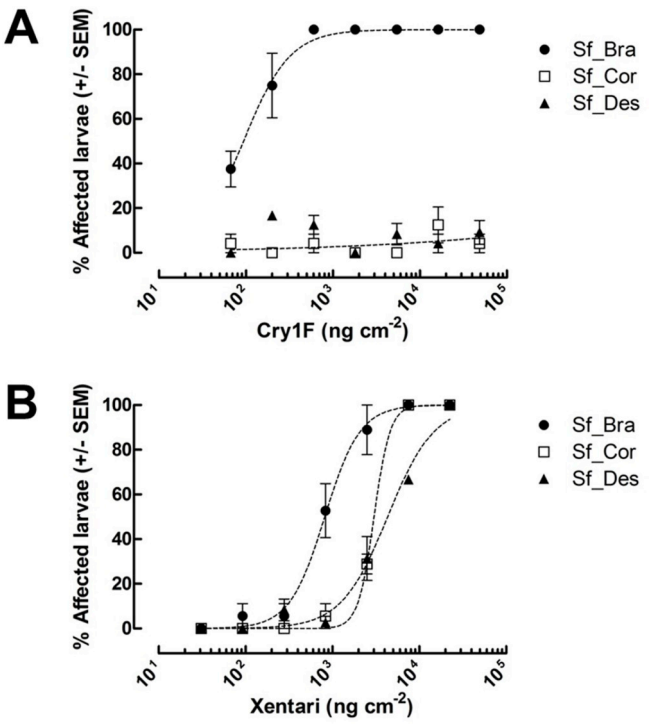

Fig. 1. Toxicity of (A) Cry1F and (B) Xentari™ towards different *Spodoptera frugiperda* strains. Sf_Bra is a susceptible reference strain and Sf_Cor and Sf_Des are strains collected in maize fields with Cry1F resistance issues.

autosomal inheritance and no significant difference in mortality between F1 progeny based overlapping 95% confidence intervals of Cry1F LC_{50}-values (Figure S1), therefore dominance was estimated based on combined data of the F1 cohorts due to the lack of sex linkage associated with the inheritance (Table S6). Estimated dominance in heterozygotes based on combined dose response data revealed a D_{LC} value of < 0.33, suggesting an incompletely recessive mode of inheritance. However estimated effective dominance (D_{ML}) of Cry1F resistance, based on a mortality endpoint in a discriminating dose bioassay, indicated highly recessive resistance (D_{ML} 0.015; Table S7). The mortality rate of F1 ♀ x Sf_Des ♂ backcross progeny exposed to a discriminating dose of 5,400 ng cm^{-2} Cry1F – killing 100% and approx. 99% of larvae of susceptible Sf_Bra and F1 parents, respectively – was 54 ± 6.5%, suggesting that a single locus (or a set of linked loci) has a major impact on Cry1F resistance in strain Sf_Des (Table 1). Testing a range of concentrations on backcross progeny would have revealed a more reliable result to support a monogenic resistance model, but a shortage in Cry1F protein amounts forced us to conduct a single concentration assay.

3.2. Reference gene selection

To find stable reference genes for accurate gene expression normalization, the expression of 10 candidate reference genes was analyzed in gut tissues and whole body from different larval stages of three *S. frugiperda* strains. The most stable genes for all tested sample groups were *L17*, *L10* and *RPS3A* with low M-values (below the default limit of M ≤ 0.5) and high expression stability (Fig. 2A; Figure S2 A, B).

3.3. Expression profile of genes potentially involved in Bt mode of action

The expression levels of 10 genes potentially involved in the Bt toxin mode of action were analyzed in neonates and gut tissue of second instar larvae from susceptible (Sf_Bra) and Cry1F-resistant strains (Sf_Cor and Sf_Des) by RT-qPCR. The most significant differences (p-value < 0.05) in the expression level were observed at neonate stage

Table 1
Mortality and genotypic composition of F1 (RS for GY deletion) x Sf_Des (RR for GY deletion) backcross progeny surviving artificial diet bioassays overlaid with a discriminating dose of Cry1F (5400 ng cm^{-2}). Control treatment was solvent only and a Chi-square test was used to analyze deviation between observed and expected genotypes.

Individual larvae were genotyped and analyzed for the presence/absence of a GY deletion at sites 788–789 of the ABCC2 transporter gene.

Backcross	Treatment	Mortality % ± SE	χ^2 (df)	P	n	SS (%)	SR (%)	RR (%)
F1 ♀ x Sf_Des ♂	Control	0 ± 0	0.36 (1)	0.55	51	4	45	51
	Cry1F	54 ± 6.5			51	0	0	100

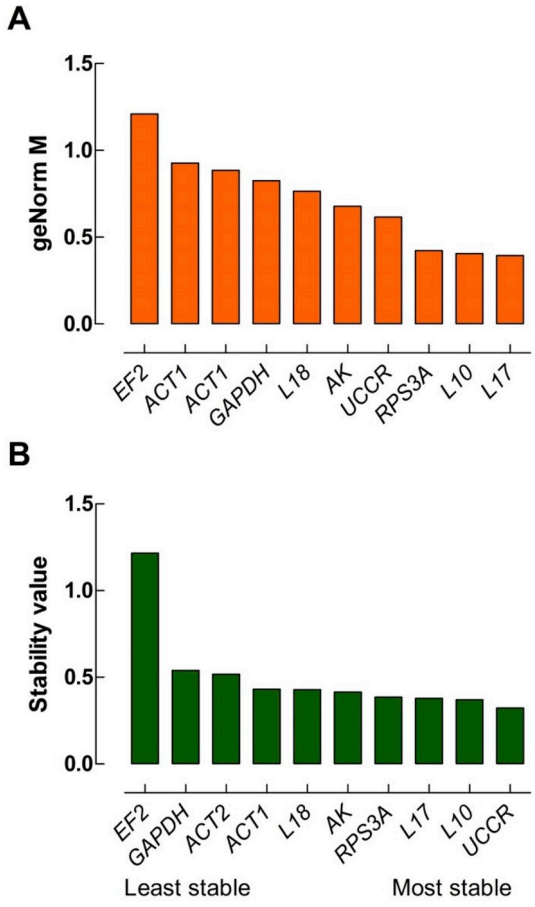

Fig. 2. Stability of genes considering expression in whole body and gut tissue throughout the larval development of three strains of *Spodoptera frugiperda*. Gene expression stability was evaluated by using methods based on (A) geNorm qBase Plus and (B) Normfinder. For further details refer to Material and Methods.

in Sf_Des strain (Fig. 3; Table S8): sALP1, mALP2, APN1 and APN2 were overexpressed while *ABCC2* and *ABCC3* were down-regulated. The expression of *APN6* gene was abundant in both resistant strains, while no expression was detected in Sf_Bra neonates. A similar result was observed for *mALP1*, which had such a low expression in all three strains at neonate stage that it could not be quantified, even after designing different primer pairs (data not shown).

From the 10 genes analyzed, three were differently expressed (p-value < 0.05) in gut tissue, *ABCC3* and *mALP1* were down-regulated in Sf_Cor, while *APN6* was over-expressed in Sf_Des. There was no significant difference in the expression level in gut tissue for *ABCC2*, *CAD*, *sALP1, mALP2, APN1*, *APN2* and *APN5* of resistant strains when compared to Sf_Bra at gut of 2nd instar larvae.

3.4. Amplification of SfABCC2 and computational modelling

The full length sequence of *SfABCC2* was amplified and aligned with published lepidopteran ABCC2 amino acid sequences (Fig. 4A) revealing two main mutations, which were present exclusively in the Cry1F resistant strains. A deletion of six nucleotides in the extracellular loop IV (EC4) between transmembrane domains VII (TM7) and VIII (TM8) resulted in the deletion of two amino acids (glycine and tyrosine) at positions 788 and 789. In the same loop, at site 799 an amino acid substitution was found as result of a non-synonymous mutation. The proline at this site was substituted with either lysine (Sf_Des) or arginine (Sf_Cor) (Fig. 4B). The obtained consensus sequences of *S. frugiperda* ABCC2 of strains Sf_Bra, Sf_Des and Sf_Cor – based on the six sequenced biological replicates of ten guts of 4th instar larvae – were submitted to GenBank (accession numbers MN 399979, MN399978 and MN 399980, respectively). Supplementary Figure S6 provides an amino acid alignment of all six replicates of the ABCC2 sequence obtained by sequencing of individuals of strain Sf_Bra.

The alignment of *S. frugiperda* ABCC2 protein sequence with bovine MRP1 (used as template in the SfABCC2 homology modelling) shows 30.8% identity (Figure S3) and at the EC4, the homology models suggest a tandem loop arrangement while the shorter region in the original crystal template (PDB-identity number: 5UJ9) exhibits only one. In this poorly defined epitope the distance between the two mutated residues is roughly 12 Å (Backbone Cα – Cα). A third mutation at position 1088 (glycine substituted to aspartic acid) was observed in the resistant strains. This mutation is located in the (intracellular) nucleotide-binding domain 2 at the outer surface of a helical linker region. As this domain does not exhibit larger gaps or deletions in *S. frugiperda* and *B. taurus*, the reliability of the position of this mutation is certainly higher than the two mutations described above. However, an amino acid alignment revealed that this region is less conserved among lepidopteran pests than the others (Fig. 4A).

3.5. Functional characterization of mutant SfABCC2 transporter in Sf9 cells

The functional characterization of the mutations found in the SfABCC2 was done by expressing the *ABCC2* wild and mutant alleles in Sf9 cells and measuring their response to Cry toxins by cell viability assays. The expression of SfABCC2 was verified by Western blot (Figure S4) using anti-FLAG tag antibody, indicating similar expression independent of the SfABCC2 type. SDS-PAGE gel analysis of ABCC2 + 3XFLAG revealed a protein of expected size (153.8 kDa).

For quantification of membrane integrity, we conducted a LDH release assay. Non-transfected Sf9 cells treated with Cry1F did not show a major effect on viability (91.6% ± 1.7), whereas Sf9 cells expressing the wild type SfABCC2 transporter showed a significant cytotoxic effect (36% ± 5.3 viability). Cell lines expressing the mutation variants did not differ statistically when compared to non-transfected Sf9 cells. These results show that the SfABCC2 wild type allele increases Cry1F toxicity in Sf9 cells as compared to the SfABCC2 mutant variants (Fig. 5A).

A second cytotoxicity test was used to complement the data obtained from the membrane integrity assay. A similar pattern of

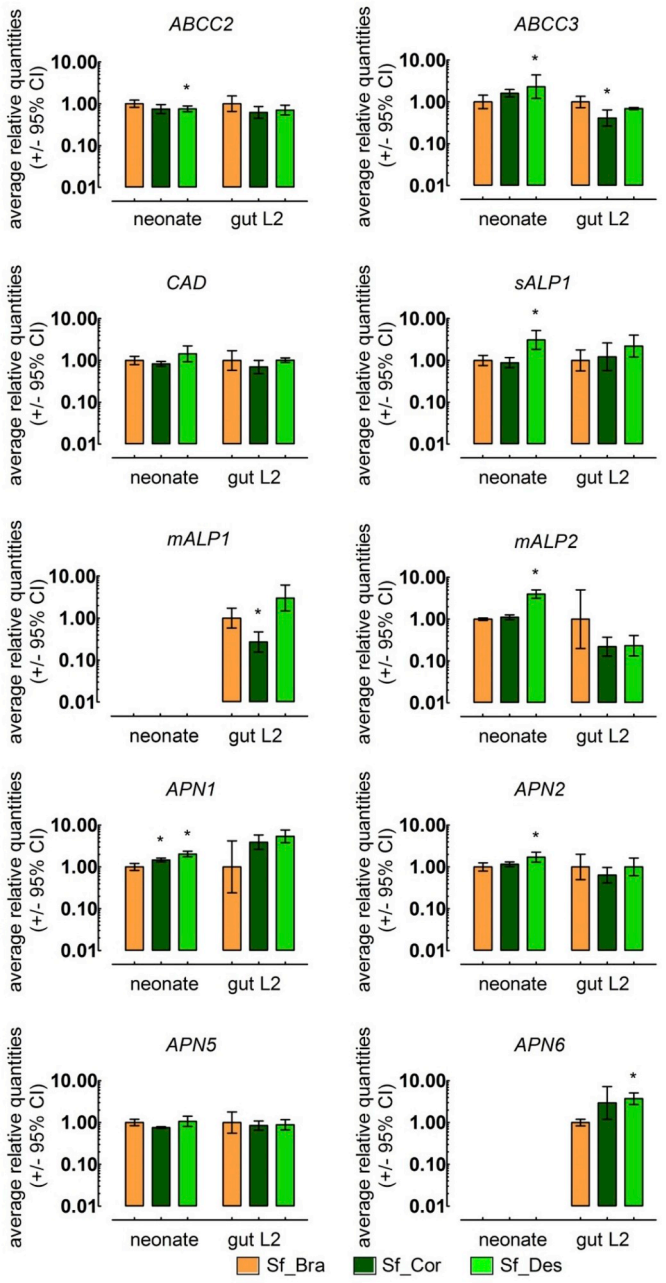

Fig. 3. Expression level (log scale) of genes potentially involved in the mode of action of Cry1F toxin in neonates and gut tissue of second instar larvae of *Spodoptera frugiperda* of strains Sf_Bra (orange), Sf_Cor (dark green) and Sf-Des (light green). The expression level was normalized to L17, L10 and RPS3A reference genes and relative to the susceptible strain (Sf_Bra). Asterisks (*) above a column denote a significant difference (unpaired *t*-test, $P < 0.05$) between one of the resistant (Sf_Des or Sf_Cor) and the susceptible (Sf_Bra) strain. Neonates and gut tissue samples were separately analyzed. CAD: cadherin; ABCC2/ABCC3: ATP- binding cassette subfamily C2/C3 transporter; mALP1/mALP2: membrane bounded alkaline phosphatase class 1/class2; APN: amino peptidase class 1, 2, 5 and 6. Data were measured from RT-qPCR from four to six biological replicates (30 individuals each for neonates and 10 individuals for the gut tissue). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cytotoxicity was observed with the second cytotoxic test using image analysis (Fig. 5B) of cells exposed to Cry1F. However, toxicity among the different cell lines did not differ when incubated with the Bt insecticide product Xentari™ (Figure S5).

3.6. Genotyping to determine resistance allele frequencies in fall armyworm field samples

A pyrosequencing diagnostic assay was designed and ten populations collected between 2017 and 2018 were investigated. The results show that the frequency of the GY deletion is widespread in different regions of maize cultivation in Brazil (Fig. 6). A population collected in Vilhena-RO (RO-VI) showed the highest frequency for the GY deletion, with 83.3% of the individuals tested being homozygotes for the mutant allele (Table S9). The P799R mutation was present in four of ten populations, showing higher frequency (> 50%, homozygote for P799R allele) in the fall armyworm population collected in Primavera do Leste-MT1 (MT-PL1) (Table S9). Interestingly, individuals collected in BA-SD population collected only one year later than Sf_Des showed susceptible genotype for both resistant alleles and only few individuals were successfully genotyped due to the low quality of starting sample material. Additionally, survivors from diet overlay assays also showed the presence of a GY deletion in homozygosity, except for one individual from Sf_Des that was heterozygote (Table S9). Interestingly all survivors of a discriminating dose bioassay of strain Sf_Cor were homozygous for P799R, whereas more than two-thirds of the survivors of strain Sf_Des were homozygous wildtype (P799) and the remainder homozygous for P799K (Table S9), indicating that the resistance mutations do not necessarily co-exist.

To further address mutations in the EC4 region we sequenced pools of 50 individuals from 30 populations spanning several key maize growing regions of Brazil. Because individuals were pooled, we cannot assess individual genotypes using this method. However, using this approach we can deeply sample alleles from the population and discover mutations that could not be detected with genetic markers described above (Schlötterer et al., 2014). The peptide for the wild type SfABCC2 allele (GenBank KY489760) in the EC4 region is as follows: TNQVDGYIQTLPEGESP (amino acids 783–799) and fully identical to the peptide in EC4 of ABCC2 of the susceptible reference strain Sf_Bra (GenBank MN399979; Figure S6), whereas the GY deletion is: TNQVDIQTLPEGESK. Among 1007 total reads from all 30 populations, we observed the wild type allele 515 times (overall allele frequency 51.1%), and the GY deletion allele 247 times (24.5%). The most common form of the GY deletion allele observed had the P799K mutation at position 799, as was found in the Sf_Des population. The remaining 245 alleles sampled (24.3%) from these 30 populations were made up of rare mutations and disruptions to the EC4 region (Table S4). The next most common allele (TNQVDGYIQTLPEGESR) was observed 22 times, and it has the P799R mutation only. This allele was found at a low frequency among 14 populations (Table S4). We also found several novel deletion alleles, such as TNQVDGYIPEGESP seen 4 times and missing the QTL residues from 791–93, or TNQVDGYIQSP seen 11 times and missing the TLPEGE residues from 792–97. In addition, there are several rare insertion alleles, such as TNQVDG**VRCQKTSPWCTLGTGN**SP (insertion in bold) which was seen 10 times and in 7 populations, or TNQVD**YNKIQTNI**QTLPEGESP, observed once in 9 separate populations. These alleles, and several others like them, share a common property that an insertion or series of insertions alter the coding frame and disrupt the GY amino acids at 788–89, but nevertheless sum to a multiple of 3 so that the overall protein coding frame remains intact.

In total we identified 125 unique rare alleles in this EC4 region that are neither the wild type or common GY deletion allele. While it is possible this figure is an overcount due to sequencing errors, 31 distinct alleles were observed more than once, and 26 were seen in more than one population, which is unlikely to occur by random error.

In Fig. 6 we display the frequency of alleles observed on a map of Brazil. Due to the large number of rare alleles, we put their counts all together as a category labelled “Other Mutations”. None of the rare alleles in this class have been isolated and tested, so we cannot say for certain which, if any, cause Cry1F resistance. However, we show that

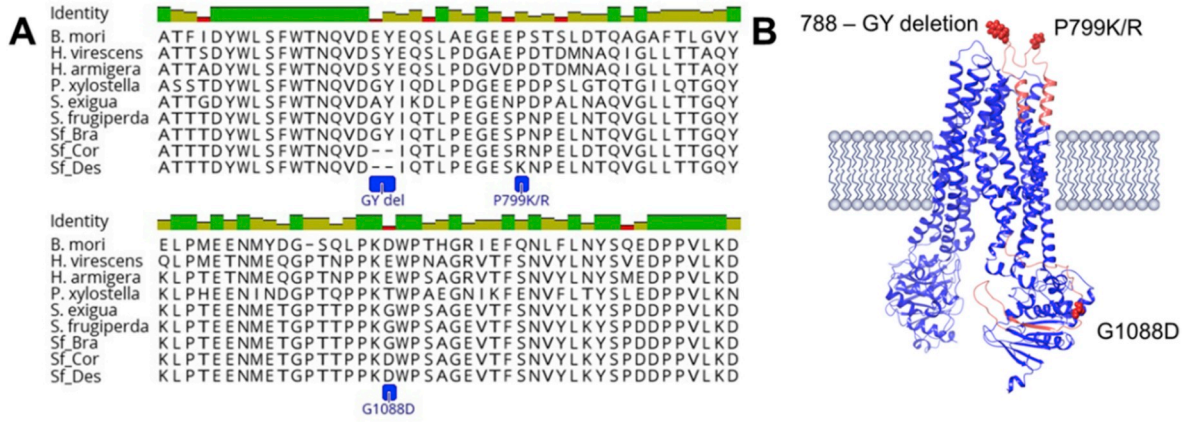

Fig. 4. (A) Alignment of the partial amino acid sequence of the extracellular loop 4 region of ABCC2 from six lepidopteran pests. *Bombyx mori* (BAK82126); *Helicoverpa armigera* (AHL68986); *Heliothis virescens* (ADH16740); *Plutella xylostella* (AEI27593), *Spodoptera exigua* (AIB06822) and *Spodoptera frugiperda* (KY489760). Sf_Bra is a susceptible strain. Sf_Cor and Sf_Des are Cry1F resistant strains.(B) Homology model of *S. frugiperda* ABCC2 based on bovine MRP1 sequence (PDB identity number: 5UJ9). The three mutations sites are highlighted in the corresponding regions in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

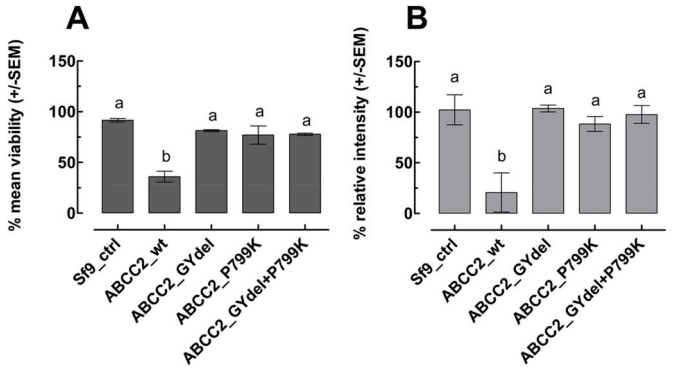

Fig. 5. Cytotoxicity of Cry1F on Sf9 non-transfected cells (Sf9_ctrl), cells expressing wild (ABCC2_wt) and mutant types (ABCC2_GYdel, ABCC2_ P799K, ABCC2_GYdel + P799K) of ABCC2 transporter from *Spodoptera frugiperda*. (A) Cell viability based on LDH release and (B) by the cell-permeant fluorescent probe Calcein-AM. Different letters denote a significant difference (One-way ANOVA; post hoc Tukey comparison, $P < 0.05$). Data shown are mean values ± SEM (n = 6).

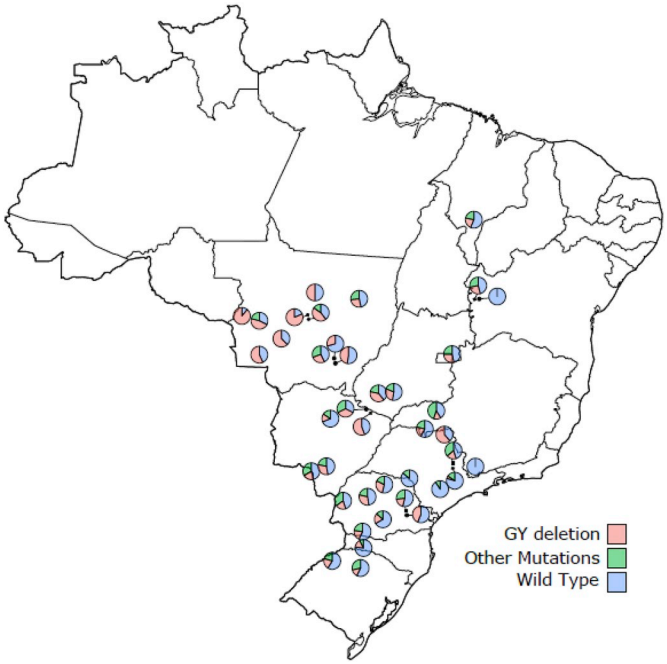

Fig. 6. Frequency of SfABCC2 alleles in 40 *Spodoptera frugiperda* populations collected from different regions in Brazil (see Table S1 for more information about the populations and Table S4 and S9 for genotyping results). For each population the frequency of the GY deletion (pink), other mutations in the EC4 region of SfABCC2 (green) and wild type SfABCC2 (blue) is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

disrupting the GY residues at position 788-89 leads to resistance, and several of these alleles disrupt these residues in novel ways, so it seems likely that some of these alleles could also cause resistance to Cry1F.

4. Discussion

One of the best-known resistance mechanisms to Bt toxins is the reduced binding to specific midgut receptors, due to reduced expression or target-site mutations (Bravo et al., 2007; Heckel et al., 2007). To characterize the Cry1F resistance mechanism in *S. frugiperda* populations from Brazil, we first analyzed the expression pattern of genes involved in the Bt mode of action among resistant (Sf_Cor and Sf_Des) and susceptible (Sf_Bra) strains. This analysis required a comprehensive validation of suitable reference genes not yet described in detail for fall armyworm, but other *Spodoptera* species, such as *S. exigua* and *S. litura* (Lu et al., 2013; Zhu et al., 2014). Our combined results determined ribosomal genes *L17*, *L10* and *RPS3A* as the most reliable for normalization when used together to analyze all gut tissue and whole body sample groups in three *S. frugiperda* strains. Ribosomal gene *L10* was already recently confirmed as a reliable reference gene in other *Spodoptera* species (Lu et al., 2013; Zhu et al., 2014).

Earlier studies revealed a significant down-regulation of *mALP2* in gut tissue in Cry1 resistant lepidopteran pests such as *H. virescens*, *H. armigera* and *S. frugiperda* (Jurat-Fuentes et al., 2011). However, more recently, Banerjee et al. (2017) did not find a co-segregation of down-regulation of *mALP2* and Cry1F resistance in fall armyworm collected in Puerto Rico. Likewise our study revealed no significant down-regulation of *mAPL2* levels in both Cry1F resistant strains when compared to strain Sf_Bra, and therefore was not further investigated in this study.

A different expression pattern of APN isoenzymes genes was detected; *APN1*, *APN2* and *APN6* were significantly overexpressed in the resistant strain Sf_Des at neonate stage and *APN1* and *APN6* in Sf_Cor; while in the gut tissue *APN6* was overexpressed in Sf_Des. Paralogues of APN have cleavage preferences for various classes of amino acids and therefore, the overexpression of a specific APN can reflect differential

affinity for various classes of Cry toxins (Van Rie and Ferré, 2000). Moreover, according to Hernández-Martínez et al. (2010), soluble APNs can contribute to Bt resistance by toxin sequestration and preventing its binding with other receptors present in the gut. Other studies investigating Cry1Ac resistance in cabbage looper (*Trichoplusia ni*) revealed a correlation of resistance with the down-regulation of *APN1*, but not with the concurrently detected up-regulation of *APN6* (Tiewsiri and Wang, 2011), however the up-regulation of *APN6* observed in our study was more than 10-fold lower as in Cry1Ac resistant cabbage looper. Therefore, further research is needed to test the relevance of APN altered expression and its impact on resistance to Cry1F in *S. frugiperda*.

Differently from APNs and ALP enzymes, there was no significant difference in the expression level of *CAD* between susceptible and resistant strains in gut tissue and at neonate stage. Larvae of *Drosophila melanogaster* expressing ABCC2 from *P. xylostella* in midgut tissue, showed a strong synergistic effect when exposed to Cry1Ac and cadherin-like protein from *Manduca sexta* (Stevens et al., 2017). However, according to Tanaka et al. (2013) cadherin proteins in *B. mori* are not relevant for susceptibility to Cry1F and to the best of our knowledge resistance to Cry toxins has not been linked to cadherin expression levels in fall armyworm.

It is important to mention that most of the genes involved in the Bt toxicity pathway have also other important biological functions, so some variation in expression pattern at different developmental stages and among populations may be expected (Yang et al., 2012). Therefore, considering the resistance level observed (RR > 490), differences obtained in the expression profile between susceptible and resistance strains in this study does not give strong evidence of linkage with Cry1F resistance. This is also supported by the fact that approx. 50% of (F1♀ x Sf_Des♂) backcross progeny surviving exposure to a discriminating dose of Cry1F were 100% RR for a GY deletion in ABCC2, suggesting that a single allele or a set of linked loci has a major impact on Cry1F resistance (Table 1). However non-exposed control larvae diverge almost equally into individuals heterozygous and homozygous for the GY deletion, with the exception of two individuals (4%) which were homozygote for the ABCC2 wildtype. This rather unexpected result might be due to equivocal genotyping analysis (Table 1).

We next focused on the presence of mutations in the ABCC2 transporter that could be potentially linked to Cry1F resistance because many of the described mutations identified in the *ABCC2* genes of lepidopteran pests such as *H. virescens*, *P. xylostella*, *B. mori*, *S. exigua* and *S. frugiperda* directly or indirectly affect the extracellular domain of the membrane protein (Gahan et al., 2010; Baxter et al., 2011; Atsumi et al., 2012; Park et al., 2014; Banerjee et al., 2017; Flagel et al., 2018). A remarkable finding in the current study is the presence of two novel mutations located in the extracellular loop 4 (EC4), between transmembrane domains (TM) 7 and 8. This region is the most prominent loop in the conformation of the ABCC2 transporter and it is highly likely to be involved in binding with Cry1F toxin in *S. frugiperda*. In *B. mori*, for example, EC4 has been confirmed by an alanine screening method and recombinantly expressed deletion mutants to be of major importance for the binding with Cry1A toxin (Tanaka et al., 2017; Endo et al., 2018). The location of the ABCC2 interaction site with Cry toxins in fall armyworm is still unknown, however, SfABCC2 has been recently reported as functional receptor for Cry1F, Cry1A and Cry1A.105 (Banerjee et al., 2017; Flagel et al., 2018). Furthermore, loop regions are frequently involved in protein-protein interactions and the EC4 is the longest loop in SfABCC2, composed of 43 amino acid residues. The GY deletion and amino acid substitution at sites 788–789 and 799, respectively, shed light on the potential interaction between ABCC2 transporter and Cry1F toxins in *S. frugiperda*. It is tempting to speculate that the substitution of a non-polar amino acid (proline) to a positively charged residue (lysine and more rarely, arginine) might also affect the interaction with Cry1F toxin, however further experimental work is needed to validate such an interaction. Moreover, among the 30 pooled population samples we observed many rare alleles which disrupt residues between sites 783–799 (Table S4). Though none of these alleles were tested in bioassays, their diversity and abundance in field collected populations lends further support to the importance of the EC4 domain for Cry1F toxicity, because the highest indel frequency was noticed on exon 14, which corresponds closely to the ABCC2 EC4 region carrying the GY deletion (Figure S7).

An additional mutation was observed in the resistant strains at position 1088, where glycine is substituted to aspartic acid. Despite the fact that this target-site mutation is close to the ATP-binding site, Cry1F is still active against *B. mori*, which has an aspartic acid at this particular corresponding site (Tanaka et al., 2013). Moreover, it has been recently reported that even truncated ABCC2 transporter from *S. exigua* lacking completely the ATP-binding site is still a functional receptor for Cry1Ac and Cry1Ab (Pinos et al., 2019). Therefore, the effect of the above mentioned mutation was not further investigated in this study.

Differently from what has been described for Cry1F-resistant fall armyworm from Puerto Rico and Florida (Banerjee et al., 2017; Flagel et al., 2018), the mutations detected in SfABCC2 transporter from Brazilian populations analyzed here, do not result in a premature stop codon or abrupt disruption of the SfABCC2. This fact may support the hypothesis that although the mutations present at EC4 confer resistance to Cry1F, they do not affect directly the ABCC2 function and therefore do not result in a strong fitness cost for insects harboring the resistant alleles (Santos-Amaya et al., 2017). However, fitness costs have been reported for resistant populations from Puerto Rico and Florida in a single study (Dangal and Huang, 2015), whereas others reported a lack of strong fitness costs associated with Cry1F resistance in fall armyworm from Puerto Rico (Jakka et al., 2014; Velez et al., 2014).

The cell cytotoxicity results showed that when SfABCC2 wild type is expressed in Sf9 cells, a toxicity effect can be observed, but this is significantly reduced when the SfABCC2 mutants are expressed, supporting the role of the mutations and Cry1F resistance. Although the presence of double mutant alleles (GYdel + P799K) did not result in lower cytotoxicity compared to single mutant allele presence in the cell-based assay, it needs to be further investigated if insects carrying both mutations may show higher Cry1F resistance levels or fitness costs.

Some studies have shown that Cry1F share binding site with Cry1A.105, Cry1Ac and Cry1Ab in *S. frugiperda*, but not with Cry1Ca (Banerjee et al., 2017; Flagel et al., 2018). Additional experiments would be necessary to confirm if the presence of the novel mutations in the SfABCC2 transporter have an impact on the above-mentioned Cry toxins and maybe explain the cross resistance pattern observed recently in a field selected population (D. Bernardi et al., 2015; Omoto et al., 2016). Our bioassay with Xentari™ showed a very low resistance ratio between Cry1F resistant and susceptible strains (RR up to 5-fold), confirming the lack of cross-resistance between Cry1F-resistant populations and this bio-insecticide (Horikoshi et al., 2019).

Nevertheless, a linkage between Cry1F resistance and GY deletion allele was supported by genotyping survivors of F1 (RS for GY deletion) x Sf_Des (RR for GY deletion) backcross progeny from overlay diet assays after exposure to a discriminating rate of Cry1F (Table 1). All survivors had the mutant allele, confirming the resistant phenotype observed in the bioassay.

The development of genomic DNA-based and pooled population sequencing assays allowed us to monitor the frequency of the resistant alleles in 40 different fall armyworm populations from Brazil. Our genotyping results show the presence of GY deletion allele in samples collected in a broad geographic range, suggesting that the GY deletion might be of high importance. In addition to the GY deletion allele, we also discovered potentially dozens of other alleles disrupting the EC4 region. Among all our samples, these alleles are rare, most less than 1% frequency; though when put together they are nearly as numerous as the GY deletion allele. If some of these alleles also confer Cry1F resistance, then the overall pattern observed here would be consistent with a soft selective sweep (Hermisson and Pennings, 2005, 2017). In a

soft selective sweep, multiple alleles arise (or exist prior to selection as standing genetic variation) and collectively increase in frequency in response to selection pressure. In contrast, under a hard selective sweep scenario, a single Cry1F resistance allele would arise and spread. The presence of so many alleles disrupting the EC4 region of SfABCC2 suggests a soft selective sweep may be in progress. Farias et al. (2014) suggested that the Cry1F resistance observed in different regions in Brazil is conferred by resistance alleles at the same locus, although the presence of resistance alleles is not always associated with field-resistant populations. Studies based on the genetic structure of fall armyworm populations from Brazil reveal a low genetic flow among populations geographically separated, but higher genetic similarity among populations sharing the same host plant (Martinelli et al., 2006; Silva-Brandão et al., 2018). Therefore, based on the genotype results presented here (Figure S7; Table S4), the resistant alleles might have evolved independently in different locations where maize fields expressing Cry1F were present. This could in turn give rise to the numerous rare alleles described above, as each locality evolves resistance in a unique way. However, in addition to this we also observe the much more common and widespread GY deletion allele. This allele appears to have spread broadly across Brazil, perhaps from the Northwest, where it is more common, to the rest of the country where it is less common (Fig. 6). This allele appears to have different dynamics than the other rare alleles, and may indicate a complex type of soft selective sweep, which includes both rare local alleles in addition to a common and broadly distributed allele.

Taking these results together, we propose that the presence of the novel GY deletion in ABCC2 at positions 788–789 and the amino acid substitution at position 799 plays an essential role in the resistance of *S. frugiperda* populations from Brazil against Cry1F, with the GY deletion in EC4 being present at higher frequency. Moreover, this work shows that the wide spread of the GY deletion, in addition to a large number of rare alleles which disrupt the EC4 region, could explain the high degree of Cry1F resistance found in Brazilian maize.

Declaration of competing interest

Some of the authors are employed by Bayer AG. Bayer AG manufactures maize containing the Cry1Fa and Cry1A.105 proteins. This work was partially financed by Bayer AG. The authors declare no additional conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103280.

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Chapter 4

Toxicological and molecular profiling of insecticide resistance in a Brazilian strain of fall armyworm resistant to Bt Cry1 proteins

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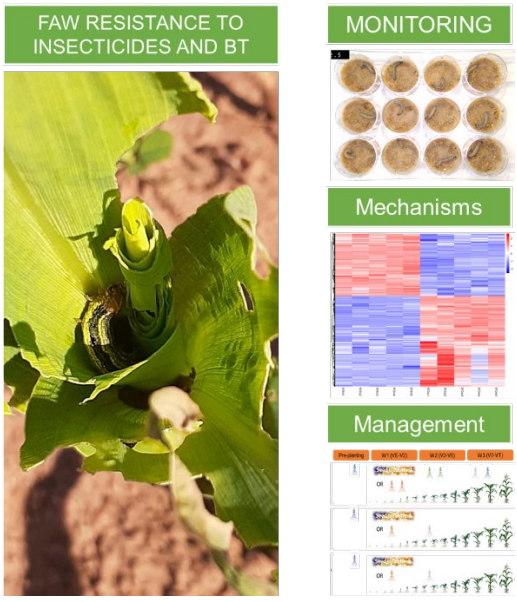

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Research Article

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Toxicological and molecular profiling of insecticide resistance in a Brazilian strain of fall armyworm resistant to Bt Cry1 proteins

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Abstract

Background: *Spodoptera frugiperda*, fall armyworm (FAW) is the major pest of maize in Brazil and has readily acquired field resistance to a broad range of synthetic insecticides and to *Bacillus thuringiensis* (Bt) insecticidal proteins expressed in important crops. This study aims to understand patterns of cross-resistance in FAW by investigating the toxicological profile of a Bt-resistant Brazilian strain (Sf_Des) in comparison to a Bt-susceptible strain (Sf_Bra).

Results: Laboratory bioassays with 15 active substances of nine mode of action classes revealed that Sf_Des has a medium level of resistance to deltamethrin and chlorpyrifos. Very high cross-resistance was observed among Cry1 toxins, but high susceptibility against Vip3A. Strain Sf_Des exhibited – depending on the substrate – up to 19-fold increased cytochrome P450 activity in comparison to Sf_Bra. RNA-Seq data support a major role of P450 enzymes in the detoxification of insecticides because we detected 85 P450 transcripts upregulated in Sf_Des. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis confirmed that *CYP9A*-like and *CYP6B39* are significantly upregulated (>200-fold) in Sf_Des in comparison to Sf_Bra strain. No target-site mutation linked to pyrethroid resistance was detected, but mutations in the AChE linked to organophosphate resistance were observed in Sf_Des. A Gene Ontology (GO) analysis of differentially expressed genes (DEG) categorized most of them into the biological process category, involved in oxidation–reduction and metabolic processes.

Conclusion: Our results indicate that multiple/cross-resistance mechanisms may have developed in the Sf_Des strain to conventional insecticides and Bt insecticidal proteins. The systematic toxicological analysis presented will help to guide recommendations for an efficient resistance management.

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

Keywords: fall armyworm; cross-resistance; detoxification enzymes; resistance management

1 INTRODUCTION

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae), is native to the American continent where it is the major pest of maize.[1, 2] However, since 2016, FAW has rapidly invaded the tropical and subtropical regions of the Eastern hemisphere, becoming a pest of global economic relevance.[3–6] FAW control has relied intensively on chemical insecticides, prompting resistance to many classes of insecticides [7, 8] and currently, FAW is among the top 15 most resistant insect pest species worldwide.[9] In Brazil, cases of insecticide resistance have been reported for different chemical classes including organophosphates, pyrethroids, spinosyns, benzoylureas and (lately) diamides.[10–14]

The commercialization of genetically engineered crops expressing insecticidal crystal (Cry) or/and vegetative (Vip) proteins derived from the bacterium *Bacillus thuringiensis* (Bt) Berliner has considerably reduced the number of insecticide applications for the control of lepidopteran pests, including FAW.[15] In Brazil, the refuge (cultivation of non-Bt nearby Bt crops) strategy is highly recommended to delay the onset of resistance to Bt crops [16] and the refuge area can be treated up to two times (including seed treatment) during the growing season with non-Bt based foliar insecticide sprays until V6 stage.[17, 18] Despite the high adoption of Bt crops in Brazil (51.3 million ha), there is rather low compliance with regard to the proposed refuge strategy.[19]

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Furthermore, not all Bt proteins are high-dose for FAW – thus, the respective protein expressed *in planta* does not cause 100% mortality of insects feeding on it.[20, 21] Therefore, the first cases of Bt protein (Cry1F) resistance were detected after only a few years of commercialization.[19, 21] Currently, Cry1F resistance is widespread in Brazil and Cry1F-resistant larvae exhibit a high level of cross-resistance to Cry1Ab, as well as maize hybrids expressing Cry1A.105/Cry2Ab, Cry1A.105/Cry2Ab2/Cry1F and Vip3Aa20/Cry1Ab.[22–24]

The fast evolution of resistance against Cry toxins has led to the need for additional insecticide applications during the cropping season.[25] In southern Brazil, for example, up to four additional insecticide applications are required to control FAW.[25] Therefore, it is essential to know the efficacy of chemical insecticides which could control Cry1F-resistant individuals present in the refuge areas, using the best synergistic approach combining Bt technology and the rotation of effective insecticides. Hence, a better understanding of insecticide susceptibility of field populations as well as involved mechanisms of resistance is important for the implementation of sustainable control strategies.

The most common mechanisms involved in insecticide (and Bt toxin) resistance are target-site mutations and enhanced detoxification. [26–28] Target-site mutations in the voltage-gated sodium channel (VGSC), acetylcholinesterase (AChE) and ryanodine receptor (RyR) have been reported in FAW populations from Brazil highly resistant to pyrethroids, organophosphates and diamide insecticides, respectively.[11, 29] Moreover, target site resistance in the ATP-binding cassette transporter subfamily C2 (ABCC2), conferred by a two amino acid deletion (glycine and tyrosine – GY-deletion) was linked to high levels of Cry1F resistance in a FAW strain (Sf_Des) collected in Brazil.[30]

In order to better understand possible multi/cross-resistance patterns, we have examined the efficacy of different insecticide modes of action, including Bt proteins towards the previously described Cry1F-resistant (Sf_Des) and a Cry1F-susceptible (Sf_Bra) strain. Furthermore, the toxicological profile of the two FAW strains (Sf_Des and Sf_Bra) was characterized at the molecular and biochemical level. Transcriptomic RNA-Seq analysis and the activity of major detoxification enzymes involved in the detoxification pathways, such as P450 enzymes, carboxylesterases (CE), glutathione S-ransferases (GST) and uridine diphosphate-glucosyltransferases (UGTs), were investigated. Results obtained here at both phenotypic and genotypic levels provide a better understanding of the detoxification process of FAW towards synthetic insecticides and Bt insecticidal proteins, and provide practical support for managing Cry1F-resistant individuals in a high-dose/refuge system.

2 MATERIAL AND METHODS

2.1 FAW strains and rearing

Two *S. frugiperda* strains, Sf_Bra (susceptible to Cry1F, collected in the state of São Paulo, 2005) and Sf_Des (field-resistant to Cry1F, collected in *São* Desidério – Bahia, 2016), described previously by Boaventura *et al.* (2020),[30] were sampled in maize-growing regions in Brazil. Larvae were fed on an artificial diet based on wheat germ and soybean powder without exposure to any Bt protein or synthetic insecticides. Adults were fed with 10% (v/v) malt solution every second day. The insects were reared under controlled conditions (25 ± 1 °C, 55 ± 5% relative humidity).

2.2 Chemicals and insecticidal proteins

All chemicals and solvents used in this study were of analytical grade unless otherwise stated. The representative active ingredient of nine different mode of action classes were of analytical grade and used according to information given in Table S1. Bradford reagent and Bovine Serum Albumin (BSA) were purchased from Bio-Rad (Hercules, CA, USA). The chemicals 1-chloro-2,4-dinitrobenzene (CDNB), L-glutathione reduced from *Saccharomyces cerevisiae* (GSH), glutathione oxidized, 1-naphthyl acetate (1-NA), 1-naphthyl butyrate (1-NB), Fast blue RR salt, NADPH, ethylenediaminetetraacetic acid (EDTA), 1,4-dithiothreitol (DTT), Triton X-100, Tween-80 and 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC) were purchased from Sigma Aldrich (Munich, Germany). The artificial substrates 7-benzyloxymethoxy-4-trifluoromethyl coumarin (BOMFC) and 7-benzyloxymethoxy resorufin (BOMR) were purchased from Vivid®, ThermoFisher Scientific (Carlsbad, MA, USA). The cOmplete™ EDTA-free proteinase inhibitor was purchased from Roche (Merck, Darmstadt, Germany).

The insecticidal toxins were produced by *B. thuringiensis* or *Escherichia coli* recombinant strains and kindly provided internally by Bayer (Chesterfield, MO, USA). Cry1Ab (91% purity) was sent as purified trypsin-activated protein in 50 mM sodium bicarbonate (pH 10.25), Cry1Ac (28.2% purity) as lyophilized material, Vip3Aa (100% purity) in 25 mM Tris–HCl, 0.25 M sodium chloride (NaCl) and 2 mM DTT (pH 8.0) buffer.

2.3 Dose–response bioassays with chemical insecticides and Bt proteins

Representative active ingredients (15 different active substances) belonging to nine different modes of action were used at concentration ranges given in Table S1. The insecticides were dissolved in 10% (v/v) acetone and 0.1% (v/v) aqueous Triton X-100 solution and the serial dilutions made in 0.1% (v/v) aqueous Triton X-100. The insecticide concentrations used varied from 722 to 0.01 ng cm^{-2} (Table S1) and a solution of 0.1% (v/v) aqueous Triton X-100 without active ingredient served as a negative control.

Artificial diet was placed in a 12-well plate (Greiner Bio-One, Austria) (2 mL diet/well) and an automated purpose-built spraying device was used to apply (12 µL/well) the different doses of insecticides in at least five different concentrations (Table S1 for concentration range). The bioassays with synthetic insecticides were conducted with 3rd instar larvae of strains Sf_Bra and Sf_Des, by adding a single larva per well on diet treated with insecticide and sealed with perforated foil. The larvae were assessed for mortality (including larvae showing symptoms of poisoning) at three (3DAT) and seven days (7DAT) after treatment. The bioassays for the insecticidal proteins were performed with neonate larvae (<24 h old) according to Boaventura *et al.* (2020)[30] and mortality was scored 5DAT. All Bt proteins were diluted in 50 mM sodium carbonate buffer (pH 10.4) and 0.1% (v/v) aqueous Triton X- 100 according to the concentrations described in Table S1.

The bioassay was replicated at least three times, each replicate consisting of 12 larvae per concentration tested. All larvae were kept at 25 ± 1 °C, 55 ± 5% relative humidity, and 16 h:8 h, light: dark photoperiod. Larvae were considered alive when they still reacted to outward stimuli and classified as affected when showing growth inhibition (1/3 of control) or strong poisoning effect, such as incomplete ecdysis for larvae exposed to triflumuron. Assays were considered valid when control mortality was ≤10%.

2.4 Preparation of enzymes and protein quantification

Pools of ten larvae (3rd instar) each of Sf_Bra and Sf_Des were homogenized on ice using a plastic mortar and 500 µL of different buffers according to the enzymatic assay to be conducted. In brief, for crude preparations of P450 enzymes, 0.1 M potassium

phosphate buffer (pH 7.6) containing 1 mM EDTA, 1 mM DTT, 200 mM sucrose and cOmplete™ EDTA-free Protease Inhibitor Cocktail tablet was used. For CE activity, tissue was homogenized in 0.1 M sodium phosphate buffer (pH 7.6) containing 0.1% (v/v) Triton X-100. For GST activity 50 mM HEPES buffer (pH 6.8) containing 0.1% (v/v) Tween-80 was used for MCB as substrate and 50 mM Tris–HCl buffer (pH 7.5) for CDNB.

The microsomal fraction for the P450 monooxygenase activity assay was obtained by centrifugation of homogenate for 5 min at 5000×*g* and 4 °C. The pellet was discarded, and the resulting supernatant was centrifuged at 4 °C for 15 min at 15 000×*g* followed by a last ultra-centrifugation step at 100 000×*g* for 60 min at 4 °C. The microsomal pellet was resuspended in 300 µL 0.1 M potassium phosphate buffer (pH 7.6), 1 mM EDTA, 1 mM DTT, 5% (v/v) glycerol and served as enzyme source. For CE and GST activity, the homogenates were centrifuged at 10 000×*g* and 4 °C for 5 min and the supernatant collected. Protein concentration was determined using Bradford reagent and BSA as a reference.

2.5 Cytochrome P450 monooxygenases

Cytochrome P450 activity was measured according to Stumpf and Nauen (2001) with slight modifications. Coumarins (BOMFC and BFC) and resorufin (BOMR) were used as model substrates and determined fluorometrically in a black flat-bottom 384-well plate format (Greiner, Essen, Germany). Each reaction consisted of 25 µL enzyme source (25 µg protein) and 25 µL of the substrate solution (50 µM of the substrate and 1 mM NADPH in 0.1 M potassium phosphate buffer pH 7.6). Control reactions without NADPH and enzyme were included. The reactions with BOMFC and BFC were incubated for 1 h at 25 °C at 300 rpm in the dark. The self-fluorescent NADPH was removed by adding 50 µL stop solution [50% (v/v) DMSO: TRIZMA-base buffer (pH 10), 5 mM glutathione oxidized, 4 U mL^{-1} glutathione-reductase] into each well. After another 30 min of incubation, fluorescence was determined in an endpoint assay at the appropriate excitation/emission wavelength settings according to manufacturer instructions. For the resorufin substrate BOMR, reactions were carried out as described above, without the addition of stop solution. The fluorescent product formation was measured using a kinetic assay for 1 h at 25 °C, with measurements taken every 5 min. All reactions were run in triplicate from four biological replicates per strain and measured using a spectrofluorometer Tecan Spark (Tecan Group Ltd., Switzerland).

2.6 Carboxylesterase activity

Enzyme substrate was prepared as described in Section 2.4 and CE activity was measured according to Grant *et al.*[31] with minor modifications. The substrate stock solution contained 100 mM of 1-NA or 1-NB dissolved in acetone and 100 µL was added to 9 mL of a filtered solution of 1.5 mM Fast blue RR salt prepared in 0.2 M sodium phosphate buffer (pH 6.0). To determine esterase activity, 10 µL diluted enzyme source (5 µg protein) and 90 µL substrate solution containing 1-NA or 1-NB (final concentration 1 mM) was added to each well of a transparent flat bottom 384-well microplate (Corning, USA). Reaction without enzyme source served as control and each reaction was run in triplicate. The esterase activity was monitored over 10 min at 25 °C with readings taken every 1.5 min using a Tecan Spark (Tecan Group Ltd., Switzerland) microplate reader at 450 nm for both substrates. The average activity was obtained from ten biological replicates per strain.

2.7 Glutathione S-transferase activity

The GST activity was measured according to Nauen and Stumpf[32] using CDNB and GSH as substrates and adapted for 384-well microplates (Corning) with minor modifications. Reactions consisted of 25 µL enzyme solution (20 µg protein) and 25 µL substrate solution (0.05 M HEPES buffer pH 6.8 containing 0.1% (v/v) Tween-80; CDNB and GSH at 0.4 mM and 4 mM final concentration, respectively). Reactions were run in triplicate for five biological replicates per strain. The change in absorbance was measured continuously for 5 min at 340 nm, and 25 °C in spectrofluorometer Tecan Spark (Tecan Group Ltd, Switzerland).

Assessment of GST activity using MCB as a substrate was performed in flat-black 384-well microplates (Greiner, Essen, Germany). The total reaction volume was 50 µL per well, consisting of 25 µL enzyme source (20 µg protein) and 25 µL substrate buffer containing MCB (final concentration 0.4 mM) and reduced glutathione (final concentration 2 mM). Measurements were taken every 2 min at kinetic modus for 20 min at 25 °C using a spectrofluorometer Tecan Spark (Tecan) at emission and excitation wavelengths of 465 nm and 410 nm, respectively. The nonenzymatic reaction of CDNB/MCB with GSH measured without enzyme served as control.

2.8 RNA extraction, RNA-Seq and cDNA synthesis

Total RNA was extracted from third instar larvae (pools of ten larvae, in total five biological replicates per strain) of Sf_Bra and Sf_Des with TRIzol® reagent (Invitrogen, USA) and followed by RNA purification using RNeasy® Plus Universal Mini Kit (QIAGEN, Germany) according to manufacturer's instruction including a DNA digestion step with DNase I (QIAGEN). The RNA was quantified by spectrophotometry (NanoQuant Infinite 200; Tecan) and its integrity verified by an automated gel electrophoresis system, according to the CM-RNA method (QIAxcel RNA QC Kit v2.0; QIAGEN). Around one µg total RNA was sent to GENEWIZ (Leipzig, Germany) and the RNA quality was checked with an Agilent 2100 BioAnalyzer. Further, an mRNA poly(A) enriched library was prepared and 150-bp paired-end reads were generated with NovaSeq Illumina sequencing platform (Illumina Inc., CA, USA).

For quantitative reverse transcription polymerase chain reaction (RT-qPCR) validation of the expression profile of selected genes, one µg total RNA was used in 20-µL reactions for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, USA), following the manufacturer's instructions for RT-qPCR analysis.

2.9 RNA-Seq transcriptomic analysis and single nucleotide polymorphism (SNP) identification

Clean reads were obtained from GENEWIZ (Leipzig, Germany) and the transcriptome assembly was accomplished using Trinity v2.8.5 and TransDecoder v5.3.0.[33, 34] Transcripts were translated using a TransDecoder v2.0.1 pipeline.[34] First, longest open reading frames (ORFs) with minimal length 30 amino acids were extracted using the TransDecoder.LongOrfs tool using a universal genetic code. Homology of ORFs to known proteins was determined by NCBI-BlastP v2.3.0+ search against the SWISSPROT database and PFAM domain prediction using hmmer v3.1b22–4. The most likely predicted ORFs were selected using TransDecoder.Predict and the longest ORF for each transcript was retained. Proteins containing interpro-domain IPR002018 (Carboxylesterase, type B) or IPR001128 (Cytochrome P450) were classified as carboxylesterases or cytochrome P450s, respectively.

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A multiple sequence alignment of 125 protein sequences identified as P450 was performed using MUSCLE v3.8.31 and FastTree v2.1.5 to create a maximum-likelihood tree using Geneious v10.2.6.

Functional annotation and gene ontology (GO) term assignment of translated longest ORFs of *de-novo* assembled transcripts was performed using Blast2GO v1.3.3. [35] Therefore, domains were predicted using InterproScan v5.17-56.0 [36] and genes were searched against Uniprot KB using NCBI-BlastP v2.2.27.[37] GO term enrichment analysis was performed on differentially regulated genes using the Bioconductor package goseq v1.28.0.[38]

Transcript quantification was determined by pseudoalignment with kallisto v0.45.0[39] and summarized at the gene level using tximport v1.12.3.[40] The Bioconductor DEseq2 package v1.16.1 [41] in the R v3.4.1 environment was used to identify differentially expressed genes.

A *P*-adjusted value (P*adjust*) ≤0.01 indicated statistical significance and Log_2-fold changes (log_2FC) of ≥1 and <1 marked up- and downregulation, respectively.

Sequences of VGSC and AChE were obtained from separate Trinity/TransDecoder assemblies of the Sf_Bra and Sf_Des samples, respectively. VGSC and AChE sequences were identified by BLAST comparison *versus* the public *Spodoptera litura* sequences XP_022824852.1 and AQQ79919.1, respectively. Multiple protein alignment of VGSC/AChE from Sf_Bra, Sf_Des strains, *S. litura* and the partial sequence of *S. frugiperda* pyrethroid resistant strain (KC435026.1) and *S. frugiperda* organophosphate resistant strain (KC435023.1) were performed for target-site identification. Sequences were compared for the presence of T929I, L932F and L1014F target-site mutations in the VGSC, numbered according to *Musca domestica* sodium channel (GenBank X96668), and A201S, G227A and F290V in the AChE, numbered according to *Torpedo californica* (PDB ID: 1EA5).

2.10 Quantitative reverse transcription polymerase chain reaction (RT-qPCR) for gene expression validation

Eleven *CYP* genes previously described by Giraudo *et al.*[42] and Nascimento *et al.*[13] to be involved in insecticide detoxification were investigated in Sf_Bra and Sf_Des strains by RT-qPCR. The ribosomal genes *rsp3A*, *L17*, and *L10* were used as reference genes (primers and accession numbers of all genes are listed in Table S2). Reactions were performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) according to the manufacturer's protocol. Briefly: reaction mixtures (10 µL) contained 2.5 µL cDNA (5 ng), 5 µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 400 nM of reverse/forward primers (Table S2), and nuclease-free water. Reactions were run in triplicate using CFX384™ Real-Time system (Bio-Rad) and nontemplate mixtures as negative controls. The PCR conditions were: 3 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A final melting-curve step was included post-PCR (ramping from 65 °C to 95 °C by 0.5 °C every 5 s) to check for nonspecific amplification. Amplification efficiencies were determined by a five-fold dilution series revealing for all primers an efficiency ≥93%.

Table 1. Log-dose mortality data obtained for 12 different insecticides against 3rd instar larvae of *Spodoptera frugiperda* strains Sf_Des and Sf_Bra in diet spray bioassays. The assessment for affected larvae was made seven days after treatment

Compound	Strain	*n*	EC_{50} (ng ai cm^{-2})	95% CI[†]	Slope (±SE)	RR[‡]
Deltamethrin	Sf_Bra	324	0.20	0.18–0.22	2.47 (0.64)	
	Sf_Des	324	2.86	1.81–4.51	1.76 (0.44)	14.23
Chlorpyrifos	Sf_Bra	288	11.67	7.47–18.22	5.07 (1.48)	
	Sf_Des	288	92.58	64.85–132.20	2.40 (0.71)	7.93
Triflumuron	Sf_Bra	252	2.20	1.57–3.07	3.46 (0.82)	
	Sf_Des	336	8.08	2.51–25.97	0.72 (0.20)	3.68
Thiodicarb	Sf_Bra	540	43.39	37.99–49.56	6.94 (1.01)	
	Sf_Des	288	105.20	76.04–145.5	1.72 (0.49)	2.42
Spinosad	Sf_Bra	396	5.13	4.08–6.45	1.60 (0.30)	
	Sf_Des	396	8.42	7.02–10.10	4.06 (0.92)	1.64
Emamectin Benzoate	Sf_Bra	401	0.03	0.02–0.03	2.89 (0.42)	
	Sf_Des	401	0.04	0.03–0.04	1.64 (0.27)	1.18
Abamectin	Sf_Bra	288	91.62	63.96–131.30	3.06 (1.02)	
	Sf_Des	252	104.50	76.95–141.80	1.80 (0.38)	1.14
Tetraniliprole	Sf_Bra	252	1.46	1.03–2.06	2.98 (1.90)	
	Sf_Des	252	1.65	1.23–2.20	2.57 (0.84)	1.13
Chlorfenapyr	Sf_Bra	401	23.97	14.20–40.45	1.62 (0.71)	
	Sf_Des	401	25.30	12.99–49.28	1.39 (0.58)	1.06
Chlorantraniliprole	Sf_Bra	252	0.37	0.18–0.78	1.22 (0.45)	
	Sf_Des	252	0.39	0.33–0.46	3.28 (0.46)	1.03
Flubendiamide	Sf_Bra	401	4.44	3.78–5.23	6.55 (1.66)	
	Sf_Des	401	4.17	3.71–4.67	4.04 (0.60)	0.94
Indoxacarb	Sf_Bra	252	4.08	3.77–4.42	4.60 (0.49)	
	Sf_Des	252	3.74	3.16–4.43	3.62 (0.86)	0.92

[†] 95% confidence interval.
[‡] Resistance ratio (EC_{50} of Sf_Des strain divided by EC_{50} of Sf_Bra).

Table 2. Log-dose mortality data obtained for insecticidal proteins from *Bacillus thuringiensis* against neonate (<24 h) larvae of *Spodoptera frugiperda* strains Sf_Des and Sf_Bra in diet overlay assays. The assessment for affected larvae was made five days after exposure

Bt protein	Strain	*n*	EC_{50} (μg ai cm^{-2})	95% CI[†]	Slope (±SE)	RR[‡]
Cry1F[§]	Sf_Bra	190	0.098	0.0811–0.1188	1.51 (0.17)	>490
	Sf_Des	190	>48.70	ND	ND	
Vip3Aa	Sf_Bra	288	0.005	0.0047–0.0056	2.23 (0.29)	1
	Sf_Des	288	0.005	0.0041–0.0051	2.01 (0.24)	
Cry1Ab	Sf_Bra	288	0.080	0.0273–0.1370	1.14 (0.37)	439
	Sf_Des	180	34.95	29.629–43.419	1.42 (0.20)	
Cry1Ac	Sf_Bra	288	0.274	0.2005–0.3664	2.34 (0.82)	111
	Sf_Des	180	30.50	18.507–66.071	0.49 (0.07)	

ND, not determined.
[†] 95% confidence interval.
[‡] Resistance ratio (EC_{50} of Sf_Des strain divided by EC_{50} of Sf_Bra).
[§] Data obtained from Boaventura *et al.* 2020.

Table 3. Comparison of enzyme activity obtained from mass homogenates (3rd instar) of *Spodoptera frugiperda* strains Sf_Bra and Sf_Des for the main detoxification enzymes, cytochrome P450-dependent monooxygenase (P450), carboxylesterase (CE) and glutathione S-transferase (GST) using different model substrates. Activities were statistically analyzed by Student's *t*-test comparing Sf_Des and Sf_Bra mean values

Enzyme	*n*	Substrate	Strain	Enzyme activity mg^{-1} (±SE)[†]	Ratio[‡]
P450	4	BOMR	Sf_Bra	5.85 (4.67)	19.3
			Sf_Des	112.72 (15.58)*	
		BOMFC	Sf_Bra	30.00 (6.86)	1.5
			Sf_Des	46.05 (9.73)	
		BFC	Sf_Bra	112.11 (13.19)	1.6
			Sf_Des	179.00 (18.90)*	
CE	10	1-NA	Sf_Bra	337.98 (34.95)	1.0
			Sf_Des	346.53 (85.45)	
		1-NB	Sf_Bra	216.72 (36.62)	1.0
			Sf_Des	223.54 (46.56)	
GST	5	CDNB	Sf_Bra	6.20 (0.50)	0.9
			Sf_Des	5.80 (0.76)	
		MCB	Sf_Bra	1310.89 (124.86)	0.8
			Sf_Des	1157.70 (294.53)	

*Indicates significant differences ($P < 0.05$, unpaired Student's *t*-test).
[†] Enzyme activity is shown as OD min^{-1} mg^{-1} or RFU min^{-1} mg^{-1}. Means in the column followed by * are significantly different ($\alpha = 0.05$, unpaired Student's *t*-test).
[‡] Mean activity obtained for Sf_Des divided by the mean activity of Sf_Bra.

2.11 Statistical analysis

Bioassay data considering dead and affected insects were fitted by a logistic regression model to calculate the EC_{50} values and 95% confidence intervals (Prism v8, GraphPad Software Inc., CA, USA). Resistance ratios (RR) were estimated by dividing the EC_{50} value obtained for Sf_Des by the EC_{50} value of the susceptible strain (Sf_Bra).

The mean kinetic velocity was calculated as the increase of RFU/OD min^{-1} in the linear phase of the enzymatic reaction. Average enzyme activity obtained from Sf_Bra and Sf_Des were

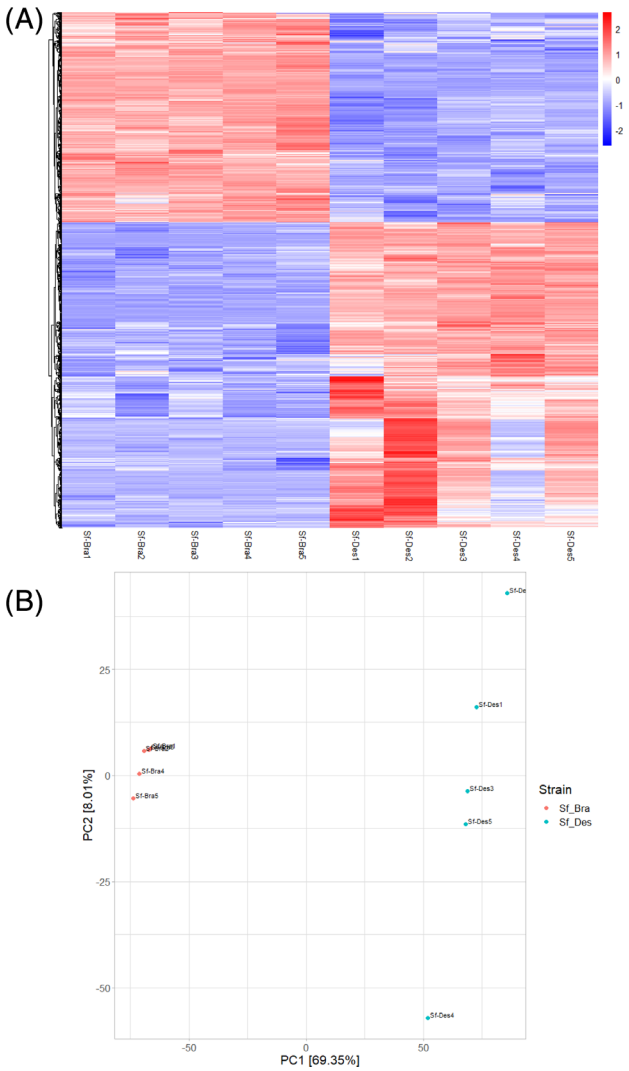

Figure 1. (A) Heatmap showing normalized differential expression level for the top 1460 genes (*Padjust* ≤ 0.01) between *Spodoptera frugiperda* strains Sf_Bra and Sf_Des based on minimal average expression across samples of 100 based on variance stabilizing transformation of DESEq2 package. (B) PCA of RNA-Seq data obtained for strains Sf_Bra and Sf_Des.

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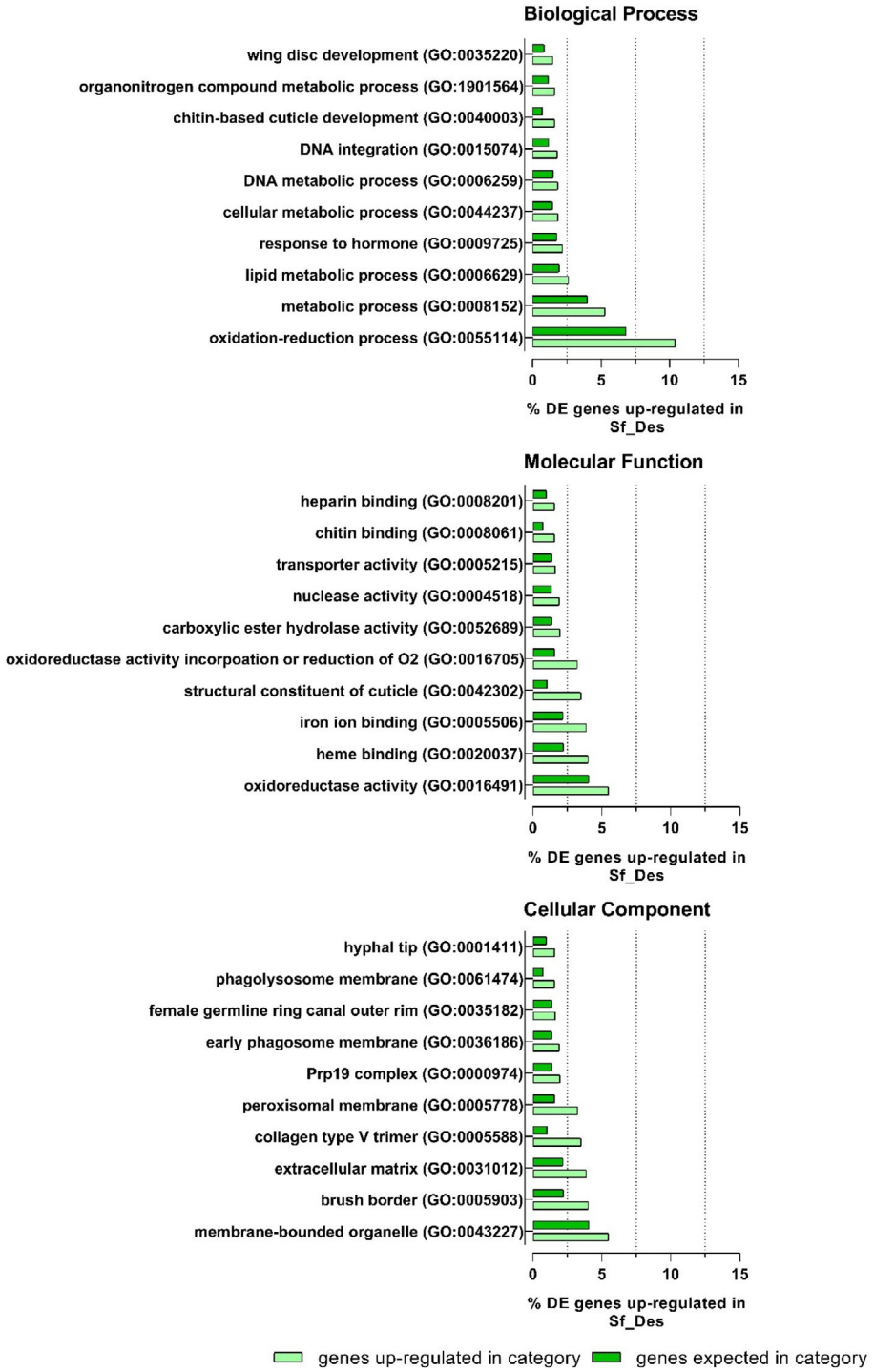

Figure 2. Overview of GO top ten categories and the respective percentage of differentially expressed transcripts (induced) of *Spodoptera frugiperda* Sf_Des strain in comparison to the susceptible reference strain Sf_Bra (P*adjust* < 0.01; DE in category ≥5) assigned to biological process, cellular component and molecular function.

statistically compared by an unpaired Student's *t*-test for each enzyme, substrate and larval development stage separately using PRISM v8.

The expression values obtained by RT-qPCR were normalized to the reference genes and Sf_Des expressions were compared to Sf_Bra and analysed for statistical differences at $P < 0.05$ by Student's *t*-test, with QBASE$^+$ v3.2software (Biogazelle, Belgium).

3 RESULTS

3.1 Bioassays

The efficacy against FAW larvae of 12 different synthetic insecticides was tested and evaluated at 3DAT and 7DAT. As not much difference was observed between the two assessments, the full set of log-dose mortality data for 7DAT is provided in Table 1 and for 3DAT in Table S3. The bioassay results indicate that the Cry1F-resistant strain

Table 4. Differentially expressed genes (DEGs) potentially involved in *Spodoptera frugiperda* detoxification of synthetic insecticides. The DEGs listed have P*adjust* values of <0.01 and an absolute value of log_2 FC ≥ 1 for upregulated genes and log_2 FC < 1 for downregulated genes

Transcripts	Total	log_2 FC ratio ≥ 1	log_2 FC ratio < 1
Cytochrome P450 monooxygenase	125	85	40
Carboxylesterase	67	36	31
Glutathione S-transferase	22	12	10
UDP-glucosyltransferases	27	16	11

Sf_Des also developed significant resistance against deltamethrin (RR_{50} = 14-fold) and chlorpyrifos (RR_{50} = 8-fold).

Almost no variation in susceptibility (RR 1-3) was seen for the individual diamide insecticides, indoxacarb, spinosad, thiodicarb, triflumuron and chlorfenapyr. However, low but significant differences in susceptibility (nonoverlapping CI 95%) were observed for thiodicarb and spinosad at 7DAT (Table 1).

Neonate larvae of strains Sf_Bra and Sf_Des also were subjected to Bt toxicity assays towards Cry1Ac, Cry1Ab and Vip3Aa. The results indicate that strain Sf_Des – known to be resistant to Cry1F[30] – shows high cross-resistance levels against Cry1Ac (>100-fold) and Cry1Ab (>400-fold), but not Vip3Aa when compared to the susceptible reference strain Sf_Bra (Table 2).

Table 5. List of top ten transcripts highly expressed in *Spodoptera frugiperda* Sf_Des strain for the main detoxification enzyme families

Transcript	Log_2FC	Description
P450		
TRINITY_DN1680_c0_g2	12.62606	*Spodoptera litura* CYP9A39-like
TRINITY_DN7188_c0_g1	10.84847	*Spodoptera litura* CYP9A39-like
TRINITY_DN2295_c0_g1	10.56542	*Spodoptera frugiperda* CYP9A59
TRINITY_DN27386_c0_g1	9.147017	*Spodoptera litura* cytochrome P450 9e2-like
TRINITY_DN36_c0_g1	9.144311	*Spodoptera litura* CYP9A39-like
TRINITY_DN2295_c0_g3	8.144188	*Spodoptera frugiperda* CYP9A58
TRINITY_DN13552_c0_g1	8.058662	*Spodoptera litura* CYP9A40-like
TRINITY_DN316_c2_g1	7.988559	*Spodoptera exigua* CYP6B31-like
TRINITY_DN9618_c0_g1	7.874711	*Spodoptera litura* cytochrome P450 4d2-like
TRINITY_DN18867_c0_g2	7.70106	*Spodoptera litura* CYP9A39-like
CE		
TRINITY_DN14758_c0_g2	8.881116	*Spodoptera littoralis* antennal esterase CXE4-like
TRINITY_DN32838_c0_g1	8.237679	*Papilio xuthus* epidermal growth factor receptor substrate 15-like
TRINITY_DN13867_c0_g1	7.814419	*Spodoptera litura* esterase FE4-like
TRINITY_DN15075_c0_g1	7.34096	*Spodoptera litura* juvenile hormone esterase-like
TRINITY_DN12727_c0_g1	7.151613	*Spodoptera litura* esterase FE4-like
TRINITY_DN11913_c0_g1	7.046612	*Spodoptera litura* acetylcholinesterase-like
TRINITY_DN13577_c0_g2	5.215824	*Spodoptera litura* acetylcholinesterase-like
TRINITY_DN40027_c1_g1	5.146553	*Spodoptera litura* juvenile hormone esterase-like
TRINITY_DN28170_c0_g1	5.011122	*Spodoptera litura* juvenile hormone esterase-like
TRINITY_DN1407_c0_g2	4.906461	*Spodoptera litura* esterase FE4-like
GST		
TRINITY_DN12777_c0_g1	7.746027	*Spodoptera frugiperda* glutathione S-transferase epsilon 9
TRINITY_DN16337_c0_g1	6.671198	*Spodoptera frugiperda* glutathione S-transferase epsilon 9
TRINITY_DN28193_c0_g1	5.735641	*Spodoptera frugiperda* glutathione S-transferase epsilon 14
TRINITY_DN22130_c0_g1	5.276331	*Spodoptera frugiperda* glutathione S-transferase epsilon 14
TRINITY_DN6508_c0_g1	4.657795	*Spodoptera frugiperda* glutathione S-transferase theta 1
TRINITY_DN32476_c0_g2	4.627718	*Spodoptera frugiperda* glutathione S-transferase delta 1
TRINITY_DN28359_c0_g1	3.949156	*Spodoptera litura* glutathione S-transferase-like
TRINITY_DN977_c0_g2	3.398927	*Drosophila melanogaster* glutathione S-transferase S1
TRINITY_DN30552_c0_g2	2.920379	*Manduca sexta* glutathione S-transferase 1
TRINITY_DN2322_c0_g1	2.086194	*Spodoptera frugiperda* glutathione S-transferase epsilon 12
UGT		
TRINITY_DN9608_c0_g2	10.6157	*Spodoptera litura* UGT 2B10-like
TRINITY_DN10206_c0_g1	8.38444	*Spodoptera exigua* UGT 33F6 mRNA
TRINITY_DN10071_c0_g1	7.69253	*Spodoptera litura* UGT 2B31-like
TRINITY_DN23825_c0_g1	7.10749	*Spodoptera litura* UGT 2B10-like
TRINITY_DN31624_c0_g1	7.05726	*Spodoptera littoralis* UGT 40 L2-like
TRINITY_DN22190_c0_g1	6.61785	*Spodoptera frugiperda* UGT 40D5
TRINITY_DN8387_c0_g1	6.20902	*Spodoptera litura* UGT 1-7C-like
TRINITY_DN1763_c2_g1	4.22764	*Spodoptera exigua* UGT 40F5-like
TRINITY_DN29556_c0_g1	3.66796	*Spodoptera littoralis* UGT 40R3-like
TRINITY_DN2969_c0_g1	3.15416	*Spodoptera exigua* UGT 33 V1-like

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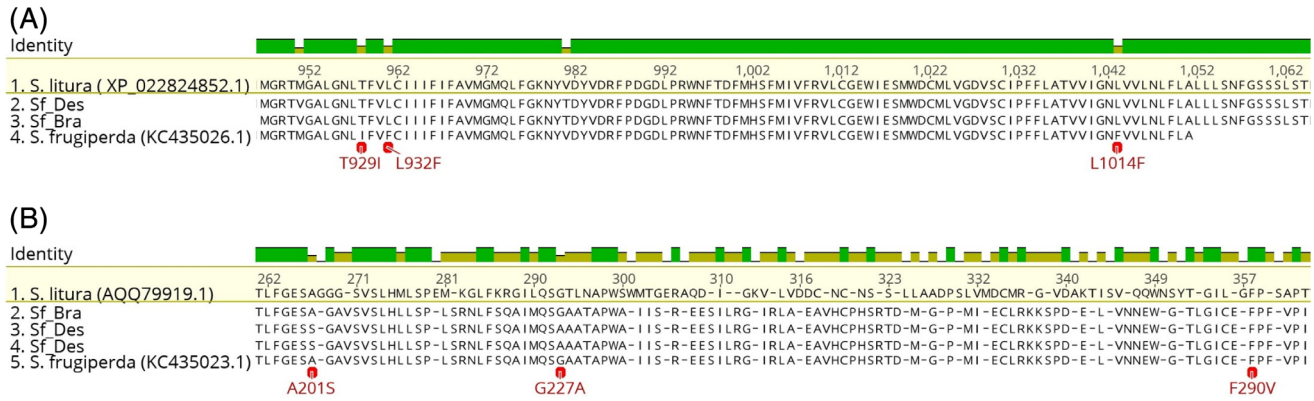

Figure 3. Sequences of VGSC and AChE were obtained from separate assemblies of the *Spodoptera frugiperda* Sf_Bra and Sf_Des strains and compared to *S. litura* sequences for VGSC (XP_022824852.1) and AChE (AQQ79919.1), and partial sequences of VGSC (KC435026.1) and AChE (KC435023.1) obtained from *S. frugiperda* strains resistant to pyrethroid and organophosphate, respectively. Sequences were compared for the presence of T929I, L932F and L1014F target-site mutations in the VGSC, numbered according to *Musca domestica* sodium channel (GenBank X96668) and A201S, G227A and F290V in the AChE, numbered according to *Torpedo californica* AChE (PDB ID: 1EA5).

Table 6. Validation of differentially expressed genes by RT-qPCR analysis. The expression level of 11 genes representing genes involved in the metabolism of insecticides was investigated in the Cry1F-resistant *Spodoptera frugiperda* strain Sf_Des by normalization to the expression of *RPS3A*, *L10* and *L17* and compared to the expression of Sf_Bra. Expressions were statistically analyzed with qbase+ software (unpaired Student's *t*-test, $P < 0.05$). The average relative expression and their respective 95% CI were obtained from five biological replicates run in triplicates

Gene	Strain	Average relative quantity	95% CI high	95% CI low	Comparison (Sf_Des/Sf_Bra)	Statistics
CYP9A-like	Sf_Bra	1.00	23.47	0.04		
	Sf_Des	267.18	385.44	185.20	267.18	*
CYP6B39	Sf_Bra	1.00	28.61	0.03		
	Sf_Des	257.60	370.30	179.19	257.60	*
CYP9A59	Sf_Bra	1.00	1.42	0.70		
	Sf_Des	3.37	5.22	2.17	3.39	**
CYP321A9	Sf_Bra	1.00	1.34	0.75		
	Sf_Des	1.41	1.92	1.03	1.41	ns
CYP333B4	Sf_Bra	1.00	1.21	0.82		
	Sf_Des	1.02	1.31	0.80	1.01	ns
CYP6B50	Sf_Bra	1.00	1.27	0.79		
	Sf_Des	0.82	1.29	0.52	0.82	ns
CYP321-like	Sf_Bra	1.00	0.63	1.58		
	Sf_Des	0.62	0.48	0.81	0.62	ns
CYP321B1	Sf_Bra	1.00	1.65	0.61		
	Sf_Des	0.48	0.61	0.39	0.48	*
CYP9A28	Sf_Bra	1.00	1.80	0.55		
	Sf_Des	0.24	0.48	0.12	0.24	**
CYP332A1	Sf_Bra	1.00	1.36	0.73		
	Sf_Des	0.12	0.14	0.10	0.12	***
CYP321A7	Sf_Bra	1.00	1.52	0.66		
	Sf_Des	0.07	0.12	0.04	0.07	***

ns, not significantly different.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ (unpaired Student's *t*-test).

3.2 Activity of detoxification enzymes (P450, CE and GST)

Cytochrome P450 activity was determined in a fluorometric assay with BOMFC, BFC and BOMR as model substrates (Table 3). The highest activity was obtained with BOMR in strain Sf_Des (112.72 ± 15.58 RFU min^{-1} mg $protein^{-1}$), which was significantly higher than in Sf_Bra (5.85 ± 4.67 RFU min^{-1} mg $protein^{-1}$), representing a 19-fold difference. The coumarin-based substrate BFC also revealed significant differences in activity, albeit at a much lower level (1.6-fold), although no difference was observed with BOMFC (Table 3).

No significant difference in CE activity was observed between Sf_Bra and Sf_Des using 1-NA and 1-NB ($P > 0.05$, two-tailed

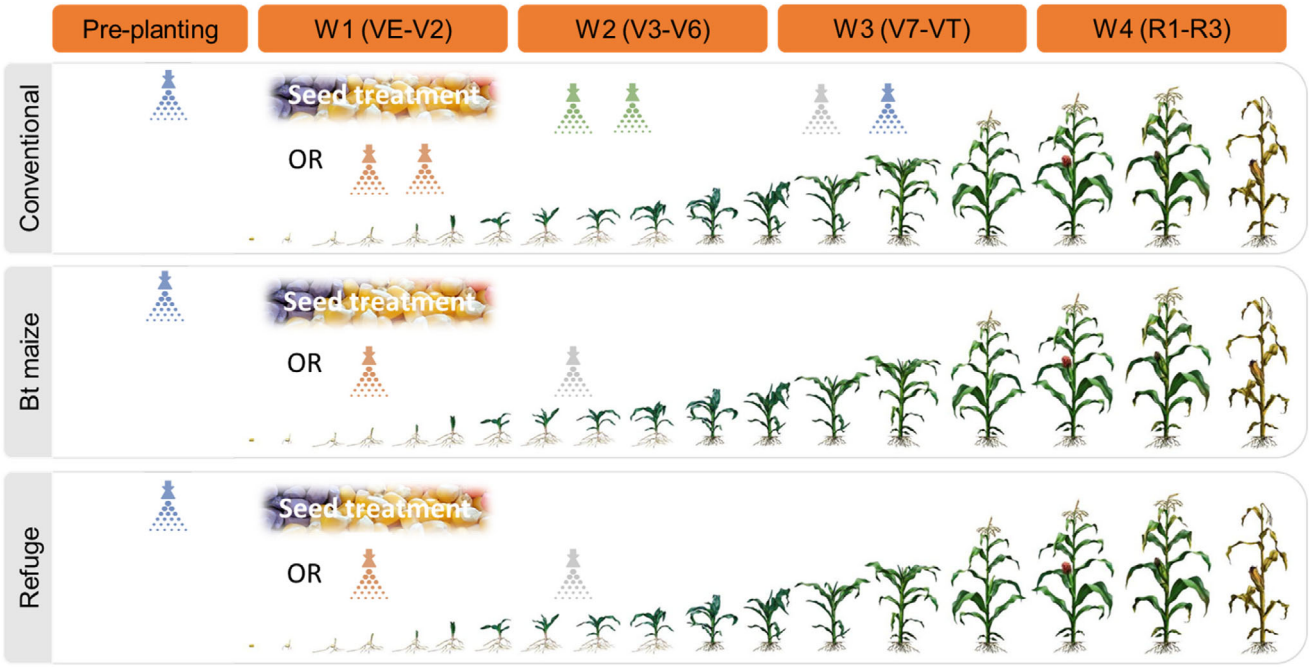

Figure 4. Proposed insecticide application scheme to control fall armyworm (FAW), *Spodoptera frugiperda*, through a growing season that avoids treating consecutive FAW generations with the same mode of action (MoA) in conventional maize, maize expressing *Bacillus thuringiensis* (Bt) insecticidal proteins and refuge areas. The shown schemes are based on the 'MoA treatment windows' approach recommended by IRAC and aim to manage FAW by different MoA in windows representing the mean duration of a single generation (30 days). Each 'spray' color represents a different MoA according to the IRAC MoA classification. Multiple applications of the same MoA are possible within a treatment window. When a treatment window is completed, a different MoA should be selected for use in the next 30 days, and if possible, a different MoA should even be applied in a third MoA treatment window. The example shown is based on a situation with four different MoA´s available and working equally good against FAW.

unpaired Student's *t*-test) as artificial substrates (Table 3), with slightly higher CE activity obtained when using 1-NA as substrate.

The average GST activity between strain Sf_Bra and strain Sf_Des did not differ significantly for both substrates, CDNB and MCB, tested (Table 3).

In conclusion, the enzyme activity measurements performed with different substrates suggest a significantly increased activity of P450 enzymes of resistant strain Sf_Des, whereas no significant differences were observed for CE and GST activities.

3.3 Transcriptomics and target-site mutations

A total of 209.969 trinity transcripts and 118.013 total trinity 'genes' were obtained from the cDNA libraries (Table S4). The average contig and median contig length were 993 and 424, respectively (Table S4). Transcript quantification was determined by pseudoalignment (Table S5), merged on gene level and filtered for genes with cumulative abundance of more than ten across all samples. The transcriptome was deposited in the NCBI Sequence Read Archive database under BioProject PRJNA641764.

A comparative gene expression analysis demonstrated that Sf_Des and Sf_Bra 3rd instar larvae have distinct gene expression profiles [Fig. 1(A)] and are well-separated by principal component analysis [Fig. 1(B)]. Among the 57 534 genes evaluated, 12 339 were differentially expressed (*Padjust* ≤ 0.01, $\log_2$FC ≥ 1 and $\log_2$FC < 1) (Fig. S1). Functional annotation was performed with BLAST2GO and GO terms could be assigned to 15 443 of 57 534 expressed genes. GO term enrichment of genes expressed at higher levels in Sf_Des revealed significant enrichment of 137 GO terms (*Padjust* ≤ 0.01, ≥5 regulated genes) distributed across all three GO domains (Biological Process: 74; Cellular Component: 12; Molecular Function: 51) (Fig. 2 and Table S6). The genes which showed the highest levels of overexpression related to detoxification processes (oxidation–reduction process, GO:0055114; metabolic process GO:008152) and cuticle development (chitin-based cuticle development, GO:0040003) in Sf_Des (Fig. 2). This also is reflected by enrichment of GO terms in cellular localization (membrane-bound organelle, GO:0043227; brush border, GO:0005903) and molecular functions (structural constituent of cuticle, GO:0042302, oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, GO:0016705). In contrast, genes expressed more highly in Sf_Bra are enriched in 111 GO terms involved in DNA integration (GO:0015074) and transposition (GO:0032196) (Table S7).

The 43 transcripts with $\log_2$FC > 10 include genes involved in cuticle proteins, P450 enzymes (CYP9A-like) and one aminopeptidase N-like (Fig. S2). Conversely, five transcripts were highly downregulated in the Sf_Des strain $\log_2$FC < −10, described as zinc finger proteins and myrosinase.

The total number of transcripts assigned as P450, CE, GST, and UGT which were up- and downregulated in Sf_Des is shown in Table 4. Among the differentially expressed genes, the top ten candidate genes involved with detoxification pathways such as P450, CE, GST and UGT were selected and are displayed in Table 5.

As our results indicated a high level of differential expression of *CYP* genes, an aligned and tree based on amino acid identity of P450 assigned transcripts was performed (Fig. S2). Highlighting the transcripts with $\log_2$FC > 5 revealed that most of them are grouped in close-related branch in the cladogram and were annotated as *CYP9A*-like genes (Fig. S3). On the one hand, alignment of the VGSC [Fig. 3(A)] and AChE [Fig. 3(B)] from consensus amino acid sequences obtained from five biological replicates of the

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Sf_Bra and Sf_Des strains, reference *S. litura* and *S. frugiperda* resistant strains revealed no target-site mutation linked to pyrethroid resistance in the VGSC of Sf_Des and Sf_Bra. On the other, A201S and G227A mutations in the AChE (numbering according to *Torpedo californica*: PDB ID: 1EA5) were observed in Sf_Des.

3.4 RT-qPCR

The RT-qPCR analysis validated the RNA-Seq data for 11 selected *CYP* genes. Among the genes tested, three were significantly upregulated in Sf_Des, *CYP9A*-like and *CYP6B39* expression were up to 260-fold higher, and *CYP9A59* was three-fold-overexpressed. however, *CYP321B1*, *CYP332A1*, *CYP321A2*, and *CYP9A28* were significantly downregulated in Sf_Des (Table 6).

4 DISCUSSION

The fast evolution of resistance to many synthetic insecticides [10–14] and insecticidal proteins [21, 22, 24, 43] challenges the control of FAW in Brazil. The increase in resistance is a result of strong selective pressure by frequent sprays with insecticides belonging to a few modes of action and high adoption of Bt crops with low compliance to refuge areas.[19] Therefore, it is of utmost importance to understand the overall toxicological profile of representative strains of resistant insects and their cross-resistance patterns.

The susceptibility of a Cry1F-resistant (Sf_Des) and Cry1F-susceptible strain (Sf_Bra) was assessed against several chemical insecticides and Bt proteins that are currently used and expressed in maize, cotton and soybean to control FAW in Brazil. Very recently we provided a detailed analysis of the mechanistic and genetic basis of Cry1F resistance in strain Sf_Des.[30] We described a GY-deletion in ABCC2 which confers high levels of Cry1F resistance and this mutant was shown to be widespread in Brazil – thus qualifying strain Sf_Des as a surrogate to investigate patterns of susceptibility and potential cross-resistance issues associated with the presence of the observed mutation. We compared the larval transcriptome of strains Sf_Bra and Sf_Des as a step towards understanding the molecular mechanisms possibly influencing the toxicological profile obtained by insecticide bioassays and the detoxification activity obtained in the biochemical assays to support the development of efficient insecticide resistance management strategies.

Although target-site mutations can have a direct effect on the susceptibility to compounds targeting the same receptor,[11, 44] metabolic resistance can affect a much broader range of compounds.[45–47] FAW adaptation to cope with many different plant allelochemicals, is driven by detoxification systems including cytochrome P450s, CE, GST, UGTs and oxidative stress genes which were shown to support its ability to detoxify a broad range of insecticides.[42, 48–51]

Moderate resistance ratios in strain Sf_Des were found for the pyrethroid deltamethrin (RR_{50} = 14-fold). In Brazil, pyrethroid resistance has been reported towards *lambda*-cyhalothrin (18-fold)[11] and shown to be conferred by target-site mutations in the VGSC (T929I, L932F and L1014F), as well as significantly upregulated GST gene expression.[11]

Our transcriptome analysis revealed that 85, 36, 12 and 36 transcripts belonging to P450s, CE, GST and UGTs, respectively, were upregulated in Sf_Des. The enzymatic assay conducted with BOMR in this study showed that P450 activity is significantly higher in Sf_Des and supported by elevated expression levels of *CYP9A*-like, *CYP6B39* (>200-fold), and *CYP9A59* (three-fold) as shown by RT-qPCR analysis. The use of fluorescent model substrates is a common methodology to quantify the activity of P450 enzymes.[52, 53] However, differences in substrate specificity need to be considered and as shown here, BOMR was the substrate showing the highest activity in Sf_Des when compared to the reference strain Sf_Bra. Moreover, the comparison of the VGSC sequence obtained for Sf_Bra and Sf_Des revealed the absence of commonly known target-site mutations and suggests a metabolic mechanism conferring pyrethroid resistance in strain Sf_Des. This is supported by the detected overexpression of some of the P450s mentioned above. High expression levels exceeding several 100-fold of individual P450s such as CYP6BQ23 were recently shown to confer pyrethroid resistance in pollen beetle (*Meligethes aeneus*).[53]

However, further bioassays with P450 inhibiting synergists and functional validation of the highly expressed candidate P450 genes in follow-up studies is essential to investigate the oxidative detoxification potential towards pyrethroids.

Our bioassays revealed a decrease in susceptibility towards chlorpyrifos (eight-fold) by comparing EC_{50} values obtained at 7DAT. Resistance towards chlorpyrifos has been described for FAW collected in Brazil,[11] and associated with an increase of CE and/or GST activities and target-site mutations in the AChE (A201S, G227A and F290V).[8, 11, 54, 55] Although the transcriptome analysis did show that few GSTs and CE were upregulated in Sf_Des, no significant differences in activity were detected with the substrates tested. The AChE sequence comparison revealed the presence of the mutations A201S and G227A in Sf_Des which could explain the resistance level observed. Heterologous expression of AChE wild-type from the silkworm (*Bombyx mori*) and AChE harboring the mutations A303S, G329A and L554S suggest reduction in AChE sensitivity to carbamate and organophosphate insecticides.[56]

For all other compounds tested throughout this study EC_{50} values obtained for Sf_Des did not differ significantly from Sf_Bra, except for a few cases with negligible levels of resistance, such as thiodicarb (2.4-fold) and spinosad (1.6-fold). Nevertheless, resistance to diamide insecticides has been observed in a laboratory-selected strain carrying the I4734M mutation in the RyR and underpins the potential of FAW to develop diamide resistance under field conditions.[29] Resistance to spinosyns was described in FAW in Brazil,[13, 14] but our study revealed a lack of resistance in Sf_Des. Also, a low level of resistance was recorded for the carbamate thiodicarb for Sf_Des supporting the recommendation of this compound for soybean seed treatment to control early damage by FAW.[57] Chlorfenapyr also has shown a lack of resistance towards the Sf_Des strain, confirming the results recently published for FAW from Brazil.[58] Chlorfenapyr is a pro-insecticide, which has to be activated by P450 enzymes.[59] Therefore, the hypothesis that the overall high activity of P450 enzymes might contribute to chlorfenapyr toxicity even in resistant insects, has been considered previously.[58, 60] Indoxacarb also is a pro-insecticide, yet it is activated by esterases through cleavage of the *N*-carbomethoxy group, resulting in an active metabolite that potently blocks the VGSC.[61]

Our bioassay results with different Bt proteins showed excellent control of the Cry1F-resistant strain Sf_Des with Vip3Aa. On the one hand, the Vip3Aa protein does not share binding sites with the Cry1 proteins and therefore crops expressing Vip3A alone or combined with other Cry proteins were shown to effectively control Cry1F-resistant *S. frugiperda*.[62, 63] On the other, there is a high level of cross-resistance among Cry1 proteins in *S. frugiperda*,[24, 64] as confirmed by our results for Cry1Ab (RR >400-fold) and Cry1Ac

(RR >100-fold). Therefore, gene-pyramiding of two or more dissimilar Bt proteins is preferred to delay insect resistance.[65]

Recently, a midgut transcriptome analysis was performed with *S. exigua* exposed to sublethal doses of Cry1Ca and among the DEG, some differences in expression of P450, CE and GST genes were observed besides Bt-related genes, such as ABC transporters.[66] Moreover, cross-resistance between pyrethroids and Cry1Ac has been reported in the diamondback moth, *Plutella xylostella*. Genetic studies suggest that possible interactions between esterases and Bt protein and/or indirect triggering of a defense metabolic pathway are involved and genetically linked at a common locus.[67] Likewise, Gunning *et al.*[68] have shown by *in vivo* assays that esterases from *Helicoverpa armigera* can bind to Cry1Ac, indicating that esterases may play a versatile role in resistance development to both Bt and conventional insecticides.[48, 69] Moreover, Zhu *et al.*[70] detected a co-development of multiple or cross-resistance to both organophosphate insecticides and Cry1F toxin in FAW, supported by high CE and GST activities in the Cry1F-resistant strain. However, in our studies no significant difference in CE or GST activities could be detected between Sf_Bra an Sf_Des.

The CYPs have been observed to respond to sublethal doses of Cry toxins in different insect species, such as *Choristoneura fumiferana*, *Manduca sexta*, *Ostrinia nubilalis* and also *S. exigua*.[66, 71, 72] In *S. exigua*, *CYP4S9*, *CYP6AB31*, *CYP6AE47* and *CYP9A* were upregulated after exposure to Cy1Ca and a similar response was observed in insects exposed to insecticides (*lambda*-cyhalothrin, chlorantraniliprole, metaflumizone and indoxacarb).[66, 73] A few studies have shown that CE is related to Cry resistance in *P. xylostella*, *O. furnacalis* and *C. medinalis* [74–76] and GST were downregulated in *O. furnacalis* to Cry1Ab and *C. medinalis* to Cry1Ac, Cry1Ab and Cry1C. [76, 77] In our studies, we have not checked the expression pattern after the exposure to Bt proteins. However, very high constitutive expression of *CYP9A*-like and *CYP6B39* (> 200-fold) were observed in Sf_Des, suggesting a role of P450 in general detoxification.

Giraudo *et al.*[42] have shown that all members of the CYP9A subfamily are detected in the midgut, fat body, and Malpighian tubules and showed a response to sublethal doses of insecticides. A *CYP9A*-like gene also was found to be upregulated in FAW resistant to lufenuron,[78] supporting the association between CYP9A and insecticide resistance. Likewise, *CYP6B39* was the gene upregulated by most compounds tested, including insecticides and plant allelochemicals.[42] As samples here were taken from the whole body, we cannot disregard the fact that genes belonging to the same subfamily can have tissue-specific expression, for instance, *CYP6AE44* was not detected in the midgut and Malpighian tubules but was present in the fat body in FAW.[42]

Recently, the variation of gene copy number in a locus which includes a cluster of P450 genes has been described to play an important role in insecticide resistance [79] and host-plant range in *S. frugiperda*.[80] *CYP9A* genes were overexpressed upon the treatment of insecticides [42] and were found in two copies clustered together with alcohol dehydrogenase in resistant FAW populations from Puerto Rico.[79]

In our experiments, *CYP321A1* and *CYP321A7* were significantly downregulated in Sf_Des, whereas expression of *CYP321A9* did not differ from Sf_Bra. However, in *Helicoverpa zea* CYP321A1 has been shown to metabolize plant toxins such as xanthotoxin as well as insecticides including aldrin, cypermethrin and diazinon.[81, 82] The next step would be to functionally validate the role of CYP9A-like and CYP6B39 enzymes in FAW and the detoxification of insecticides by their recombinant expression.

The diversity of UGTs in lepidopterans also has indicated their contribution to the process of detoxification through glycosylation[83, 84] and there are several indications in which an increase in the expression level of UGTs have been related to resistance to insecticides such as DDT.[85, 86] In strain Sf_Des studied here, 16 UGT related genes were upregulated and are candidates for a more detailed investigation.

Results of this study, in conjunction with those reported elsewhere [8, 87, 88] demonstrate that FAW insecticide resistance is conferred by multiple biochemical and molecular mechanisms, although most of the chemical classes of insecticides, except two – pyrethroids and organophosphates – worked well against a Brazilian strain highly resistant to Cry1 toxins, suggesting that the GY-deletion in ABCC2 conferring Cry1F resistance in Sf_Des does not result in significant resistant issues towards many chemical classes of insecticides. The high expression levels associated with many genes encoding detoxification enzymes, mainly P450s, even in the absence of insecticide pressure underpins the constitutive nature of the overexpression in strain Sf_Des compared to Sf_Bra, a strain maintained under laboratory conditions for 15 years. The metabolism of insecticides in insects certainly involves a series of complex metabolic processes and there are important gaps such as the multiple roles of detoxification enzymes related to the physiological and molecular mechanisms that control the processes of detoxification.[89, 90]

Our study provides a global transcriptomic profile with special emphasis on detoxification genes in a Bt-resistant Brazilian FAW strain and identified candidate genes to explore further regarding their role in insecticide metabolism. Our results support the significant difference between Sf_Des and Sf_Bra in expression and activity of P450 genes possibly involved in xenobiotic (including insecticide) metabolism, which could support some of the phenotypical resistance observations in the bioassays (e.g. against pyrethroids in the absence of target-site mutation). However, our study does not suggest cross-resistance to many synthetic insecticides in strain Sf_Des shown to be highly resistant to Cry1 toxins conferred by a mutation in ABCC2.

The use of chemical insecticides in refuge areas should be chosen and rotated based on insecticides with good efficacy against Bt-resistant insects, such as those identified here (e.g. triflumuron, thiodicarb, chlorfenapyr, emamectin benzoate, indoxacarb and diamides). A typical resistance management scenario as recommended by IRAC is proposed in Fig. 4 in alignment with the so-called 'mode-of-action treatment windows approach' to ensure that successive generations of the pest are not exposed to the same insecticide or insecticides showing cross-resistance through a growing season. Moreover, pyramided maize expressing Bt toxins with low cross-resistance to Cry1F might be preferred. Therefore, the results presented here for chemical and Bt-based insecticides have important implications for resistance management in Bt crops and IPM programs.

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SUPPORTING INFORMATION

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

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Chapter 5

Monitoring of target-site mutations conferring insecticide resistance in *Spodoptera frugiperda*

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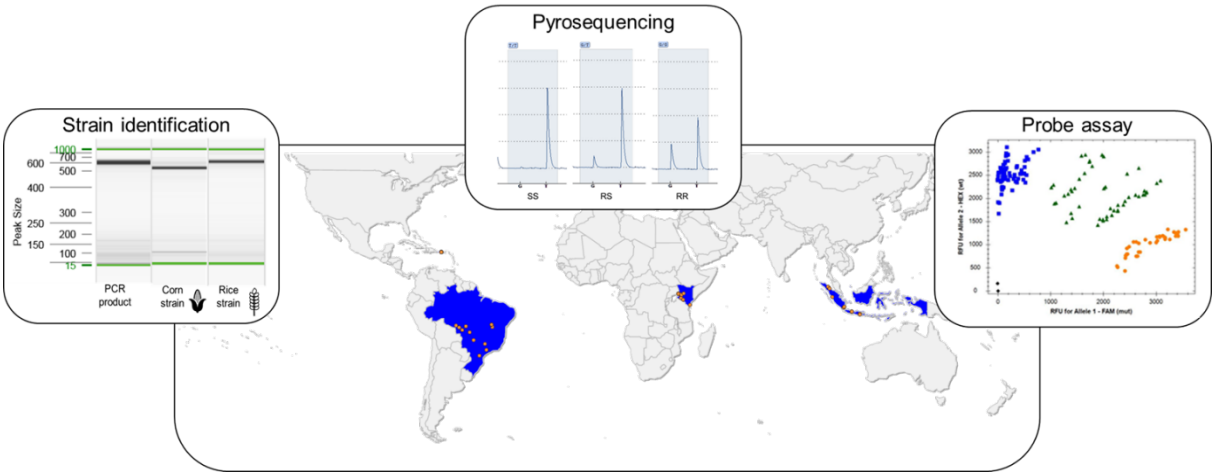

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Monitoring of Target-Site Mutations Conferring Insecticide Resistance in *Spodoptera frugiperda*

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Simple Summary: Fall armyworm, *Spodoptera frugiperda*, is an invasive moth species and one of the most destructive pests of maize. It is native to the Americas but recently invaded (sub)tropical regions in Africa, Asia and Oceania. Fall armyworm larvae feeding on maize plants cause substantial economic damage and are usually controlled by the application of insecticides and genetically modified (GM) maize expressing *Bacillus thuringiensis* (Bt) proteins, selectively targeting fall armyworm. It has developed resistance to many different classes of insecticides and Bt proteins as well; therefore, it is important to check field populations for the presence of mutations in target proteins conferring resistance. Here, we developed molecular diagnostic tools allowing us to test the frequency of resistance alleles in field-collected populations, either alive or preserved in alcohol. We tested 34 different populations collected on four different continents for the presence of mutations conferring resistance to common classes of insecticides and Bt proteins. We detected resistance mutations which are quite widespread, whereas others are restricted to certain geographies or even completely absent. The established molecular methods show robust results in samples collected across a broad geographical range and can be used to support decisions for sustainable fall armyworm control and applied resistance management.

Abstract: Fall armyworm (FAW), *Spodoptera frugiperda*, a major pest of corn and native to the Americas, recently invaded (sub)tropical regions worldwide. The intensive use of insecticides and the high adoption of crops expressing *Bacillus thuringiensis* (Bt) proteins has led to many cases of resistance. Target-site mutations are among the main mechanisms of resistance and monitoring their frequency is of great value for insecticide resistance management. Pyrosequencing and PCR-based allelic discrimination assays were developed and used to genotype target-site resistance alleles in 34 FAW populations from different continents. The diagnostic methods revealed a high frequency of mutations in acetylcholinesterase, conferring resistance to organophosphates and carbamates. In voltage-gated sodium channels targeted by pyrethroids, only one population from Indonesia showed a mutation. No mutations were detected in the ryanodine receptor, suggesting susceptibility to diamides. Indels in the ATP-binding cassette transporter C2 associated with Bt-resistance were observed in samples collected in Puerto Rico and Brazil. Additionally, we analyzed all samples for the presence of markers associated with two sympatric FAW host plant strains. The molecular methods established show robust results in FAW samples collected across a broad geographical range and can be used to support decisions for sustainable FAW control and applied resistance management.

Keywords: fall armyworm; insecticide resistance; target-site mutations; Bt resistance; corn strain; rice strain; resistance management; Indonesia; Kenya

1. Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is an important agricultural pest of several crops in the western hemisphere [1,2]. Since 2016, FAW distribution expanded globally by invading different continents, first reported in Africa and later reaching Southeast Asia and, more recently, Australia, totalizing its presence in 107 countries worldwide [3–6]. The success of FAW spread is due to many factors, such as the high reproductive capacity, long-distance migration and high polyphagia [7,8].

Two sympatric host plant strains of *S. frugiperda* have been previously described: the corn strain, which feeds on large grasses such as corn and sorghum, and the rice strain, preferring small grasses such as rice [9,10]. The two strains differ not only in their host preferences but also regarding their physiology [11], insecticide susceptibility [12] and composition of genes involved in chemoreception, detoxification and digestion [13].

In South America, both strains have been already identified in field populations using molecular markers, and most populations were structured in agreement with their host preferences [14,15]. Although initial studies on the genetic structure of FAW populations from the newly invaded countries suggest a common source of origin, probably from Florida or the Caribbean [16,17], there are differences in the strain haplotypes and disagreements regarding the molecular marker and host plant that may imply inter-population movement of FAW populations from African and Asian countries [18].

An understanding of the genetic background of FAW is essential for resistance management strategies in different regions. Besides the intrinsic variation in insecticide susceptibility associated with FAW strains [11,12,19,20], the impact of migration on insecticide resistance will depend on the pre-existence of resistance alleles in the starting population and selection pressure on the newly invaded areas and spread [14,17].

At present, the Arthropod Pesticide Resistance Database (APRD) reports 144 cases of insecticide resistance in FAW globally. Among the 41 different active substances affected, 45% of the cases belong to proteins produced by *Bacillus thuringiensis* (Bt), 26% and 19% to insecticides targeting the voltage-gated sodium channel (VGSC), and acetylcholinesterase (AChE), respectively [21]. The high number of cases reported for Bt proteins, particularly those expressed in transgenic corn, reflects the intensive adoption of transgenic crops, which corresponded to 191.7 million ha worldwide in 2018 [22]. The adoption of transgenic crops expressing insect-resistant traits to control lepidopteran pests is most advanced in the United States and Brazil. Nevertheless, in Asia, the adoption of Bt-corn is high, particularly in China and India, while it is rather limited to just a few countries in Africa [22].

Many resistance cases are reported for pyrethroid insecticides targeting the VGSC and inhibitors of AChE (i.e., carbamates and organophosphates). This is due to low application costs, a high number of compounds registered for decades and frequent applications [23]. Nevertheless, together, they still account for around 30% of the global insecticide market share [23]. The most modern chemical class used to control lepidopteran pests are the diamide insecticides, acting on the ryanodine receptor (RyR) and used in different agronomic settings [23].

It is unclear whether FAW populations present in Africa were already resistant to old chemical compounds [24]. However, farmers have complained about the efficacy of pyrethroids and organophosphate insecticides under field conditions [25]. Hence, this has led to misuse by increasing rates, application frequency or even the use of unregistered compounds [25,26]. On the other hand, if no control measures against FAW are adopted, the yield losses for corn could reach up to 20.6 m tons per annum for only 12 corn-producing countries in Africa [24].

Insecticide resistance is usually conferred by the insensitivity of the target receptor and/or pharmacokinetic processes modifying the rate or the properties of the insecticides delivered to the target site [27]. Amino acid substitutions/indels at the VGSC (T929I, L932F, and L1014F), AChE (A201S,

G227A, and F290V), RyR (I4790M and G4946E) and ATP-binding cassette subfamily C2 transporter (ABCC2) (GC insertion and GY deletion) have been linked to resistance in *S. frugiperda* to pyrethroids, carbamates and organophosphates, diamides and Bt proteins (e.g., Cry1F), respectively [28–30].

In the present study, we monitored the frequency of the above-mentioned target-site mutations in the VGSC, AChE, RyR and ABCC2 in 34 populations of *S. frugiperda* collected in Brazil, Puerto Rico, Kenya and Indonesia by PCR-based allelic discrimination assays as well as pyrosequencing diagnostics. We validated and established robust diagnostic tools based on genomic DNA, which can be implemented to support decisions for appropriate resistance management strategies.

2. Materials and Methods

2.1. Insect Collection

Larvae of FAW were collected from different sites in Brazil, Puerto Rico, Kenya and Indonesia (Figure 1 and Table S1) and kept in 70% ethanol or RNAlater® (Life Technology, Carlsbad, CA, USA) until DNA extraction and genotyping.

Figure 1. Map showing the origin of 34 fall armyworm populations collected in Brazil, Puerto Rico, Kenya and Indonesia (more details about collection sites in Table S1). All samples were used for the genotyping of target-site mutations. The schematic map was created using EasyMap software (Lutum + Tappert DVBeratung GmbH, Bonn, Germany).

2.2. DNA Extraction

Genomic DNA was extracted from individual larvae (whole body for second/third instar and abdominal fragments of fifth instar larvae). At least five individuals per FAW population (Table S1) were used for gDNA extraction and the total sample number used for the genotyping analysis is shown in Table 1. Agencourt DNAdvance™ (Beckmann Coulter, Beverly, CA, USA) and DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) were used to extract gDNA for the pyrosequencing and fluorescent probe assay, respectively. Both kits were used according to the suppliers' recommended protocols.

Table 1. Genotyping by pyrosequencing for different target-site mutations in major insecticide targets. In total, larvae of 34 populations from Brazil, Puerto Rico, Kenya and Indonesia were analyzed. Homozygous susceptible (SS), heterozygotes (RS) and homozygous resistant (RR).

Target	Country	Mutation	N	SS (%)	RS (%)	RR (%)
Voltage-gated sodium channel (VGSC)	Brazil	L1014F	140	100.0	0.0	0.0
	Puerto Rico		70	100.0	0.0	0.0
	Kenya		76	100.0	0.0	0.0
	Indonesia		110	98.2	1.8	0.0
	Brazil	L932F	143	100.0	0.0	0.0
	Puerto Rico		64	100.0	0.0	0.0
	Kenya		75	100.0	0.0	0.0
	Indonesia		88	100.0	0.0	0.0
	Brazil	T929I	143	100.0	0.0	0.0
	Puerto Rico		64	100.0	0.0	0.0
	Kenya		75	100.0	0.0	0.0
	Indonesia		88	100.0	0.0	0.0
Acetylcholinesterase (AChE)	Brazil	A201S	147	92.5	4.1	3.4
	Puerto Rico		29	100.0	0.0	0.0
	Kenya		76	89.5	10.5	0.0
	Indonesia		85	77.6	22.4	0.0
	Brazil	F290V	127	55.1	44.9	0.0
	Puerto Rico		70	4.3	10.0	85.7
	Kenya		76	26.3	47.4	26.3
	Indonesia		86	19.8	55.8	24.4
	Brazil	G227A	161	55.3	32.3	12.4
	Puerto Rico		29	100.0	0.0	0.0
	Kenya		76	100.0	0.0	0.0
	Indonesia		86	83.7	16.3	0.0
Ryanodine receptor (RyR)	Brazil	G4946E	140	100.0	0.0	0.0
	Puerto Rico		70	100.0	0.0	0.0
	Kenya		76	100.0	0.0	0.0
	Indonesia		90	100.0	0.0	0.0
	Brazil [a]	I4790M	140	100.0	0.0	0.0
	Puerto Rico		70	100.0	0.0	0.0
	Kenya		76	100.0	0.0	0.0
	Indonesia		90	100.0	0.0	0.0
ATP-binding cassette transporter subfamily C (ABCC2)	Brazil [b]	GY del	211	39.83	14.30	45.87
	Puerto Rico		19	100.0	0.0	0.0
	Kenya		70	100.0	0.0	0.0
	Indonesia		79	100.0	0.0	0.0

[a] Data published by Boaventura et al. (2020a) [30]; [b] Data published by Boaventura et al. (2020b) [28].

2.3. PCR and qPCR Conditions

PCR for pyrosequencing, PCR-RFLP and PCR for sequencing were performed in 30 µL reaction mixture containing 15 µL JumpStart™ Taq ReadyMix™ (Sigma-Aldrich, St. Louis, MO, USA), 500 nM of forward and reverse primers (Table S2), around 20 to 50 ng gDNA and nuclease-free water. The cycling conditions comprised 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, the respective annealing temperature according to Table S2 for 30 s and 72 °C for 45 s, and a final elongation step at 72 °C for 5 min.

The fluorescent probe assays for detection of mutations F290V, I4790M and a GC insertion in the ABCC2 consisted of reactions set up at a final volume of 10 µL, with 5 µL SsoAdvanced™ Universal Probes Supermix (Bio-Rad, Hercules, CA, USA), 700 nM of forward and reverse primers (Table S2), 200 nM of probes, 20–50 ng of gDNA and nuclease-free water, and the reactions were run in duplicate.

The conditions of PCR amplification were 95 °C for 5 min and 40 cycles at 95 °C for 15 s and 60 °C for 30 s. The real-time PCR was conducted in a CFX-384 real-time thermocycler (Bio-Rad, Hercules, CA, USA) and the end-point fluorescence values, taking cycle 35 as a threshold, were plotted in a scatter-plot using Bio-Rad qPCR analysis software CFX Maestro 1.0 (Bio-Rad, Hercules, CA, USA).

2.4. Characterization of S. frugiperda Strains

2.4.1. Characterization of COI Haplotypes Using PCR-RFLP

Corn and rice strain genotyping were performed using the molecular markers based on mitochondrial cytochrome oxidase subunit I (COI) with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), according to Nagoshi et al. (2007, 2012) [31,32]. Three to five individuals from different populations of *S. frugiperda* collected in Kenya (EP-K, KV-K, NJ-K, MJ-K and MD-K), Indonesia (WS-I, DS-I, S-I, WC-I and BC-I), Brazil (Sf_Bra, Sf_Cor, MT-PL1-2, BA-SD and PR-PG) and Puerto Rico (PR60, PR61, PR62, PR63 and PR64) (Table S1) were characterized. PCR reactions were carried out according to Section 2.3, using primer JM76 and JM77 (Table S2). After amplification, 1.0 µL of FastDigest MspI (Thermo Scientific, Vilnius, Lithuania) was added to 10 µL of each PCR reaction and incubated at 37 °C for 10 min. The PCR products were verified by an automated gel electrophoresis system, according to the AL320 method (QIAxcel DNA Screening Kit v2.0, QIAGEN, Hilden, Germany). In order to validate the results, a second PCR spanning another restriction site was performed using designed forward (891F_COI) and reverse (c1303R_COI) primers (Table S2). After the amplification, the digestion step was performed by adding EcoRV (New England Biolabs, Frankfurt, Germany), according to the manufacturer's instructions.

2.4.2. Characterization of Tpi Haplotypes Using DNA Sequencing

Plant host strain identification was additionally performed using the triosephosphate isomerase (*Tpi*) gene as a genetic marker, according to Nagoshi et al. (2019) [18]. The PCR amplification was performed according to Section 2.3, using the forward (TpiE4) and reverse (850R) primers described in Table S2. The PCR products were verified by an automated gel electrophoresis system, according to the OM500 method (QIAxcel DNA Screening Kit v2.0, QIAGEN), purified using PCR Clean-up Gel Extraction kit (Macherey-Nagel, Düren, Germany) and Sanger-sequenced by Eurofins Genomics (Cologne, Germany). The obtained *S. frugiperda Tpi* nucleotide sequences were aligned with the *Tpi* sequences for corn and rice variants according to the reference genome [13] (https://bipaa.genouest.org/data/public/sfrudb/), using Geneious software v. 10.2.3 (Biomatters Ltd., Auckland, New Zealand).

2.5. Target-Site Resistance Diagnostics by Pyrosequencing

Amino acid substitutions in the VGSC (T929I, L932F and L1014F), AChE (A201S, G227A and F290V), RyR (I4790M and G4946E) and ABCC2 (GC insertion and GY deletion) result in resistance to pyrethroid, carbamate/organophosphate, diamide and Cry1F Bt protein, respectively. Mutation sites in the VGSC, AChE and RyR are numbered according to *Musca domestica* (GenBank X96668), *Torpedo californica* (PDB ID: 1EA5) and *Plutella xylostella* (GenBank AET09964), respectively.

A pyrosequencing based genotyping assay was designed for targeting each mutation separately and performed across 34 FAW populations (see Table S1 for details about FAW populations).

Primer pairs were designed with Assay Design Software (QIAGEN, Hilden, Germany), according to sequences deposited at the National Center for Biotechnology Information (NCBI) for FAW para-type *VGSC* (GenBank KC435025) and *ace-1* (GenBank KC435023). Primers targeting FAW *RyR* (GenBank MK226188) and *ABCC2* (GenBank KY489760) were described elsewhere [28,30], as indicated in Table S2.

The PCR conditions for pyrosequencing were performed as described in Section 2.3, using primers given in Table S2. The pyrosequencing reaction was carried out as described elsewhere [33], using a sequencing primer specific for every target-site mutation analyzed, according to Table S2.

2.6. Fluorescence Based Allelic Discrimination Assays

2.6.1. F290V Mutation in AChE

Primers were designed using the OligoArchitect™ Assay Design (Sigma-Aldrich, St. Louis, MO, USA) for the detection of the F290V mutation in ace1. Allele-specific probes were labeled with FAM (Sf_F290_FAM) or HEX (Sf_F290_mut_HEX) at the 5′ end for the detection of the wildtype and mutant allele, respectively (Table S2). Five individuals from populations from Brazil (Sf_Bra, MT-PL1 and PR-PG), Puerto Rico (PR60, PR61, PR62 and PR63), Kenya (EP-K, KV-K, MJ-K, KF-K and NW-K) and Indonesia (WS-I, DS-I, S-I, WC-I and JL-I) were tested (Table S1). PCR reactions and allele discrimination analysis were performed as described in Section 2.3.

2.6.2. GC Insertion in ABCC2

The GC insertion in ABCC2 was detected according to Banerjee et al. (2017) [34], with slight modifications. Briefly, reactions were composed of a HEX-labeled probe (SfABCC2mut allele) that is *SfABCC2* mutant allele-specific and a FAM-labeled probe (SfABCC2), specific to the *SfABCC2* wildtype allele, gDNA (around 50 ng), the forward (Sf_ABCC2_F) and the reverse (Sf_ABCC2_R) primers (Table S2). The populations tested were the same as described in Section 2.6.1 and PCR reactions were prepared as mentioned in Section 2.3.

2.6.3. I4790M Mutation in the RyR

The detection of the RyR I4790M mutation was performed as described by Boaventura et al. (2020) [30] using forward (Sf_taq_I4790_F) and reverse (Sf_taq_I4790_R) primers, the mutant allele-specific FAM-labeled probe (Sf_I4790_mut_FAM) and a HEX-labeled probe (Sf_I4790_HEX) that is wildtype allele-specific (Table S2). Individuals with known genotype from strain Chlorant-R (homozygote for M4790) as well as artificial heterozygotes (a mixture of gDNA from Chlorant-R and Sf_Bra individuals) were used as internal controls. The assay was validated with populations collected in Brazil, Puerto Rico, Kenya and Indonesia, as described above (Section 2.6.1).

3. Results

3.1. Characterization of S. frugiperda Strains

The mitochondrial COI and nuclear Tpi molecular markers were employed for the identification of sympatric FAW rice and corn strain according to Nagoshi et al. [17,31,32]. The amplification of the respective COI fragment resulted in a PCR product of around 569 bp for both strains, but the fragment amplified from corn strain contained a MspI restriction site; therefore, after digestion, the PCR product was cut into two fragments (approximately 487 and 72 bp) (Figure 2A and Figure S1A). According to this method, all samples tested from Brazil and Puerto Rico were characterized as corn strain, whereas in Kenya and Indonesia, most of the individuals were characterized as rice strain, i.e., 70% and 91.7%, respectively (Figure 2A).

On the other hand, when using the EcoRV restriction site, the rice strain fragment was cut into two bands of around 350 and 150 bp and the corn strain fragment of 500 bp remained uncut (Figure 2B and Figure S1B). Again, all samples tested from Brazil and Puerto Rico were corn strain and most of the samples from Kenya and Indonesia were rice strain, i.e., 61 and 92%, respectively.

Host plant strain characterization using the *Tpi* gene was performed according to Nagoshi et al. (2019) [17]. The genetic markers used in this method are all single nucleotide substitutions present in the *TpiE4* exon. When using the primers 412F and 850R (Table S2), most of the *TpiE4* exon is amplified, producing a fragment of about 199 bp. Trimmed sequences were deposited in NCBI (GenBank MT706015–MT706018). The strain is defined by the gTpi183Y site, where the corn strain has a cytosine and the rice strain a thymine. All the samples tested from the four countries were corn strain, having a cytosine at the position gTpi183Y. It is worth mentioning that, at the position gTpi192Y,

an adenine or thymine was observed in some samples from Brazil, Puerto Rico and Kenya (1, 1 and 2, respectively), while Nagoshi et al. (2019) [17] reported only a cytosine or a thymine at position Tpi192Y.

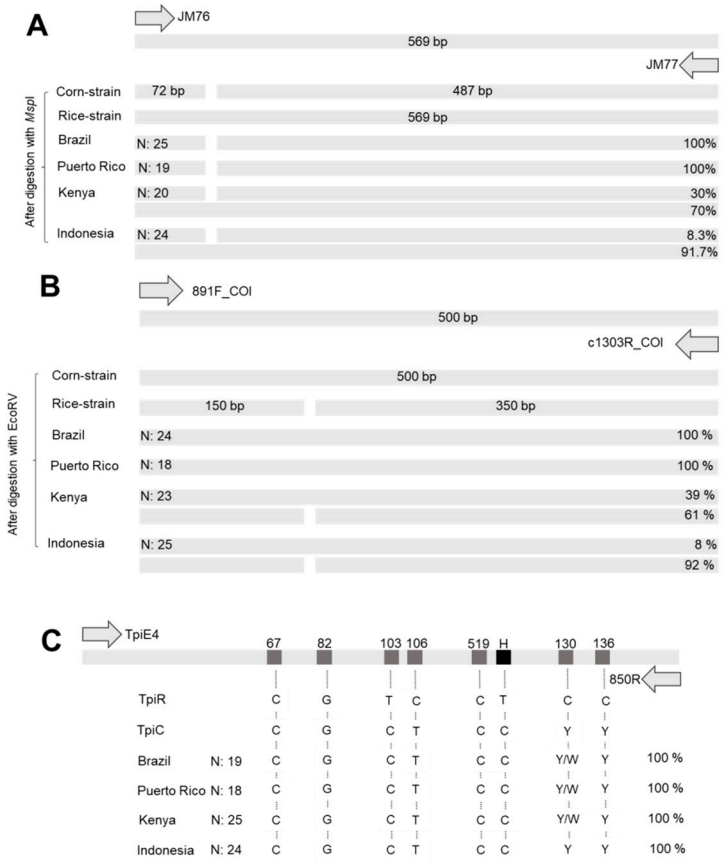

Figure 2. Schematic representation of amplified *COI* and *TpiE4* fragments used for *Spodoptera frugiperda* host plant strain identification in field samples collected in Brazil, Puerto Rico, Kenya and Indonesia. *COI* polymorphism in *S. frugiperda* was determined by RFLP-PCR. (**A**) PCR product containing a MspI restriction site in the corn strain and PCR fragments obtained after digestion with FastDigest MspI. (**B**) PCR product that contains an EcoRV strain-specific site. After digestion with EcoRV, the corn strain remains uncut, whereas the rice strain is cut. (**C**) *TpiE4* fragment with different polymorphic sites was Sanger-sequenced. Position marked H defines whether it is a rice strain (thymine; TpiR) or a corn strain (cytosine; TpiC).

3.2. Detection of Target-Site Mutations by Pyrosequencing

The pyrosequencing assay used to genotype the mutations in the VGSC revealed that almost all analyzed larvae (n = 396) were wildtype, with no mutations at those sites analyzed. Only strain NB-KA from Indonesia included a few individuals heterozygous for the L1014F mutation, corresponding to 1.8% of all samples analyzed from Indonesia (Table 1). On the other hand, the mutations T929I and L932F were not detected at all in any population tested (Table 1), suggesting the lack of target-site resistance to pyrethroids in almost all samples analyzed. Resistant AChE alleles were found at much higher frequencies across countries in many populations analyzed. The mutation F290V was detected at the highest frequency (Table 1). In Brazil, 45% of the samples genotyped (57 out of 127 larvae) were heterozygote, whereas most samples from Puerto Rico (except strain PR65) were homozygote for V290, representing 85.7% of all samples tested (60 out of 70 larvae). Populations collected in Kenya and Indonesia also carried the F290V mutation in AChE and, on average, 47% and 56% of the samples were heterozygotes, respectively. The other AChE mutation sites analyzed, A201S and G227A, were not detected in Puerto Rico, while G227A was absent also in Kenya. RyR mutations G4946E and

I4790M, conferring resistance to diamide insecticides, were not detected in any of the populations tested (Table 1); all individuals tested were homozygous wildtype at both positions. We also tested 379 individuals for the presence of a GY deletion in ABCC2, known to confer resistance to Cry1F in FAW [28]. This functionally validated target-site mutation was absent in samples collected in Puerto Rico, Kenya and Indonesia but detected in many of the tested Brazilian larvae, as recently described [28] (Table 1).

3.3. Detection of Target-Site Mutations by Fluorescence Based Allelic Discrimination Assays

As the target-site, mutation F290V in AChE was the most frequent mutation found in all populations tested. We decided to develop a PCR-based allelic discrimination assay using fluorescent probes, which could be performed at larger-scale worldwide using a qPCR machine, because pyrosequencing-based diagnosis is more expensive and less common. All larvae analyzed from Puerto Rico were homozygote for the V290 resistance allele (Figure 3A). In Kenya and Indonesia, five populations were tested and, on average, 40% and 56% of the larvae were heterozygotes, respectively (Figure 3C,D). Individuals from the two field populations from Brazil (MT-PL1 and PR-PG) were homozygous for the V290 resistance allele (Figure 3B). The population Sf_Bra was kept for 15 years under laboratory conditions without insecticide exposure and all larvae were homozygotes for the susceptible wildtype allele F290 (Figure 3B).

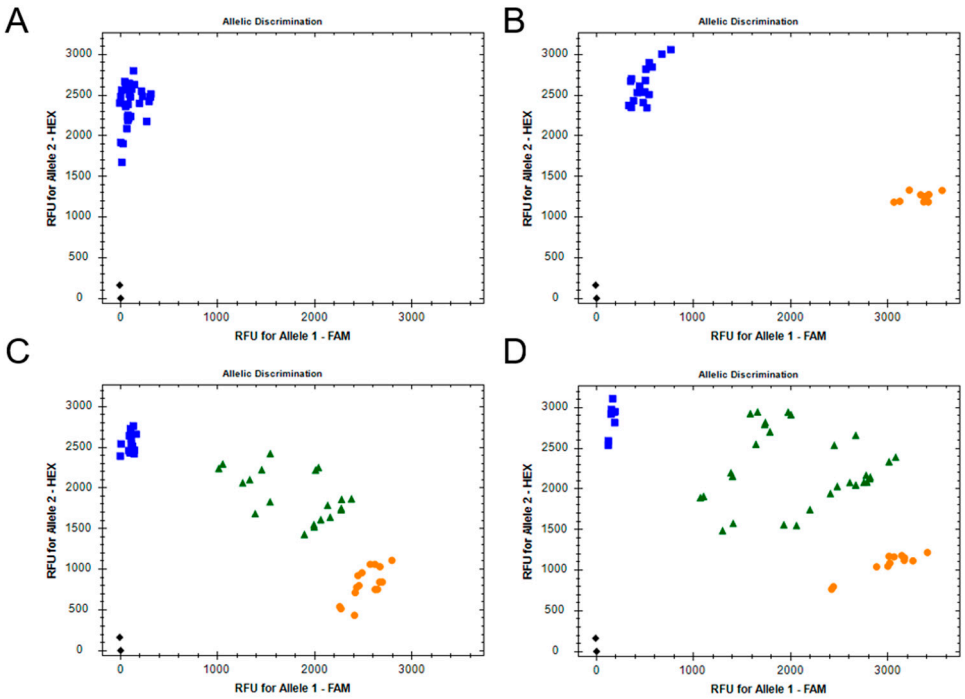

Figure 3. Bivariate plot showing the discrimination of different acetylcholinesterase alleles in *Spodoptera frugiperda* samples by an allele-specific real-time PCR fluorescent probe assay. Each dot represents a single larva. Blue squares represent mutant RR homozygotes (V290; allele 1), orange circles susceptible SS homozygotes (F290; allele 2) and green triangles SR heterozygotes (F290/V290). Analysis of fall armyworm field samples collected in (**A**) Puerto Rico, (**B**) Brazil, (**C**) Kenya and (**D**) Indonesia.

The I4790M mutation in the RyR was assessed using Chlorant-R resistant FAW larvae as a positive control for M4790. The resistant allele was not present in any other sample analyzed (Figure 4). For the detection of GC insertion at the ABCC2 causing resistance to Cry1F protein in *S. frugiperda* in Puerto Rico, the assay described by Banerjee et al. (2017) [34] was used, however substituting the VIC fluorescent probe with FAM, as described in Section 2.6.2. The GC insertion was only observed in Puerto Rican samples (Figure S2).

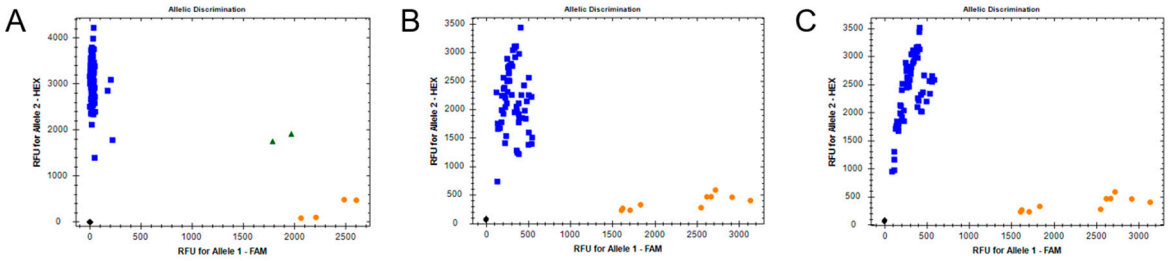

Figure 4. Detection of the RyR I4790M mutation using an allele-specific real-time PCR fluorescent probe assay, as recently described by Boaventura et al. (2020). (**A**) Genotyping of *Spodoptera frugiperda* collected in Puerto Rico represented by blue squares (wildtype SS homozygotes, I4790 allele), orange circles represent strain Chlorant-R mutant RR homozygotes from Brazil (M4790; allele 1) and green triangles artificial SR heterozygotes (I4790/M4790). All individuals tested from (**B**) Kenya and (**C**) Indonesia were susceptible homozygotes for I4790 (blue squares).

4. Discussion

The highly invasive nature and the potential economic impact of FAW have raised a lot of concerns across continents. Changes in agricultural practices and biological control are among a diverse range of measures implemented in recently invaded African countries and India by smallholder farmers and at rather low FAW infestation levels [25,26,35–39]. In countries with significant agricultural input subsidy programs, synthetic insecticides have been used to control FAW outbreaks [25,26,38]. However, in some countries, farmers claimed rather low efficacy of some of the insecticide classes used, such as organophosphates and pyrethroids [25,35]. It remains unclear whether the low field efficacy of insecticides against FAW in Africa is due to resistance or poor application technology affecting plant coverage.

Our genotyping study was conducted to shed some light on the presence of target-site insecticide resistance mechanisms in 34 populations collected in Kenya, Indonesia, Puerto Rico and Brazil. Our results from FAW populations collected in Kenya showed a relatively high frequency of the F290V mutation in AChE, the target of organophosphate and carbamate insecticides. The chance that alleles conferring resistance to these rather old chemical classes were already present at high frequency in the invasive population is quite high. Their frequency was likely augmented by further selection, using applications of cheap products based on organophosphate and carbamate chemistries. Similar findings have been reported for the tomato leafminer (*Tuta absoluta*) in Iran, where this pest has been recently introduced. Resistance to pyrethroids and organophosphates was expected in the invading populations and this expectation was supported by the identification of target-site mutations in VGSC and ace1, respectively [40].

As a result of frequent insecticide applications, multiple resistance cases have been described for field populations in regions where FAW is native [41,42]. In Brazil and Puerto Rico, for instance, resistance has been reported to pyrethroids, organophosphates, carbamates, spinosyns, benzoylureas and, most recently, diamides [29,42–47]. The genetic inheritance of insecticide resistance in FAW has been investigated, and cases of FAW resistance were described as polygenic and metabolic [46–48].

Pyrethroid insecticides are supposed to bind in the domain IIS4-S5 linker and domain IIIS6 of *para*-type sodium channels [49] and the common L1014F mutation has been reported to confer pyrethroid resistance ratios of 10-20-fold [29,50–52]. More than 30 unique resistance-associated mutations including L1014F or combinations thereof have been described in VGSC in many other different species [53]. Three mutations (T929I, L932F and L1014F) at the VGSC have been recently described in pyrethroid-resistant *S. frugiperda* from Brazil [29] and one of the mutations, L932F, was detected in FAW populations from China [54]. Our genotyping results revealed the absence of the L1014F mutation in almost all analyzed samples, except for one population from Indonesia (K-I, Table S1), where only two heterozygotes out of 30 individuals were detected. No other mutation conferring pyrethroid resistance and described for *S. frugiperda* was detected in the populations tested.

However, other mechanisms such as enhanced metabolism by elevated levels of cytochrome P450 monooxygenases are known to confer pyrethroid resistance in FAW [29] but were not tested in our study as we used gDNA of alcohol preserved FAW samples as we did not have access to living insects.

Organophosphates and carbamates target AChE and resistance is often associated with mutations in the *ace-1* gene, leading to amino acid substitutions at the enzyme's active site [55]. Our genotyping results confirmed the presence of the following amino acid substitutions A201S, G227A and F290V in populations collected in Brazil, as described by Carvalho et al. (2013) [29]. The detected point mutations co-exist, at least in heterozygous individuals, in populations BA-SD, PR-PG and MT-PL1-2. Moreover, we also detected the F290V mutation in samples from Puerto Rico, Indonesia and Kenya. Point mutations linked to organophosphate resistance have been described for *Cydia pomonella* (F399V), *Chilo suppressalis* (A314S) and *P. xylostella* (D131G, A201S, G227A and A441G) [56–60]. Moreover, heterologous expression of AChE mutants (A303S, G329A and L554S) from the silkworm (*Bombyx mori*) have supported the reduction in AChE sensitivity towards carbamate and organophosphate insecticides [61].

Diamide insecticides comprised two chemotypes, the phthalic (flubendiamide) and anthranilic acid diamides (e.g., chlorantraniliprole), which were shown to be affected differently by the presence of point mutations leading to amino acid substitutions, particularly G4946E and I4790M in the lepidopteran RyR (numbering according to the *P. xylostella* RyR)—recently reviewed by Richardson et al. [62]. Phthalic diamides are less potent against pests carrying a methionine at position 4790 [63]. In Puerto Rico, resistance ratios of 160 to 500- fold have been reported to chlorantraniliprole and flubendiamide, respectively, but the mechanisms of resistance were not studied in detail [42]. However, in our genotyping assays, G4946E and I4790M were not detected in samples from Puerto Rico or any other country. So far, the I4790M mutation in *S. frugiperda* has been detected only in one FAW strain (Chlorant-R) from Brazil, selected with chlorantraniliprole under laboratory conditions and showing resistance ratios of >230 and >42,000-fold against chlorantraniliprole and flubendiamide, respectively [30,43]. Our genotyping data suggest that the frequency of those resistance alleles (G4946E and I4790M) is low under field conditions in those locations here investigated.

Mutations in the ABCC2 transporter have been associated with Cry1F and Cry1A.105 resistance in FAW: a GC insertion causing a premature stop codon has been found in Cry1F-resistant FAW strains from Puerto Rico [34,64], whereas a functionally validated GY deletion was very recently described in Cry1F-resistant populations from Brazil [28]. While we identified the described GC insertion and GY deletion in many samples from Puerto Rico and Brazil, respectively, none of the above-mentioned mutations were found in populations from Kenya or Indonesia, supporting the absence/very low frequency of these mutations in the field and a lack of selection pressure by transgenic corn expressing Cry1F in those countries. Recent whole-genome sequencing of FAW samples collected in China, Malawi, Uganda and Brazil revealed a novel ABCC2 resistance allele in FAW collected in Brazil, leading to a truncated and likely non-functional protein [65].

Two sympatric host plant strains of *S. frugiperda* have been previously described: the corn strain and the rice strain, which prefers forage grasses and rice [9–11]. Recent studies have reported that *S. frugiperda* populations present in Asia and Africa are an inter-strain hybrid, with the genetic background mostly from the corn strain [54,66]. Therefore, we were interested in the host-plant strain composition of our samples and analyzed individual larvae of different populations using recently described markers by RFLP and PCR. Our results using COI and Tpi genetic markers confirmed that the corn strain is the most abundant in Brazil and Puerto Rico, as shown in previous studies [14,15,67,68]. The COI genetic marker used in this study revealed dominance of the rice strain in those populations that we collected in Kenya (70%, though collected from corn plants) and Indonesia (91.7%). However, to avoid any identification bias, we used a second marker, *Tpi*, and the obtained data revealed that all samples, including those from Kenya and Indonesia, resemble corn strain and none rice strain. This discrepancy between COI and *Tpi* markers has already been noticed by other authors, especially with samples from Africa and Asia, where strain characterization is dependent on the molecular marker

used [16,18,65]. However, the exclusive identification of the corn strain in our samples by using the *Tpi* marker is in accordance with the preferred host, suggesting that it is a more accurate strain marker than COI. In terms of insecticide susceptibility, there is not much difference between host-plant strains, at least when considering the efficacy of recommended label rates of many insecticides. A slightly higher level of cytochrome P450 activity in corn-adopted FAW [9] may render the corn strain slightly more tolerant, but this is unlikely to result in reduced efficacy of insecticides at their recommended label rates in the absence of resistance. The rice strain has been reported to be more susceptible to diazinon, carbaryl and Bt toxins, whereas corn strain larvae were shown to be more susceptible to the carbamate carbofuran [12,19,20]. Recently, Arias et al. (2019) [14] have tested the possible influence caused by the migration of individuals from hot spots—characterized by higher LC_{50} values against flubendiamide and lufenuron. The authors concluded that migration did not play the key role but, rather, the pest management measures adopted and cropping strategies in the respective region. Therefore, we want to reinforce that, although high frequencies of alleles conferring resistance to organophosphate and carbamates were detected, the choice of the appropriate management strategy to be adopted based on regionally registered insecticides and alternative measures is likely to be the key factor for sustainable FAW control. The practical relevance of the presence of alleles conferring resistance is determined by the selection pressure adopted in the field and whether the mutations present carry any fitness cost. The resistance alleles might decrease in frequency in the absence of selection pressure or increase when the application of specific insecticides increases [14]. Therefore, strategies to slow down the development of insecticide resistance should be driven by the application of insecticides with different modes of action [14,69]. Compounds such as diamides, emamectin benzoate and spinosyns [25,70–72] have mostly shown good control of several lepidopteran pests and would be valuable tools in FAW resistance management strategies in the newly invaded countries.

5. Conclusions

Based on our genotyping results described in this study, the field efficacy of organophosphate and carbamate insecticides is likely to be compromised by the presence of the AChE V290 allele in hetero- and homozygous form in Brazil, Kenya, Indonesia and Puerto Rico. To achieve successful integrated pest management of FAW and reduce the risk of economic losses, resistance management strategies will need to be implemented at regional levels in the newly invaded countries and can be supported by using the presented diagnostic tools to detect and monitor the early presence of resistance alleles in the field.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/11/8/545/s1, Table S1: Populations of *Spodoptera frugiperda* collected in different countries and years used for genotyping of target-site mutations, Table S2: List of primers for pyrosequencing and dual fluorescence probe assay used for the identification of different target-site mutations and Spodoptera frugiperda strain identification by RFLP-PCR and Sanger sequencing, Figure S1: Automated analysis of DNA fragments showing COI polymorphism in Spodoptera frugiperda. (A) PCR product containing a strain specific MspI site that was amplified using the JM76 and JM77 primers (Table S2) followed by products obtained after the digestion with FastDigest MspI. Corn-strain is cut and rice-strain remains uncut as it does not have the MspI site. (B) PCR product amplified with the primers 891F_COI and c1303R_COI (Table S2) that contains a EcoRV strain specific site. After digestion with EcoRV the corn-strain amplicon remains uncut whereas it is cut in the rice-strain. Details about samples, see Table S1, Figure S2: Detection of GC insertion allele at the ATP-binding cassette subfamily C2 (ABCC2) conferring resistance to Bacillus thuringiensis Cry1F toxin using PCR fluorescent probe assay described by Banerjee et al [34]; Blue squares represent mutant ABCC2 homozygotes for the GC insertion, orange circles ABCC2 wildtype SS homozygotes, and green triangles SR representing heterozygotes. Analysis of fall armyworm field samples collected in (A) Brazil, (B) Puerto Rico, (C) Kenya, and (D) Indonesia.

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Chapter 6

General Discussion

This thesis aimed to investigate at the molecular and biochemical level the different mechanisms involved in the resistance to synthetic insecticides and insecticidal proteins in FAW.

Mechanisms conferring resistance to diamide insecticides (Chapter 2) and Cry1F (Chapter 3) were here for the first time reported and characterized for *S. frugiperda* collected in Brazil. Considering the global economic importance of FAW as a pest, diagnostic methods for the identification of resistance alleles were performed in 34 different populations of FAW collected in four different countries (Chapter 4), providing monitoring tools for early detection of resistance alleles in the newly invaded countries in Africa and Asia. To achieve a broader understanding of the toxicological and detoxification profile of FAW, we investigated a Cry1F-resistant strain from Brazil (Sf_Des) in comparison to a reference strain (Sf_Bra). The efficacy of different insecticides and Bt proteins were tested and the detoxification pathways were investigated by molecular and biochemical means (Chapter 5). This thesis contributes to a broader understanding of molecular mechanisms conferring resistance to a range of insecticides and provides tools to design insecticide resistance management strategies by supporting integrated pest management at different geographies.

6.1 Resistance to synthetic insecticides

FAW has been controlled in the American continent historically by several applications of synthetic insecticides and more recently also by the adoption of crops expressing Bt proteins (Resende et al., 2016; Storer et al., 2012). However, cases of field resistance to commercially available insecticides and crops expressing Bt such as maize, cotton, and soybean have hindered FAW control. Only in Brazil, cases of resistance to 24 compounds have been reported (“APRD,” 2020) and currently FAW is among the top 15 most resistant pests worldwide (Sparks et al., 2020).

The elucidation of the mechanisms conferring resistance is an essential step for supporting recommendations for the control of FAW, based on the current resistance status and insecticide efficacy. Resistance management strategies are not only relevant in the newly invaded countries, but essential for keeping sustainable strategies and product life-cycle management where many resistance cases are reported, for instance in Brazil.

Inheritance of resistance has been extensively investigated for many insecticides (Bernardi et al., 2016; Nascimento et al., 2016; Okuma et al., 2017). Understanding the dominance of resistance supports recommendations regarding field rates and speed of resistance development (Bourguet et al., 2000). When resistance is functionally recessive or incompletely recessive, heterozygote individuals might be controlled at field rates (Nascimento et al., 2016; Okuma et al., 2017). The dominance of resistance is also important for Bt traits. However, in this case, the expression *in planta* may be constant during the vegetative development and high-dose (25-fold the dose needed to kill all homozygous susceptible larvae) (Roush, 1998).

Very few studies have been carried out on the molecular mechanism underlying insecticide resistance in FAW. Limitations regarding infrastructure can play a role, but also depending on the nature of resistance, i. e. polygenic resistance might require much more complex studies.

The first case of insecticide resistance reported to *S. frugiperda* was for the carbamate carbaryl (Young and McMillian, 1979). Carbamates and OP insecticides are for a long time in the market and are represented by a large number of compounds (Sparks et al., 2020), favoring the evolution of resistance of FAW to these chemical classes. Earlier studies have shown a significant decrease in AChE sensitivity in FAW resistant to carbamates and OP´s (Yu, 2006; Yu et al., 2003). Ten years later, point mutations in the AChE (A201S, G227A, and F290V) have been linked to FAW resistance to OP´s in FAW from Brazil (Carvalho et al., 2013). Currently, OP-resistant alleles have been reported in FAW from a wide geographic range (China, Malawi, Uganda, and Brazil) (Guan et al., 2020; Zhao et al., 2020). These results were also confirmed in this study (Chapter 4), by genotyping 34 FAW populations collected in Brazil, Puerto Rico, Kenya, and Indonesia. These results lead us to conclude that mutations in the AChE have been fixed in different populations of FAW and were carried to the newly invaded countries with the migrated population. The frequency of the F290V mutation in Kenya (47.4 % heterozygotes and 26.3 % homozygotes) and Indonesia (55.8 % heterozygotes and 26.3 % homozygotes) (Chapter 4) might support the relatively low efficacy of OP´s reported in the field (Sisay et al., 2019).

Another chemical class that has been intensively used to control lepidopteran pests are the pyrethroid insecticides and resistance cases in FAW have been reported (Carvalho et al., 2013; Diez-Rodríguez and Omoto, 2001; Rabelo et al., 2020).

Mechanisms commonly involved in pyrethroid resistance are target-site mutations in the VGSC (Williamson et al., 1996) and P450-mediated metabolic resistance, followed by CE (Z.-G. Wang et al., 2019; Wheelock et al., 2005).

So far, target-site mutations (T929I, L932F, and L1014F) in the VGSC have been observed in pyrethroid-resistant FAW from Brazil (Carvalho et al., 2013). In Chapter 4, only one FAW population from Indonesia had two individuals heterozygous for the L1014F, while none of the three mutations linked to pyrethroid resistance was detected in FAW populations in Asia and

Africa (Guan et al., 2020; Zhao et al., 2020). Even in Puerto Rico where FAW populations have evolved resistance to pyrethroids (Gutiérrez-Moreno et al., 2019), all individuals from five different strains were homozygous for the susceptible allele (Chapter 4). Those results suggest that the frequency of resistance alleles is relatively low in the field and that detoxification enzymes might play a major role in pyrethroid resistance in FAW. This is supported by synergist studies in FAW populations from China (Zhao et al., 2020) and the results shown in Chapter 5. The Cry1F-resistant strain (Sf_Des) showed cross-resistance to deltamethrin (14-fold), absence of target-site mutations linked to pyrethroid resistance in the VGSC, and considerably high expression and activity of P450 enzymes in comparison to a reference strain Sf_Bra (Chapter 5).

The RNA-Seq data obtained for Sf_Des revealed that many P450 genes are up-regulated (mainly *CYP9A*-like identified transcripts) in comparison to the susceptible strain Sf_Bra. The results were also confirmed by gene expression anaylsis (RT-qPCR) for CYP9A-like (up to 200-fold) and at enzymatic levels with up to 19-fold higher activity in Sf_Des using BOMR as substrate. On the other hand, the number of GST´s differentially up or down-regulated in Sf_Des was not prominent, confirmed by similar enzymatic pattern as the reference strain Sf_Bra (Chapter 5). Therefore, P450s might play a major role in the overall detoxification profile of Sf_Des towards insecticides.

The high constitutive expression of specific genes might be more prompt to confer resistance at field conditions, although exposure to sublethal doses might result in changes at transcriptional levels and help the identification of candidate genes involved in the insecticide detoxification (Nascimento et al., 2016). In Sf_Des, *CYP9A9*-like, *CYP6B39*, and *CYP959* seem to be involved in insecticide metabolism as evidenced previously, and described in Chapter 5 (Giraudo et al., 2015; Nascimento et al., 2015). However, further functional evidence with the selected candidate genes would be needed to support the cross-resistance pattern among insecticides.

In China, the susceptibility of *S. frugiperda* to indoxacarb was significantly different (10-fold for LC_{50}) across populations (Zhao et al., 2020). However, in our bioassays (Chapter 5), no significant differences between Sf_Bra and Sf_Des were observed towards indoxacarb, confirming the lack of cross-resistance between indoxacarb and pyrethroids as already expected (Yu and McCord, 2007).

Diamide insecticides are used to control lepidopteran pests in different agronomic settings worldwide (Richardson et al., 2020). Although in the last decade resistance has been reported and characterized for several lepidopteran pests (Jouraku et al., 2020; Richardson et al., 2020; Troczka et al., 2012). In Chapter 2, the mechanism of diamide resistance in *S. frugiperda* was the first time described. The target-site mutation I4734M (corresponding to I4790M in *P. xylostella*) observed in the RyR in FAW (Chlorant-R) confer different levels of resistance

among diamide chemotypes, the methionine at this particular site results in higher resistance towards flubendiamide (>5400-fold) than chlorantraniliprole (225-fold) (Bolzan et al., 2019).

6.2 Resistance to Bt proteins

Regarding the mechanisms conferring Bt resistance, target-site mutations and differences in the expression of Bt receptors in the insect gut play a major role (Guo et al., 2015; Jakka et al., 2016; Jurat-Fuentes et al., 2011). Mutations in the ABCC2 transporter have been previously linked to resistance to different Cry proteins, including Cry1F resistance in FAW populations from Puerto Rico (Banerjee et al., 2017; Flagel et al., 2018). However, the mechanism described in those populations seems to be something specific for the island as it has not been described for any other FAW population so far (Nagoshi et al., 2017). The genotyping results (Chapter 4) for FAW populations from Puerto Rico, Brazil, Kenya, and Indonesia confirmed that the GC insertion in ABCC2 was not present anywhere else besides Puerto Rico.

Cry1F resistance in FAW has evolved after few years of Bt-maize commercialization in Brazil (Farias et al., 2014) and in Chapter 3 the mechanism of resistance was for the first time identified and characterized for FAW from Brazil.

The deletion of glycine and tyrosine (GY deletion), the amino acid substitution from proline to lysine (P799K) and other rare mutations at the extracellular loop 4 in the ABCC2 are present across Brazil in different frequencies (Chapter 3). More recently, a novel resistance allele in the ABCC2 (resulting in a truncated protein) was also described for FAW collected in Brazil, though no further functional validation and linkage study has been provided (Guan et al., 2020). The high number of ABCC2 resistance alleles reflect the effect of soft selective sweeps, where multiple adaptive alleles at the same locus sweep through the population at the same time (Hermisson and Pennings, 2005). Soft sweeps are also possible as a result of parallel adaptation in geographically structured populations when several mutations emerge independently in distant locations before one has spread over a broader geographical range (Arendt and Reznick, 2008).

Recombinantly expressed ABCC2 harboring the GY deletion and P799K were exposed to Cry1F in cytotoxic assays (Chapter 3). Cells exposed to Cry1F toxin provided *in vitro* functional evidence that the GY deletion and P799K in fact confer resistance to Cry1F (Chapter 3). Moreover, linkage studies by crossing individuals from the susceptible (Sf_Bra) and Cry1F-resistant (Sf_Des) strains suggest that resistance to Cry1F is incompletely recessive (D_{LC} value <0.33). Backcrossings between F1 and Sf_Des revealed almost 50 % survivorship at a Cry1F discriminatory dose (killing 99% of heterozygotes) and all surviving insects were homozygous for the GY deletion (Chapter 3). Under field conditions cross-resistance between Cry1F and

Cry1Ab/Cry1Ac has been described (Bernardi et al., 2015; Horikoshi et al., 2016; Santos-Amaya et al., 2016) and confirmed in Chapter 5 by the bioassays with Cry1Ab and Cry1Ac.
On the other hand, GY deletion and P799K in the ABCC2 do not confer resistance to all Cry1 toxins. Bioassays with the Xentari™, a commercially available product based on Cry1Aa, Cry1Ab, Cry1C, and Cry1D showed low resistance (5-fold) in bioassays (Chapter 3) and no difference in the cytotoxic assays (Chapter 3). The Cry1C and Cry1D present in the Xentari™ formulation still control Cry1F-resistant insects (Horikoshi et al., 2019). The bioassay results against other Cry toxins (Chapter 5) also reinforced the fact that Cry toxins with similar homology mainly at domain III, such as Cry1F and Cry1Ab/Cry1Ac share binding sites at the ABCC2 (Bravo et al., 2007). Therefore, resulting in high levels (up to 400-fold) of cross-resistance towards Cry1Ab and Cry1Ac in Sf_Des, while Vip3A still shows efficient control of Cry1-resistant FAW (Chapter 5).
ABC transporters are involved in phase III of detoxification (Yu, 2008) and some Bt-linked mutations reported in the ABC transporters result in a truncated protein (Banerjee et al., 2017; Guan et al., 2020). The loss of functionality of ABC transporters could hypothetically alter the efflux/translocation of xenobiotics (including synthetic insecticides) and affecting their elimination, but detailed studies are lacking. Although the GY deletion and P799K substitution (Chapter 3) do not create any premature stop codon, the toxicological profile and cross-resistance pattern in the Cry1F-resistant strain (Sf_Des) in comparison to a Bt-susceptible strain (Sf_Bra) were investigated in Chapter 5.

6.3 Cross/multi-resistance cases

Cross-resistance in Cry1F-resistant FAW has been observed to OP´s (Zhu et al., 2015) and an overall reduction in susceptibility to a range of synthetic insecticides in Bt-resistant FAW under laboratory and field-conditions (Muraro et al., 2019).
Multiple mechanisms of resistance have been previously described in FAW populations conferred by an increase in activity of detoxification enzymes and a decrease in AChE sensitivity to OP´s in resistant insects (Yu, 1992, 1991). The broad investigation of the toxicological profile described in this thesis, qualify Sf_Des as an appropriate surrogate to better understand the possible control strategies using the best synergistic approach combining Bt technology and the rotation of effective insecticides.
Sf_Des has evolved high levels of resistance to Cry1F, Cry1Ac, and Cry1Ab (Chapter 5). Among the 12 synthetic insecticides tested, Sf_Des showed significant levels of resistance only to the pyrethroid deltamethrin (14-fold) and the OP chlorpyrifos (8-fold). However, it should not be neglected that resistance alleles to spinosyns, diamides, and lufenuron have been selected in field strains, although at low frequency (Boaventura et al., 2020a; Lira et al., 2020;

Nascimento et al., 2016; Okuma et al., 2017). The protein alignment of AChE from Sf_Bra and Sf_Des, showed point mutations A201S and G227A only in Sf_Des, revealing that multi mechanisms of resistance can be found in one unique population.

The role of detoxification enzymes conferring cross-resistance to Bt proteins remains unclear. However, differences in P450, CE, and GST expression were observed after exposure to sublethal doses of Cry proteins (Ren et al., 2020). Genetic and *in vivo* studies suggest possible interactions between esterases and Bt protein (Sayyed et al., 2008). Moreover, cross-resistance between pyrethroids and Cry1Ac has been reported in *P. xylostella* and OP insecticides and Cry1F in FAW (Zhu et al., 2015), supported by high CE and GST activities. However, in this study, Sf_Des showed similar CE and GST activities to the susceptible strain. It would be interesting to validate the role of *CYP9A-like* genes in FAW as P450s from this family were also overexpressed upon the treatment of insecticides and Cry1Ca in *S. exigua* (Hu et al., 2019; Ren et al., 2020). The expression of P450 enzymes was not checked after the exposure to Bt proteins, however, the very high constitutive expression of *CYP9A9*-like and *CYP6B39* (> 200-fold) in Sf_Des, suggest a role of these genes in general detoxification. *CYP9A9*-like gene was found to be up-regulated in FAW resistant to lufenuron (Nascimento et al., 2015) and after exposure to insecticides, such as up-regulation (14-fold) of *CYP9A30* after exposure to deltamethrin (Giraudo et al., 2015), supporting the association between CYP9A and insecticide resistance. Likewise, *CYP6B39* was the gene upregulated by most compounds tested, including insecticides and plant allelochemicals (Giraudo et al., 2015).

The next step would be to functionally validate the role of CYP9A9-like and CYP6B39 enzymes in FAW and the detoxification of insecticides by their recombinant expression.

6.4 Resistance diagnostic methods

The understanding of mechanisms conferring resistance can predict cross-resistance cases and therefore support efficient recommendations for sustainable pest control. Bioassays with insecticides, although very laborious, have been historically used for the detection of resistance in field populations. Currently, molecular techniques for the detection of insecticide resistance are gradually being developed and adopted, mostly established for target-site mutation identification rather than metabolic resistance.

Target-site mutations can be detected at the genomic level using more simple and less expensive methods such as allele-specific PCR (AS-PCR) and restriction fragment length polymorphism (PCR-RFLP) (Black IV and Vontas, 2007). Allelic discriminatory probe-assay (i.e TaqMan®), pyrosequencing (Fakhrai-Rad et al., 2002; Nauen et al., 2012), and KASP assay (Wosula et al., 2020) have been also developed for target-site mutations identification.

As an example of the practical importance of such methods are the pyrosequencing assays developed in this study, which allowed a higher-throughput genotyping and could support the the use of diamide insecticides such as tetraniliprole in Indonesia. However, the pyrosequencing instrument and maintainance cost is rather high and less suited to be implemented at a broader scale. Therefore, additionally, PCR-based assays designed here for the identification of mutations in the RyR, ABCC2, and AChE conferring resistance to diamides, Cry1 proteins, and OP, respectively (Chapter 4) can be of great help in different locations.
More than 30 years ago the increased metabolic activity involved in insecticide resistance in mosquitoes, for instance, was measured by a filter-based system allowing the detection of esterase activity using 1-NA as a substrate (Pasteur and Georghiou, 1989). A more modern approach has been used to detect the enzyme amount of CYP6CM1- a P450 which metabolizes neonicotinoids and pymetrozine - in the whitefly *B. tabaci* (Nauen et al., 2013). A lateral flow kit similar to a pregnancy test based on specific CYP6CM1-antibodies was developed and used in Spain and Turkey for the detection of resistance in the field (Nauen et al., 2015). Gene expression analysis of genes known to confer resistance can be investigated by RT-qPCR or microarrays (Carvalho et al., 2013). More modern variants are the Droplet Digital PCR (Zink et al., 2017) or Oxford Nanopore MinION (MinION) sequencing (Bronzato Badial et al., 2018) which could be used for metabolic and target-site resistance detection.

6.5 Future of synthetic insecticides and biotech crops in controlling FAW

The development of multi/cross-resistance in FAW impairs its control with the current tactics available. The control of FAW might be focused in early larvae stages when susceptibility towards synthetic insecticides and Bt toxins is higher (Waquil et al., 2013). Moreover, not all Bt proteins currently expressed in the major crops display a full high-dose control of FAW in the field (Herrero et al., 2016). Therefore, the intrinsic activity towards FAW should be taken into account already at the early phase of trait or insecticide development.
Besides FAW, other related *Spodoptera* species such as *S. cosmioides* and *S. eridania,* are also present in the field and need eventually to be controlled (Bernardi et al., 2014). Therefore, instead of focusing on a single species, there is a trend to consider a group of pests which is targeted by Bt crops (Ricroch and Hénard-Damave, 2015). The adoption of Bt pyramided (expressing more than one Bt protein) is preferred to delay the development of resistance and improve efficacy against a broadening spectrum of pests (Carrière et al., 2015).
Alleles conferring resistance to Cry1F are broadly present in Brazil (Chapter 3). Therefore, the choice of which crop variety to sow should take into account cross-resistance patterns among Cry1F, Cry1Ac, and Cry1Ab as confirmed in this study (Chapter 5) (Hernández-Martínez et al., 2012; Hernández-Rodríguez et al., 2013; Luo et al., 1999; Sena et al., 2009). It is important to

mention that resistance or cross-resistance to a particular Bt protein can lead also to cross-crop resistance, considering that the same Bt toxins are expressed in more than one crop (Machado et al., 2020). The Vip3A protein does not show cross-resistance to Cry1 proteins in FAW and thus exhibits an excellent efficacy against Cry1F-resistant FAW (Chapter 5) (Bergamasco et al., 2013; Chakroun and Ferré, 2014; Sena et al., 2009). Nevertheless, resistance to Vip3A has been selected in FAW under laboratory conditions recently in the United States and Brazil (Fatoretto, 2017; Yang et al., 2018). The mode of action of Vip3A seems to involve different binding proteins in the insect gut and is yet not completely understood. Here again, efforts on the elucidation of Vip mode of action are extremely important to anticipate and support early resistance strategies.

Recently, a field study in the South of Brazil reveals that maize hybrids expressing Cry proteins failed to control FAW requiring up to four insecticidal applications (Burtet et al., 2017). Therefore, knowledge on the efficacy of insecticides to be used in an agronomic system where Bt is adopted and field resistance occurs is really important. In Chapter 5, almost all insecticides tested (except deltamethrin and chlorpyrifos) showed efficient control of Cry1F-resistant insects. Kanno et al. (2019) have shown great control of FAW resistant to insecticides and Bt with chlorfenapyr, this result was also confirmed by the bioassays shown in Chapter 5. The adoption of Bt-expressing crops is still limited for many of the newly invaded countries, even more for small farmers in these countries. Nevertheless, the government of countries such as Ghana is subsiding the distribution of Bt-based sprayable products to control FAW (Babendreier et al., 2020), supporting that Bt technology is an important component of IPM.

Moreover, baseline studies on the efficacy of different chemicals in the newly invaded countries are of utmost importance (Deshmukh et al., 2020; Sisay et al., 2019; Zhao et al., 2020). Such studies can support practical decisions on spraying windows against FAW, composed of insecticides belonging to different modes of action and therefore, delaying resistance. In the Eastern hemisphere, the performance of different compounds seems to vary depending on the country. In a field study in Ethiopia, spinosyns, *lambda*-cyhalothrin, a commercial mixture of *lambda*-cyhalothrin with chlorantraniliprole, and botanical compounds such as neem extract (*Azadirachta indica*) showed satisfactory control of FAW (Sisay et al., 2019). A recent study with FAW populations from China revealed that emamectin benzoate, spinetoram, chlorantraniliprole, chlorfenapyr, and lufenuron are effective active substances against FAW, while *lambda*-cyhalothrin and azadirachtin exhibited lower toxicity (Zhao et al., 2020).

Efforts in identifying the dominant FAW host-plant strains have been taken in the newly invaded countries (Nagoshi et al., 2020, 2019), because rice- or corn-strain are different regarding their susceptibility to insecticides and their biology, which can have practical influence in the management of this pest (Gouin et al., 2017). The molecular markers currently available for strain identification (*COI* and *Tpi*) have shown controversial results (Tay et al., 2020), as shown

here in Chapter 4. Although some studies suggest that the corn- and rice-strain could differ in their sexual communication (Unbehend et al., 2013), the use of pheromone traps in Benin and Nigeria, where FAW populations consisted of a mixture of both strains, showed no strain-specific sexual communication differences (Haenniger et al., 2020).

It is important to note that field failures are not always due to resistance but the performance of the insecticides can be affected by other factors such as incorrect application rates, formulation issues, or poor application coverage (Kranthi et al., 2002). Therefore, the education and training of farmers by technical support is also essential for the successful implementation of IPM.

6.6 Integrated management of FAW and IRM globally

Ideally, the development and implementation of insecticide resistance management strategies commence before the development of resistance. Unfortunately, a pest such as FAW with many host plants, many generations per year, and high reproductive rate favors the evolution of resistance. Also, the spread of resistance alleles can be faster due to the ability of adults to fly long distances.

Studies on IRM strategies were in the past restricted to the American continent. However, FAW became a pest of global economic relevance leading to joint efforts among agrochemical industries and international organizations in seeking FAW control measures which could be applied in different agronomic contexts. The maize-producing regions differ significantly in their agricultural system in Asia, Africa, and Latin America. Therefore, more locally-based control measures should be considered.

The monitoring of FAW infestation is essential for early control, though insecticide application should be based on an economic threshold, rather than protective. The economic threshold might vary among countries, based on the crop cultivar, environmental conditions, and pest dynamics. Therefore, farmers should follow local recommendations also regarding label rates and water volumes.

In the American continent, the control of FAW relies mainly on Bt crops, and the structured refuge strategy is highly recommended to delay the evolution of resistance. The refuge strategy consists of a minimum of 10 % of the area of non-Bt (within 800 m of Bt crop). Moreover, IRAC-Brazil recommends the application of (non-Bt) foliar insecticides in the refuge area if percent damaged plants reach 20 % (Davis Scale 3) (Davis et al., 1992; “IRAC,” 2018). The number of sprays in the refuge should not exceed two sprays and should be before V6 (up to 60 days post-sowing). When the refuge is sprayed, the Bt field should also be scouted and sprayed at the same time as the refuge if the level of damage in the Bt field exceeds the threshold provided by the seed supplier (“IRAC,” 2018).

The rotation of modes of action in a “window-treatment” scheme is highly recommended for insecticide application, to minimize exposure of consecutive generations to the same insecticide modes of action. Each window should be approximately 30 days covering a single generation of the target insects, as recommended in Chapter 5 based on the study with a FAW strain from Brazil. Insecticide seed treatments may also be an option to control FAW not only in maize but also in early infestation in soybean fields (Pes et al., 2020; Triboni et al., 2019).

In case, more than one insecticide application is required during an application window (30 days), it is principally recommended to apply an insecticide from a different MoA (though block applications of the same MoA within a window are possible), also considering field history regarding resistance and cross-resistance patterns (“IRAC,” 2018). The use of insecticides with a safe toxicological profile to non-target and beneficial organisms might be considered, particularly during crop flowering. Moreover, the use of insecticides and their respective harvest interval might be followed rather the crop is for feed or food end proposes (“IRAC,” 2018).

Insecticide mixtures may offer benefits for pest control and/or IRM when appropriately incorporated into rotation strategies with additional MoA. Good cultural practices, such as field monitoring before sowing, management of crop post-harvest stubble, and volunteers is highly recommended to keep FAW infestations below economic thresholds.

Differences in FAW susceptibility towards insecticides/Bt are expected depending on the resistance pressure at field conditions and also the frequency of resistance alleles in the population. As recently reported, instead of a unique FAW introduction in the Eastern hemisphere, Tay et al. (2020) suggest that multiple entries are responsible for the fast spread of FAW in Africa and Asia. The heterogeny in the genetic background could also support the differences in susceptibility to control measures reflected by the inherited resistance coming from different populations' sources (Tay et al., 2020).

Bt crops are not commonly adopted in Africa, but for many years, maize expressing Cry1Ab and Cry1A.105 + Cry2Ab2 in South Africa has been used to control stem borers and since 2018 also FAW (Kruger et al., 2012). A recent study showed moderate overall survivorship of FAW towards Cry1Ab, while Cry1A.105 + Cry2Ab2 showed sufficient FAW control and might be adopted gradually in more African countries (Botha et al., 2019).

Resistance to insecticides and Bt proteins are likely to be in higher frequency in regions of intense agriculture such as in Bahia state in Brazil, where Sf_Des was collected. Maize, soybean, and cotton are cultivated all year long favoring FAW development. The polyphagous habit of FAW hinders crop rotation as an alternative control option in countries where the extensive agriculture of staple food takes place. However, in smaller agronomic settings such as in African countries where farms are small (< 2 ha), the push-pull strategy with Napier

grasses as a trap crop or intercropping with beans has shown satisfactory FAW control (Guera et al., 2020; Hailu et al., 2018; Midega et al., 2018).

Synthetic insecticides are an important control tactic against FAW. However, the lack of appropriate protective equipment or adapted sprayers among smallholder farmers and resistance issues reinforce the need to search for alternatives and IPM approaches (Tambo et al., 2019).

Biological control options have been used to control FAW at smaller farms, such as the egg parasitoid *Telenomus remus* that is naturally occurring in some African countries (Shylesha et al., 2018). The rearing of this wasp and other endogenous parasitoids can be potentially implemented as inundative biological control agents in Africa (Bateman et al., 2018; Hruska, 2019; Kansiime et al., 2019). Other cultural tactics might be also implemented to control and reduce FAW infestation such as early planting, balanced fertilization, soil management, and habitat diversification (Harrison et al., 2019; Prasanna et al., 2018). Intercropping maize with legumes (e.g. beans that are not host plants for FAW) may also reduce FAW damage (Hailu et al., 2018). Potential microbial substances, including entomopathogenic fungi and nematodes, bacteria, baculoviruses, and botanical extracts have been intensively investigated (Dougoud et al., 2019; Tambo et al., 2019). In general, all of the above alternative solutions to chemical insecticides barely have a pre-harvest interval, have no relevant residues, and are safe for applicators and consumers.

Baculovirus insecticide (SfMNPV) is an efficient tool for IRM. It has been registered for use in maize and shown great control of insects resistant to a broad range of synthetic insecticides in Brazil (Bentivenha et al., 2018; “IRAC,” 2018). This option has been extensively promoted also in African countries as the production of baculovirus can be easily performed in small-scale and at low cost (Bentivenha et al., 2018).

Government support and subsidies can impact positively IRM strategies, such as in Ghana specifically, where the government has switched from supporting chemical pesticides to free distribution of Bt-based products (Babendreier et al., 2020).

Computer-based decision support systems considering climate, pest biology, natural enemies, and the crop in combination with the economic threshold allows optimized use of insecticides (Garcia et al., 2019). As the late identification of FAW in African countries has been pointed as one of the reasons for the fast FAW spread in the continent, many mobile-based programs have been developed aiming to support the management in many languages (“FAO - News Article,” 2020).

6.7 Future studies

The understanding of mechanisms of resistance and their implications for cross-resistance patterns can provide great support for the seed industry to improve their traits and also the choice of combinations to be included in pyramided seed (Carrière et al., 2016, 2015).

The discovery of insecticidal proteins is crucial to maintain or improve the longevity of the next generation of Bt traits. This is especially true for insects with high risk to evolve resistance such as FAW, which requires hundreds of candidates to be screened to get a FAW-active protein (Jerga et al., 2019). Moreover, considering the broad resistance spectrum of FAW to the Bt insecticidal proteins commercially available, the engineering, including modification and optimization of proteins that act also in resistant strains, probably by binding to different gut receptors is really important (Y. Wang et al., 2019). Some cases of cross-resistance have been associated with the structural similarity between Cry protein domains II and III (Bravo et al., 2007; Hernández-Rodríguez et al., 2013). For instance, field-evolved resistance of FAW to Cry1F expressed in maize hybrids, rapidly evolved resistance to a pyramid of Cry1A.105 + Cry2Ab at the laboratory and more recently also in the field (Santos-Amaya et al., 2016). Cry1Fa and Cry1A.105 are closely related, therefore the risk of rapid FAW resistance to Cry1A.105 + Cry2Ab corn in Brazil is also high because this pyramid is being used remedially to counter resistance to Cry1Fa (Santos-Amaya et al., 2016). Resistance to Bt proteins can be overcome by modifications at Cry domains, such as the newly modified Cry1Da_7 and Cry1B.868. These Cry proteins bind to different receptors at the insect midgut and therefore showed good control of resistant FAW strains (Y. Wang et al., 2019).

According to Carrière et al. (2016) to have long-lasting pyramiding traits, the industry should prioritize the rapid phase-out of single-protein crops and replace them by pyramided seeds while all proteins are still active towards the main target pest in the field.

There are several methods to study the activity of Bt proteins, including ligand blots (Chakroun and Ferré, 2014; Keeton et al., 1998; Martinezramirez et al., 1994), *in vitro* binding experiments with labeled Bt proteins (Adegawa et al., 2017; Endo et al., 2017), and binding with BBMV preparations (Martin and Wolfersberger, 1995). Recently, a novel technique utilizing a *Disabled Insecticidal Protein* (DIP) was described to forecast cross-resistance issues among Cry proteins. The DIP is structurally modified in a way that is not toxic to the insect but has binding properties. When the insect is exposed to a DIP and a particular Cry protein in a feeding competitive-like assay, scientists can preview shared binding properties and therefore move forward in Bt research in a more assertive and targeted way (Jerga et al., 2019).

Genome-editing technologies are also modern tools to validate the function of Bt and insecticide receptors in insect pests (Gui et al., 2020; Guo et al., 2019; Huang et al., 2020; Zuo et al., 2020). Unfortunately, RNA interference (RNAi) has shown controversial results in lepidopteran insects (Terenius et al., 2011). However, CRISPR/Cas technology seems to be

more effective and promising for functional MoA validation in FAW. Recently, ABCC2 from FAW has been knocked-out using the CRISPR/Cas technology, and resistance towards Cry1F was observed (118-fold), providing additional functional evidence of ABCC2 mediated Cry1F toxicity (Ming-hui et al., 2019). On the other hand, cadherin has also been knocked out in FAW and no increase in tolerance towards Cry1F and Cry1Ac was observed (Zhang et al., 2020). CRISPR/Cas9 could be for instance used to investigate the role of mutations observed in the ABCC2 in FAW from Brazil, by introducing them in a susceptible background and accessing the susceptibility towards different Bt proteins.

Transcriptomic analyses have been used to identify genes involved in Bt intoxication (Lei et al., 2014; Oppert et al., 2012; Ren et al., 2020; Song et al., 2016; Wei et al., 2018; Xu et al., 2015). Some groups of genes and expression profiles have been consistently linked to putative functions such as up-regulation of antimicrobial peptides (AMPs) (Crava et al., 2015), and up-regulation REPATs (Herrero et al., 2007; Navarro-Cerrillo et al., 2012). As many of those genes are involved in many other biological processes, conclusions, and interpretations of such RNA-Seq analysis is difficult. Nevertheless, from a more holistic analysis, unprecedented results can surge. More recently, the hypothesis that the MAPK pathway is activated and modulated by the coordinate expression of two insect growth hormones (20-hydroxyecdysone and juvenile hormone) and consequently the expression of some Bt receptors has been proposed (Guo et al., 2020).

Higher throughput cytotoxic assays, such as the one described in Chapter 3, where specific Bt receptors are expressed in cell lines and incubated with Bt proteins are also advanced methods that can support the development of new active Bt proteins. The heterologous expression of the highly expressed CYP9-like genes and pharmacokinetic studies with different insecticides can provide further information on their role in insecticide resistance in the field.

The general understanding of the Bt mode of action in insect pests could help to improve Bt insecticides, for example, through the identification of new receptors for Vip3 proteins. Some Bt proteins have already been modified in such a way that the binding affinity is increased or do not depend on a receptor for pore formation (Pardo-López et al., 2009, 2006). Moreover, insecticide resistance could be potentially overcome through the computational support of respective docking models helping to predict efficacy (Correy et al., 2019).

6.8 Summary

The main aim of this thesis was to characterize the mechanisms of resistance evolved to chemical and biotechnological technologies currently used to control FAW, *S. frugiperda*. Although native to the American continent, since 2016 FAW has reached Africa, Asia and Australia and became a global threat for economically important crops, particularly maize. A better understanding of the mechanisms conferring resistance can support efficient pest control and resistance management strategies.

Compounds from the diamide chemical class of insecticides are essential for the control of many lepidopteran pests, including FAW. Therefore, their sustainable use to delay resistance development is essential to farmers world-wide. In Chapter 2 the mechanism conferring high levels of diamide cross-resistance is characterized in FAW collected in Brazil. A target-site mutation (I4734M) in the RyR was for the first time reported in FAW. This mechanism is well studied also in other species and it is known to confer different resistance levels among diamide insecticides. The pyrosequencing and fluorescent probe assay developed here allowed the genotyping of field-collected populations from Brazil, Puerto Rico, Kenya, and Indonesia (Chapter 4). Although this resistance allele has not been detected in any FAW field population tested, the developed assay has shown robust results and renders it as a useful tool to monitor diamide resistance allele frequency.

The adoption of crops expressing Bt proteins is one of the major measures to control FAW in the American continent. However, practical resistance to Cry1F has evolved after a few years of commercialization, but the resistance mechanism remained elusive. In Chapter 3 the mechanism conferring resistance to Cry1F in FAW collected in Brazil was for the first time described. Novel mutations in the Bt receptor ABCC2 were identified in the resistant strain Sf_Des. The most frequent mutations detected (GY deletion and P799K) are located at the EC4 domain, which has been suggested as crucial for the binding of Cry1F to ABCC2. Functional *in vivo* cytotoxic assays and genetic linkage studies confirmed that the detected GY deletion is conferring high levels of resistance to Cry1F (Chapter 3). The sequencing of ABCC2 and genotyping by pyrosequencing revealed that mutations in the ABCC2 are broadly distributed in FAW in Brazil, but not in Puerto Rico, Kenya, and Indonesia (Chapter 5).

Another objective was to understand the toxicological and detoxification gene profile of a Cry1F-resistant strain, taking Sf_Des as an example, particularly to identify insecticide cross-resistance patterns helping to define efficient control recommendations. The Sf_Des strain has been collected in 2016 one of the most intensive maize production regions in Brazil, the Bahia state. So, it has been historically exposed to different chemical treatments as well as Bt proteins expressed in different crops. A population that has been reared under laboratory conditions since 2005 (Sf_Bra) without exposure to any insecticide served as a susceptible reference. The larval susceptibility of both strains to different insecticides that are currently

used in the refuge areas or conventional cropping systems was assessed and confirmed moderate levels of cross-resistance to the pyrethroid deltamethrin (14-fold) and the OP chlorpyrifos (8-fold). Very high levels of cross-resistance were observed against Cry1Ac and Cry1Ab, supporting previous reports. Further biochemical and molecular investigations with strain Sf_Des revealed high activity of P450 enzymes. Exemplified by the up-regulation of CYP9A-like and CYP6B39 transcripts (>200-fold) measured by RT-qPCR. Therefore, it is suggested that P450´s might play an essential role and supporting the data obtained in toxicological profiling. Nevertheless, the candidate P450´s and their insecticide metabolic ability need further investigation.

The last objective was to develop robust and rapid genotyping methods allowing the identification of known target-site mutations conferring resistance to pyrethroids, organophosphates, diamides and, Cry1F insecticidal protein. In total 34 populations of FAW collected in different geographies were genotyped for 10 mutations sites in different insecticide receptors. The obtained results confirmed that mutations in the VGSC occur at very low frequency, because only two samples from Indonesia were heterozygous for the mutation L1014F, while the F290V mutation in the AChE was present in all countries. On the other hand, the I4734M mutation in the RyR was not detected in any FAW population as well as the mutations in the ABCC2 conferring resistance to Cry1F (GC insertion detected in FAW populations in Puerto Rico and GY deletion present in FAW from Brazil).

The results presented in this thesis uncovered two novel mechanisms conferring resistance to diamides and Bt proteins in FAW from Brazil not yet described. Moreover, the development of robust diagnostic tools would help to genotype the frequency of resistance alleles in different countries in future monitoring programmes. Overall, this study contributes to the efficient and sustainable control of FAW, helps to implement appropriate insecticide resistance management strategies and provide insights for future research to close knowledge gaps to further facilitate evidence-based FAW control.

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Appendix A supporting information (Chapter 2)

Detection of a ryanodine receptor target-site mutation in diamide insecticide resistant fall armyworm, *Spodoptera frugiperda*

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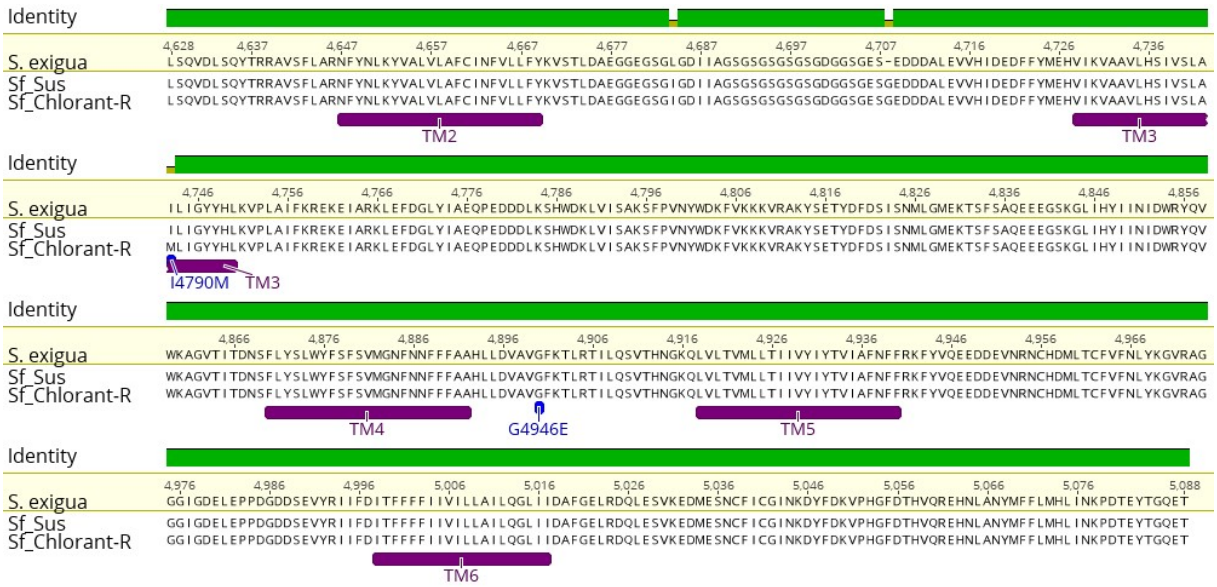
Table S1. List of primers used for Sanger sequencing, pyrosequencing and fluorescent PCR-based allelic discrimination assay.

Primer	Sequence	
Sf. 1-F	5'-TCAAGGTGGCTGCAGTACTG-3'	Partial sequencing RyR - Sanger
Sf. 2-F	5'-GCCATCGAAGCTGAGAGCAA-3'	
Sf. 3-F	5'-AGGCAGCAAGGGACTGATTC-3'	
Sf. 1-R	5'-GTTCCTGTTGACCTCGTCGT-3'	
Sf. 2-R	5'-GAAGAAGTCCCAGCATCGCT-3'	
Sf. G4946-F	5'-GTGATGGGCAACTTCAAC	Pyrosequencing
Sf. G4946-R.btn	5'-[btn]TTTTCCGTTATGCGTGAC-3'	
Sf. G4946-F.Seq	5'-ATTTGCTAGATGTCGCT-3'	
Sf. I4790-F.btn	5'-[btn]CGAGGACTTCTTCTACATGG-3'	
Sf. I4790-R	5'-AATTTACGGGCAATCTCC-3'	
Sf. I4790-R.Seq	5'-ATGGTAGTACCCGATGA-3'	
Sf.taq_I4790M_F	5'-ACGACGATGCACTAGAAG-3'	PCR-based assay
Sf.taq_I4790M_R	5'-CACCTTGAGATGATAGTACC-3'	
Sf.I4790M_HEX	5'-[HEX]TGTCGCTCGCTATACTCATCG[BHQ1]-3'	
Sf.I4790M_mut_FAM	5'-[6FAM]CTCGCTATGCTCATCGGGT[BHQ1]-3'	

Table S2. Genotyping by pyrosequencing for RyR target-site mutation I4790M (numbering according to *P. xylostella*) in *Spodoptera frugiperda* larvae collected in corn fields in Brazil, crop season 2018. Additional information on collection sites is given in Table 1.

Sample	Number of larvae analysed	I4790M genotype (n)			I4790M genotype (%)		
		RR	SR	SS	RR	SR	SS
PR-PG	10	0	0	10	0.0	0.0	100.0
SP-IT	17	0	0	17	0.0	0.0	100.0
MS-CS	10	0	0	10	0.0	0.0	100.0
MT-SZ	12	0	0	12	0.0	0.0	100.0
MT-TS	13	0	0	13	0.0	0.0	100.0
MT-PL	23	0	0	23	0.0	0.0	100.0
MT-LV	18	0	0	18	0.0	0.0	100.0
BA-SD	21	0	0	21	0.0	0.0	100.0
RO-VI	20	0	0	20	0.0	0.0	100.0

Figure S1. Multiple amino acid alignment of RyR sequences covering transmembrane domains (TM) II to VI in *Spodoptera exigua* (GenBank: AFC36359) and *Spodoptera frugiperda* (Sf) strains Sus (GenBank: MK226188) and Chlorant-R. Transmembrane domains are highlighted in purple and respective mutations sites are indicated by blue squares.



Appendix B supporting information (Chapter 3)

Molecular characterization of Cry1F resistance in fall armyworm, *Spodoptera frugiperda* from Brazil

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Table S1. Populations of *Spodoptera frugiperda* collected in different states and years in Brazil. Field strains designated FAW_Pop were collected in non-Bt maize fields, maintained in the laboratory for one generation and F1 neonates were bioassayed for survivorship on TC1507 and non-Bt maize (5d).

Code[a]	City, State	Year	% Survivorship	
			TC1507 maize	Non-Bt maize
Sf_Bra	Unknown, São Paulo	2005	nt[b]	nt
Sf_Cor	Correntina, Bahia	2016	nt	nt
Sf_Des	São Desidério, Bahia	2016	nt	nt
PR-PG	Ponta Grossa, Paraná	2018	nt	nt
SP-IT	Ituverava, São Paulo	2018	nt	nt
MS-CS	Chapadão do Sul, Mato Grosso do Sul	2017	nt	nt
MT-SZ	Sapezal, Mato Grosso	2017	nt	nt
MT-TS	Tangará da Serra, Mato Grosso	2017	nt	nt
MT-PL1-2	Primavera do Leste, Mato Grosso	2017	nt	nt
MT-LV	Lucas do Rio Verde, Mato Grosso	2017	nt	nt
BA-SD	São Desidério, Bahia	2017	nt	nt
RO-VI	Vilhena, Rondônia	2017	nt	nt
FAW_Pop1	Luis Eduardo Magalhães, Bahia	2016	90.00	93.33
FAW_Pop11	Uberlândia, Minas Gerais	2016	94.17	91.67
FAW_Pop12	Pouso Alegre de Minas, Minas Gerais	2016	95.00	95.00
FAW_Pop13	Frutal, Minas Gerais	2016	91.67	90.83
FAW_Pop14	São Gabriel do Oeste, Mato Grosso do Sul	2016	94.17	94.17
FAW_Pop15	Ponta Porã, Mato Grosso do Sul	2016	89.17	94.17
FAW_Pop16	Dourados, Mato Grosso do Sul	2016	97.50	95.00
FAW_Pop18	Chapadão do Sul, Mato Grosso do Sul	2016	100	92.50
FAW_Pop19	Campo Verde, Mato Grosso	2016	94.17	95.83
FAW_Pop20	Lucas do Rio Verde, Mato Grosso	2016	93.33	99.17
FAW_Pop21	Sapezal, Mato Grosso	2016	94.17	97.50
FAW_Pop23	Querencia, Mato Grosso	2016	90.83	90.00
FAW_Pop24	Sinop, Mato Grosso	2016	91.67	95.00
FAW_Pop26	Campo Mourão, Paraná	2016	91.67	90.00
FAW_Pop27	Palotina, Paraná	2016	93.52	87.96
FAW_Pop28	Ponta Grossa, Paraná	2016	95.83	96.67
FAW_Pop29	Pato Branco, Paraná	2016	95.83	95.83
FAW_Pop3	Formosa, Goiás	2016	94.17	96.67
FAW_Pop30	Rolândia, Paraná	2016	93.33	93.33
FAW_Pop31	Guarapuava, Paraná	2016	89.17	87.50
FAW_Pop32	Santa Rosa, Rio Grande do Sul	2016	93.33	94.79
FAW_Pop33	Carazinho, Rio Grande do Sul	2016	86.46	94.44
FAW_Pop34	Chapecó, Santa Catarina	2016	91.67	90.83
FAW_Pop35	Ourinhos, São Paulo	2016	90.00	95.00
FAW_Pop36	Itapetininga, São Paulo	2016	93.33	94.17
FAW_Pop37	Santa Cruz das Palmeiras, São Paulo	2016	91.67	93.52
FAW_Pop38	Conchal, São Paulo	2016	89.17	93.33
FAW_Pop4	Jataí, Goiás	2016	88.33	92.50
FAW_Pop5	Rio Verde, Goiás	2016	95.00	95.00
FAW_Pop9	Balsas, Maranhão	2016	97.50	93.33

[a] Collecting and Access to Genetic Heritage Permits: 41766; 40380; 56686; 010663/2015-0; RC142D8; RB0AEEB; AB3B059

[b] nt = not tested (collected for genotyping by pyrosequencing only)

Table S2. List of primer pairs used for the screening of reference genes; amplification of genes involved in the Cry1F mode of action; *ABCC2* site-directed mutagenesis, amplification of *ABCC2* from *Spodoptera frugiperda* and pyrosequencing.

Primers	Forward primer (5' to 3')	Reverse primer (5' to 3')
Sf_ABCC2_GYdeletion	ATCCAGACTCTGCCTGAG	GTCCACCTGGTTGGTCCA
Sf_ABCC2_P799K	GGGCGAGTCCAAGAATCCTGAGC	TCAGGCAGAGTCTGGATG
Sf_ABCC2_CDS[3]	ATGATGGACAAATCTAATAAAAATACCGCGGC	CTAAGCGGTTTTGGAATCACTTTCAT
Sf_cadherin[1]	GAGAGCTGAGGGTCACTTGG	AGCGGACTCGGTTGTAGAGA
Sf_ABCC2[1]	CCTCAGACGGATGCTTTG	GTCGCCTGTTTCCTTCAC
Sf_ABCC3[2]	TTGAGGACCGTGTTCTTAG	AACGATAGCACCATAGGC
Sf_mALP1[1]	CACTGCCGCTACTGTGCTG	CCTGTGCCTTATCATTCCAAA
Sf_mALP2[1]	GGCTTTCTGCCCAACTGT	TCTACGAGCCAATCAACG
Sf_sALP1[1]	ACGAGCGAGACGTGTATCACAA	CGCCCAGGAACATGACCAC
Sf_APN1[1]	TCTCAGTTTCTTCACTTTGCTA	ACTTGGGCAAAGGTGTTC
Sf_APN3[1]	TCTCAGGCAATGAAGCCAATA	CACCCATGCTTTGAAATCCTC
Sf_APN4[1]	GAAGTGGTTCCCCTGCTA	CGAGACGACAACGACATG
Sf_APN6[1]	ATCTTGGGACCGATTCTA	TTGTCATGGGACCTAACT
Sf_ACT1	CTACACTGTCAGAAGGACG	CACACCTGGTAGAACTCC
Sf_ACT2	GAGAGTGCTCAAGAACGAC	CTAGAGAGCCAGAAGTTGTC
Sf_AK	GAACAACTGTCCGTGCCTC	GAGATGTCGTAGACTCCAC
Sf_EF2	GTGTTCGACGCCATCATG	CATCACCACCTGAGCAGAG
Sf_GAPDH[2]	GTCATCTCCAACGCTTCC	CAGAGGGTCCGTCAACAG
Sf_RPS3A[2]	CAACTCTGAACTTCGTGAG	CTACCACCCTCTCCATGAA
Sf_L10	GTCGTGCCAAGTTCAAGTTC	GTCCTCACGCAGCTTCTC
Sf_L17	GTGACGGAAGCTATCAAGAC	ACTTGTTGCCGAGGACAC
SfL18[1]	CGTATCAACCGACCTCCACT	AGGCACCTTGTAGAGCCTCA
Sf_UCCR	CAACCAGCGCACACAGAAC	GAAGCGTCGTAGTGGGATC
Sf_788-GYdeletion	[btn] CCGACTACTGGCTTAGTTT	GCTCGCATAGTCATCACT
Sf_788-GYdeletion_seq		CTTCGGGTAAAGTTTGT
Sf_P799K/R1	ACCAGGTGGATGGATACATA	
Sf_P799K/R2	ACCGACTACTGGCTTAGTT	[btn] ACCGACTTGAGTGTTCAAC
Sf_P799K/R_seq	ACCCGAAGGAGAAAG	

[1] primer pairs described by Jakka et al., 2016;
[2] primer pairs described by Li et al., 2017;
[3] primer pair described by Flagel et al., 2018.

Table S3. Gene names, species and GenBank accession numbers for the 10 candidate reference genes used in this study.

Gene Name (Abbreviation)	Species	Accession No.	Species	Accession No.
β-Actin 1 (*ACT1*)	*S. exigua*	JF728812.1	*S. frugiperda*	MN044625
β-Actin 2 (*ACT2*)	*S. exigua*	JF728814.1	*S. frugiperda*	MN044626
Arginine kinase (*AK*)	*S. litura*	HQ840714	*S. frugiperda*	MN044627
Elongation Factor 2 (*EF2*)	*S. exigua*	AY078407.1	*S. frugiperda*	MN044631
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	*S. litura*	HQ012003	*S. frugiperda*	KC262638.1
Ribosomal Protein L10(*L10*)	*S. litura*	KC866373	*S. frugiperda*	MN044628
Ribosomal Protein L17(*L17*)	*S. exigua*	EU259814.1	*S. frugiperda*	MN044629
Ribosomal Protein L18(*L18*)	*S. litura*	XM022974624	*S. frugiperda*	AF395587.1
Ribosomal Protein S3A(*RPS3A*)	*S. litura*	KC866374	*S. frugiperda*	AF429977
Ubiquinol-cytochrome C-reductase (*UCCR*)	*S. litura*	HQ599193	*S. frugiperda*	MN044630

Table S4. Illumina sequencing libraries corresponding to the extracellular loop 4, corresponding to 14th exon of SfABCC2 for 40 fall armyworm populations collected in maize fields in Brazil. The data in the table is given by following: exon14_peptide sequence, classification (Wild type, other mutation; GY deletion), city of collection, population code and read_name,read_seq.

Table S4 is provided as a separate file “Table S4_Data pooled population sequencing_other mutations.csv”.

The data file can be downloaded at
https://www.sciencedirect.com/science/article/pii/S0965174819303947?via%3Dihub#appsec1

Table S5. Log-dose probit-mortality data for Cry1F toxin tested against neonates of three different strains of *Spodoptera frugiperda*.

Toxin	Strain	n	LC_{50} (ng cm^{-2})	95% CI[a]	Slope (±SE)	RR[b]
Cry1F	Sf_Bra	190	98.17	81.13-118.8	1.51 (0.17)	1
	Sf_Cor	192	>48700			>490
	Sf_Des	190	>48700			>490
Xentari	Sf_Bra	168	818	649-1032	2.14 (0.048)	1
	Sf_Cor	167	3024	2480-3887	4.71 (0.041)	3.7
	Sf_Des	168	4329	3472-5399	1.62 (0.045)	5.3

[a] Confidence interval, 95%

[b] Resistance ratio (LC_{50} of Sf_Cor/Des strain divided by LC_{50} of Sf_Bra)

Table S6. Log-dose probit-mortality data for Cry1F toxin tested against neonates of resistant (Sf_Des), susceptible (Sf_Bra) and reciprocal F1 crosses of *Spodoptera frugiperda*.

Strain	n	LC_{50} (ng cm^{-2})	95% CI[a]	Slope (±SE)	RR[b]	D_{LC}[c]
Sf_Bra	190	98.2	81.1-119	1.51 (0.17)	1	
Sf_Des	190	>400000[d]			>4070	
F1 pooled	654	1580	1360-1850	1.79 (0.24)	16	<0.33

[a] Confidence interval, 95%

[b] Resistance ratio (LC_{50} of Sf_Des or F1 strain divided by LC_{50} of Sf_Bra)

[c] Degree of dominance (according to Bourguet *et al*., 1996)

[d] Extrapolated value

Table S7. Estimated dominance of Cry1F resistance by single concentration methods based on survival data obtained in Cry1F discriminating dose bioassays with crosses among Sf_Des (resistant) and Sf_Bra (susceptible). Percent survival data are mean values ± SE (n=96 larvae for Sf_Des and Sf_Bra; n=192 larvae for F1 pooled)).

[Cry1F]	Sf_Des	Sf_Bra	F1 pooled	D_{ML}[a]
5400 ng/cm²	91.7 ± 4.8	0 ± 0	1.39 ± 1.3	0.015

[a] Degree of dominance (according to Bourguet *et al*., 2000)

Table S8. Expression level of different genes in neonates and gut tissue of 2nd instar larvae of *Spodoptera frugiperda* of strains Sf_Bra, Sf_Cor and Sf_Des. The expression level was normalized to *L17*, *L10* and *RPS3A* reference genes and relative to the susceptible strain (Sf_Bra). Significant differences between resistant (Sf_Des or Sf_Cor) and susceptible (Sf_Bra) strains for the expression of individual genes was analysed by an unpaired t-test (ND=not detected; for further details refer to materials and methods).

Gene	Stage	Strains	Average relative quantity	95% CI low	95% CI high	Comparison	P<0.05
ABCC2	neonate	Sf_Bra	1.000	0.825	1.212		
		Sf_Cor	0.748	0.586	0.956	Sf_Cor/Sf_Bra	NO
		Sf_Des	0.758	0.648	0.886	Sf_Des/Sf_Bra	YES
	gut L2	Sf_Bra	1.000	0.651	1.536		
		Sf_Cor	0.623	0.452	0.859	Sf_Cor/Sf_Bra	NO
		Sf_Des	0.709	0.544	0.923	Sf_Des/Sf_Bra	NO
ABCC3	neonate	Sf_Bra	1.000	0.690	1.450		
		Sf_Cor	1.612	1.313	1.980	Sf_Cor/Sf_Bra	NO
		Sf_Des	2.313	1.208	4.429	Sf_Des/Sf_Bra	YES
	gut L2	Sf_Bra	1.000	0.731	1.367		
		Sf_Cor	0.413	0.266	0.643	Sf_Cor/Sf_Bra	YES
		Sf_Des	0.696	0.656	0.738	Sf_Des/Sf_Bra	NO
CAD	neonate	Sf_Bra	1.000	0.798	1.254		
		Sf_Cor	0.832	0.730	0.949	Sf_Cor/Sf_Bra	NO
		Sf_Des	1.442	0.934	2.228	Sf_Des/Sf_Bra	NO
	gut L2	Sf_Bra	1.000	0.580	1.723		
		Sf_Cor	0.699	0.486	1.004	Sf_Cor/Sf_Bra	NO
		Sf_Des	1.016	0.899	1.148	Sf_Des/Sf_Bra	NO
sALP1	neonate	Sf_Bra	1.000	0.755	1.325		
		Sf_Cor	0.885	0.670	1.170	Sf_Cor/Sf_Bra	NO
		Sf_Des	3.096	1.851	5.180	Sf_Des/Sf_Bra	YES
	gut L2	Sf_Bra	1.000	0.561	1.784		
		Sf_Cor	1.224	0.573	2.612	Sf_Cor/Sf_Bra	NO
		Sf_Des	2.205	1.210	4.018	Sf_Des/Sf_Bra	NO
mALP1	neonate	Sf_Bra	ND				
		Sf_Cor	ND				
		Sf_Des	ND				
	gut L2	Sf_Bra	1.000	0.580	1.724		
		Sf_Cor	0.269	0.154	0.469	Sf_Cor/Sf_Bra	YES
		Sf_Des	3.015	1.486	6.117	Sf_Des/Sf_Bra	NO
mALP2	neonate	Sf_Bra	1.000	0.932	1.073		
		Sf_Cor	1.110	0.967	1.273	Sf_Cor/Sf_Bra	NO
		Sf_Des	3.975	3.161	4.997	Sf_Des/Sf_Bra	YES
	gut L2	Sf_Bra	1.000	0.199	5.034		
		Sf_Cor	0.220	0.131	0.369	Sf_Cor/Sf_Bra	NO
		Sf_Des	0.232	0.132	0.406	Sf_Des/Sf_Bra	NO
APN1	neonate	Sf_Bra	1.000	0.824	1.213		
		Sf_Cor	1.467	1.321	1.630	Sf_Cor/Sf_Bra	YES
		Sf_Des	2.035	1.747	2.370	Sf_Des/Sf_Bra	YES
	gut L2	Sf_Bra	1.000	0.239	4.181		
		Sf_Cor	3.917	2.626	5.842	Sf_Cor/Sf_Bra	NO
		Sf_Des	5.401	3.824	7.629	Sf_Des/Sf_Bra	NO
APN2	neonate	Sf_Bra	1.000	0.800	1.250		
		Sf_Cor	1.161	1.019	1.323	Sf_Cor/Sf_Bra	NO
		Sf_Des	1.721	1.307	2.267	Sf_Des/Sf_Bra	YES
	gut L2	Sf_Bra	1.000	0.496	2.017		
		Sf_Cor	0.636	0.415	0.974	Sf_Cor/Sf_Bra	NO
		Sf_Des	1.004	0.621	1.625	Sf_Des/Sf_Bra	NO
APN5	neonate	Sf_Bra	1.000	0.836	1.196		
		Sf_Cor	0.760	0.724	0.799	Sf_Cor/Sf_Bra	NO
		Sf_Des	1.068	0.802	1.423	Sf_Des/Sf_Bra	NO
	gut L2	Sf_Bra	1.000	0.559	1.790		
		Sf_Cor	0.848	0.660	1.088	Sf_Cor/Sf_Bra	NO
		Sf_Des	0.886	0.665	1.180	Sf_Des/Sf_Bra	NO
APN6	neonate	Sf_Bra	ND				
		Sf_Cor	ND				
		Sf_Des	ND				
	gut L2	Sf_Bra	1.000	0.836	1.197		
		Sf_Cor	2.962	1.205	7.280	Sf_Cor/Sf_Bra	NO
		Sf_Des	3.723	2.709	5.116	Sf_Des/Sf_Bra	YES

Table S9. Pyrosequencing results on the frequency of the GY deletion and the amino acid substitution P799R in *Spodoptera frugiperda* populations collected in Brazil as well as from Sf_Des and Sf_Cor larvae surviving Cry1F discriminating dose treatments (5400 ng/cm²).

Population	GY deletion				P799R[a]			
	N	RR (%)	RS (%)	SS (%)	N	RR (%)	RS (%)	SS (%)
RO-VI	18	83.3	11.1	5.6	12	0.0	0.0	100.0
MT-SZ	21	42.9	28.6	28.6	14	0.0	0.0	100.0
MT-PL2	22	22.7	13.6	63.7	16	25.0	0.0	75.0
MT-TS	20	50.0	25.0	25.0	15	0.0	0.0	100.0
MS-CS	17	52.9	5.9	41.2	12	0.0	0.0	100.0
SP-IT	22	45.5	31.8	22.7	12	16.7	0.0	83.3
PR-PG	16	37.5	12.5	50.0	11	0.0	0.0	100.0
MT-PL1	20	25.0	45.0	30.0	11	54.5	0.0	45.5
MT-LV	16	75.0	12.5	12.5	18	11.1	0.0	88.9
BA-SD	9	0.0	0.0	100.0	22	0.0	0.0	100.0
Sf_Des survivors	21	90.5	9.5	0.0	22	27.3	0.0	72.7
Sf_Cor survivors	15	100.0	0.0	0.0	15	100.0	0.0	0.0

[a]P799K in Sf_Des (see also sequence alignment in Figure 4A)

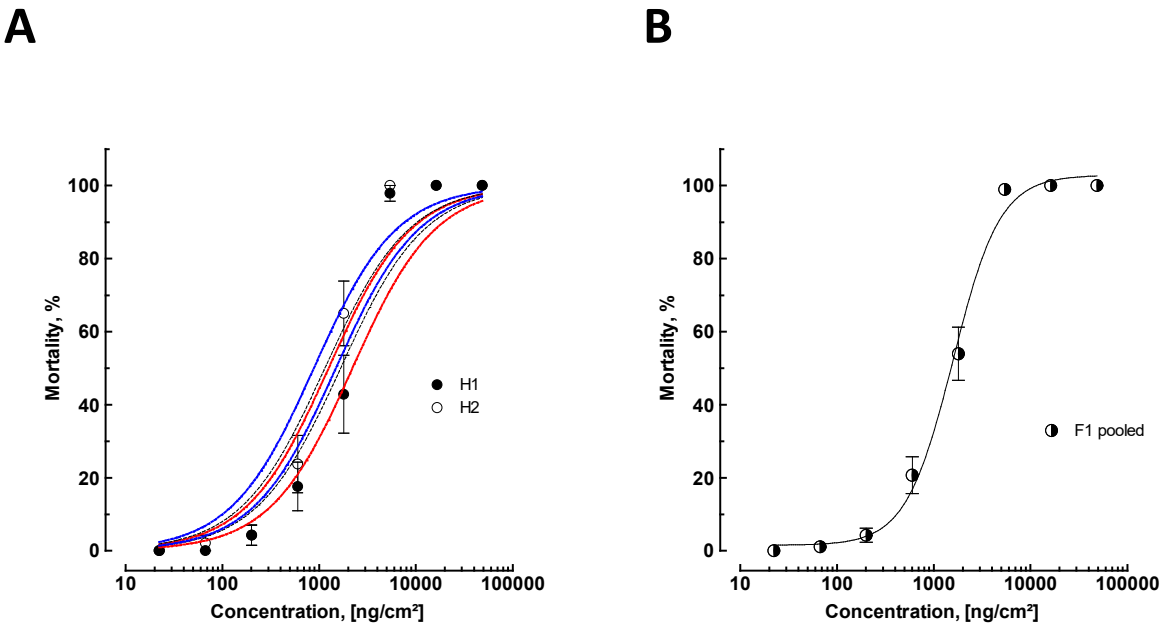

Figure S1. Efficacy of Cry1F against heterozygotes obtained from reciprocal crosses of fall armyworm strains Sf_Des ♀ x Sf_Bra ♂ (H1) and Sf_Bra ♀ x Sf_Des ♂ (H2). **(A)** Reciprocal crosses revealed similar results (overlapping 95% confidence intervals (CI)) suggesting an autosomal inheritance of resistance to Cry1F. H1: LC_{50} 1671 ng cm^{-2} (95% CI: 1254-2227 ng cm^{-2}) and H2: LC_{50} 1151 ng cm^{-2} (95% CI: 884-1500 ng cm^{-2}) **(B)** Combined dose-response data of the F1 cohorts (F1 pooled).

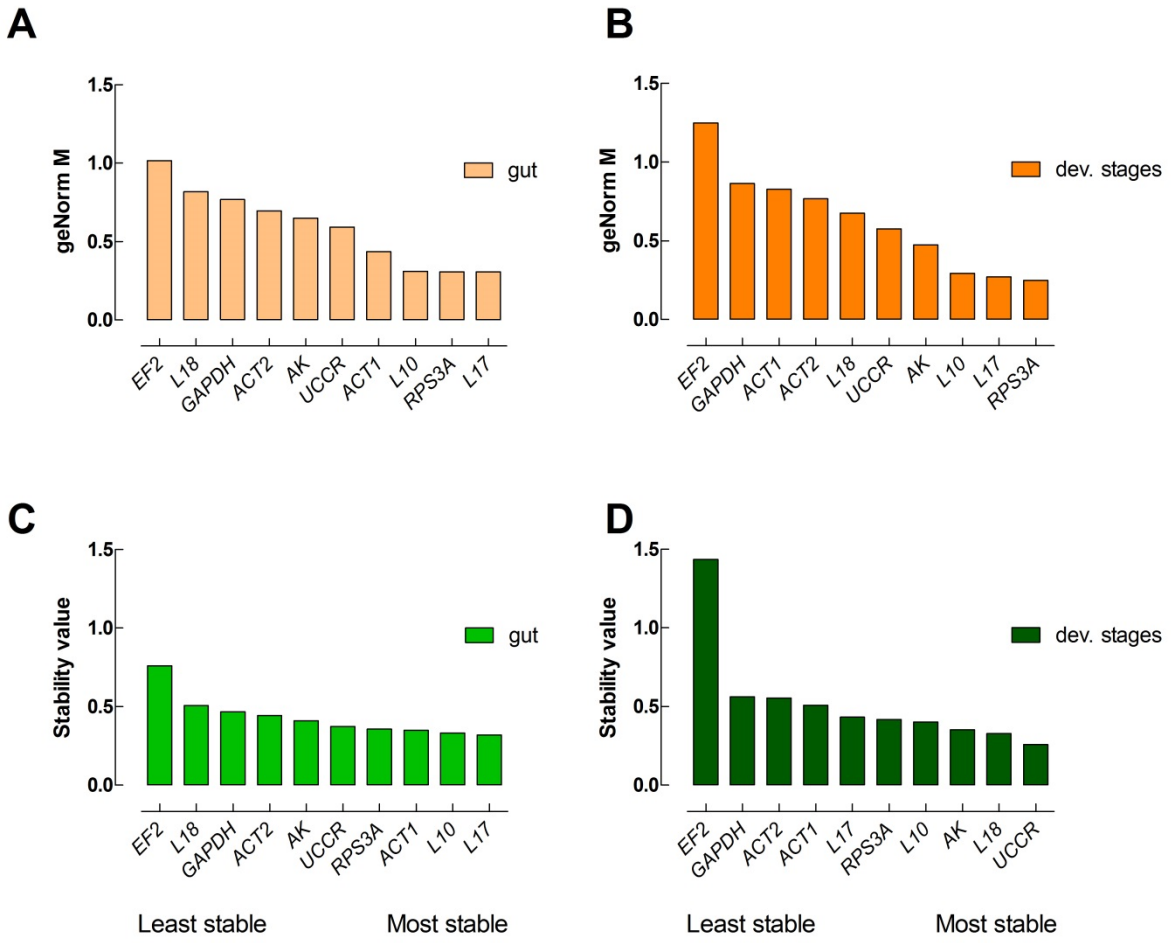

Figure S2. Ranking and stability of reference genes based on the expression in gut tissues and whole body throughout the larval development of three strains of *Spodoptera frugiperda*. **(A)** and **(B)** represent ranking based on the average expression stability value M evaluated by geNorm qBase Plus v3.1. **(C)** and **(D)** represent ranking calculated by Normfinder. The lower the stability value (≤ 0.5), the more stable is the candidate reference gene.

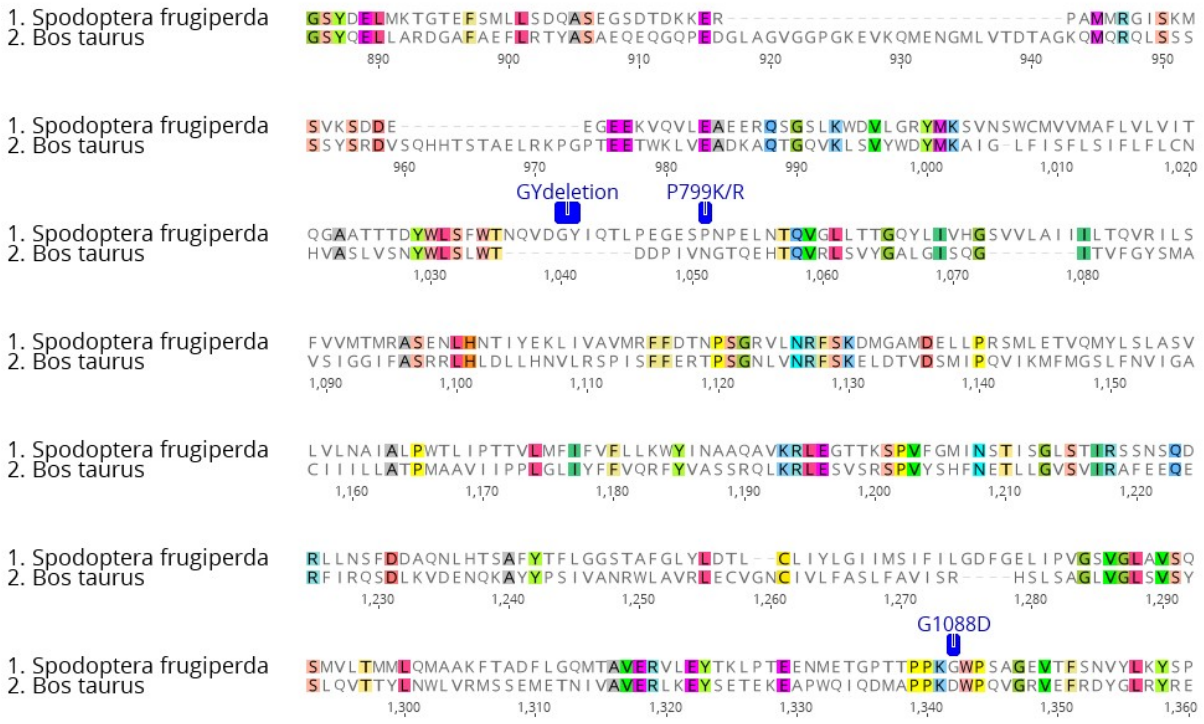

Figure S3. Alignment of ABCC2 amino acid sequence from *Spodoptera frugiperda* (GenBank accession number: KY489760) with bovine (*Bos taurus*) Multidrug Resistance Protein (MRP1) sequence (PDB identity number: 5UJ9) by Geneious version 10.2.

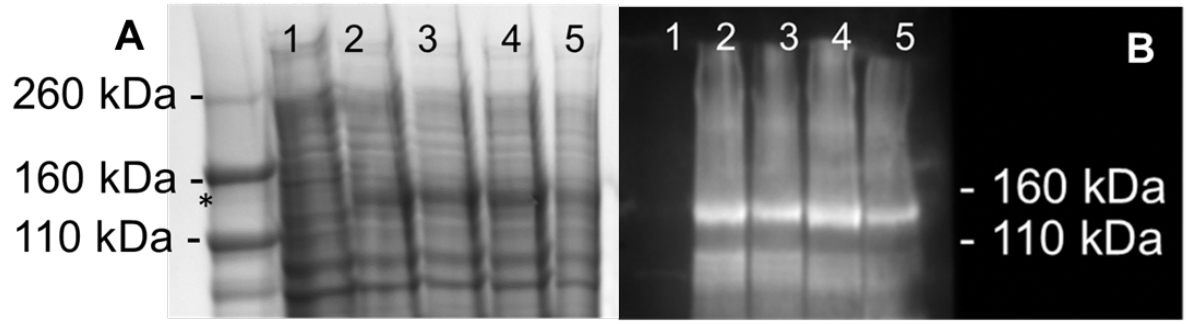

Figure S4. Cell lysate of Sf9 cells expressing ABCC2 of *Spodoptera frugiperda* in (**A**) SDS-PAGE gel and (**B)** Western blot using anti-FLAG antibody. Lane **1**: Sf9 cells without ABCC2 expression; lane **2**: ABCC2 wild type; lane **3**: ABCC2_GYdeletion; lane **4**: ABCC2 _P799K; lane **5**: ABCC2_GYdel+P799K. The wild type ABCC2 and 3XFLAG tag has an expected molecular weight of 153.794 kDa.

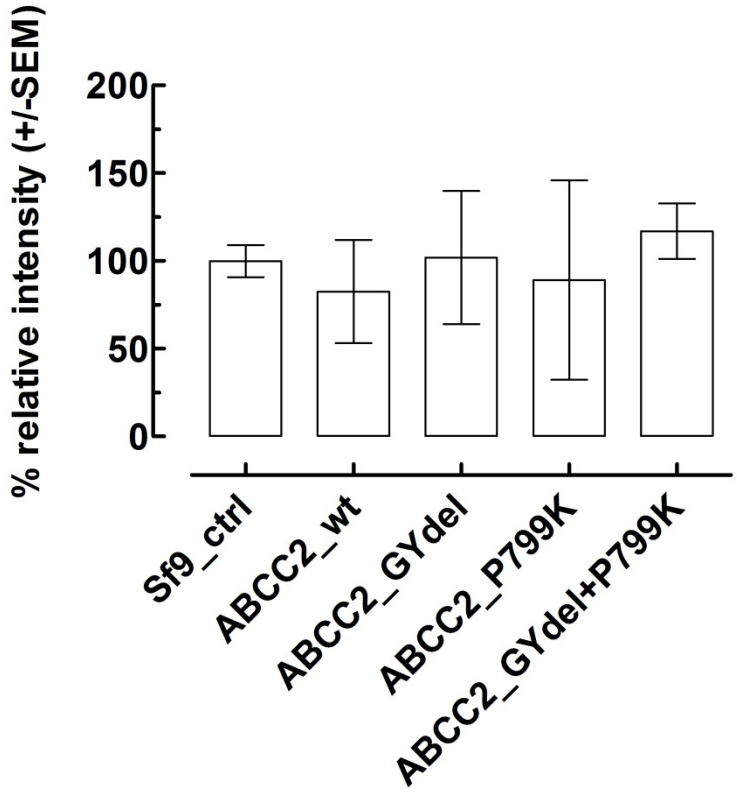

Figure S5. Sf9 cells without baculovirus infection (Sf9_ctrl), expressing wildtype (ABCC2_wt) and mutant types (ABCC2_ GYdel, ABCC2_ P799K, ABCC2_P799K+GYdel) of ABCC2 transporter from *Spodoptera frugiperda* after 1h incubation with 0.2 µg mL^{-1} of Xentari™ toxin. The average intensity relative to PBS treatment, calculated by imaging analysis under DAPI-filter with MetaView® imaging system (Universal Imaging Co., Westchester, PA).

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3_Sf_Bra    GPTTPPKGWPSAGEVTFSNVYLKYSPDDPPVLKDLNFAIKSGWKVGVVGRTGAGKSSLIS
4_Sf_Bra    GPTTPPKGWPSAGEVTFSNVYLKYSPDDPPVLKDLNFAIKSGWKVGVVGRTGAGKSSLIS
5_Sf_Bra    GPTTPPKGWPSAGEVTFSNVYLKYSPDDPPVLKDLNFAIKSGWKVGVVGRTGAGKSSLIS
6_Sf_Bra    GPTTPPKGWPSAGEVTFSNVYLKYSPDDPPVLKDLNFAIKSGWKVGVVGRTGAGKSSLIS

KY489760    ALFRLSDITGSIKIDGLDTQGIAKKLLRSKISIIPQEPVLFSASLRYNLDPFDNYNDEDI
MN399979    ALFRLSDITGSIKIDGLDTQGIAKKLLRSKISIIPQEPVLFSASLRYNLDPFDNYNDDDI
1_Sf_Bra    ALFRLSDITGSIKIDGLDTQGIAKKLLRSKISIIPQEPVLFSASLRYNLDPFDNYNDDDI
2_Sf_Bra    ALFRLSDITGSIKIDGLDTQGIAKKLLRSKISIIPQEPVLFSASLRYNLDPFDNYNDDDI
3_Sf_Bra    ALFRLSDITGSIKIDGLDTQGIAKKLLRSKISIIPQEPVLFSASLRYNLDPFDNYNDDDI
4_Sf_Bra    ALFRLSDITGSIKIDGLDTQGIAKKLLRSKISIIPQEPVLFSASLRYNLDPFDNYNDDDI
5_Sf_Bra    ALFRLSDITGSIKIDGLDTQGIAKKLLRSKISIIPQEPVLFSASLRYNLDPFDNYNDDDI
6_Sf_Bra    ALFRLSDITGSIKIDGLDTQGIAKKLLRSKISIIPQEPVLFSASLRYNLDPFDNYNDDDI

KY489760    WRALEQVELKESIPALDYKVSEGGTNFSMGQRQLVCLARAILRSNKILIMDEATANVDPQ
MN399979    WRALEQVELKESIPALDYKVSEGGTNFSMGQRQLVCLARAILRSNKILIMDEATANVDPQ
1_Sf_Bra    WRALEQVELKESIPALDYKVSEGGTNFSMGQRQLVCLARAILRSNKILIMDEATANVDPQ
2_Sf_Bra    WRALEQVELKESIPALDYKVSEGGTNFSMGQRQLVCLARAILRSNKILIMDEATANVDPQ
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3_Sf_Bra   WRALEQVELKESIPALDYKVSEGGTNFSMGQRQLVCLARAILRSNKILIMDEATANVDPQ
4_Sf_Bra   WRALEQVELKESIPALDYKVSEGGTNFSMGQRQLVCLARAILRSNKILIMDEATANVDPQ
5_Sf_Bra   WRALEQVELKESIPALDYKVSEGGTNFSMGQRQLVCLARAILRSNKILIMDEATANVDPQ
6_Sf_Bra   WRALEQVELKESIPALDYKVSEGGTNFSMGQRQLVCLARAILRSNKILIMDEATANVDPQ

KY489760   TDALIQKTIRKQFATCTVLTIAHRLNTIMDSDRVLVMDQGVAAEFDHPYILLSNPNSKFS
MN399979   TDALIQKTIRKQFATCTVLTIAHRLNTIMDSDRVLVMDQGVAAEFDHPYILLSNPNSKFS
1_Sf_Bra   TDALIQKTIRKQFATCTVLTIAHRLNTIMDSDRVLVMDQGVAAEFDHPYILLSNPNSKFS
2_Sf_Bra   TDALIQKTIRKQFATCTVLTIAHRLNTIMDSDRVLVMDQGVAAEFDHPYILLSNPNSKFS
3_Sf_Bra   TDALIQKTIRKQFATCTVLTIAHRLNTIMDSDRVLVMDQGVAAEFDHPYILLSNPNSKFS
4_Sf_Bra   TDALIQKTIRKQFATCTVLTIAHRLNTIMDSDRVLVMDQGVAAEFDHPYILLSNPNSKFS
5_Sf_Bra   TDALIQKTIRKQFATCTVLTIAHRLNTIMDSDRVLVMDQGVAAEFDHPYILLSNPNSKFS
6_Sf_Bra   TDALIQKTIRKQFATCTVLTIAHRLNTIMDSDRVLVMDQGVAAEFDHPYILLSNPNSKFS

KY489760   SMVKETGDNMSRILFEVAKTKYESDSKTA
MN399979   SMVKETGDNMSRILFEVAKTKYESDSKTA
1_Sf_Bra   SMVKETGDNMSRILFEVAKTKYESDSKTA
2_Sf_Bra   SMVKETGDNMSRILFEVAKTKYESDSKTA
3_Sf_Bra   SMVKETGDNMSRILFEVAKTKYESDSKTA
4_Sf_Bra   SMVKETGDNMSRILFEVAKTKYESDSKTA
5_Sf_Bra   SMVKETGDNMSRILFEVAKTKYESDSKTA
6_Sf_Bra   SMVKETGDNMSRILFEVAKTKYESDSKTA
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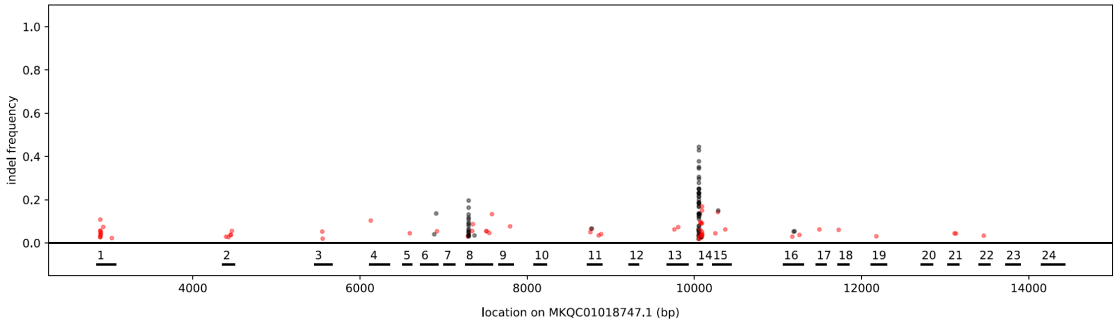
Figure S6. Amino acid alignment of the fall army worm ABCC2 transporter (GenBank KY489760; Banerjee et al., 2017), and Sf_Bra ABCC2 consensus (GenBank MN399979) based on six individually sequenced *ABCC2* genes of different larvae (1_Sf_Bra – 6_Sf_Bra aligned underneath). Amino acid polymorphisms are highlighted in yellow and the EC4 region is marked in blue, grey and green for “wild type” (KY489760), Sf_Bra consensus (MN399979) and six individual Sf_Bra larvae, respectively. The mutation sites are marked above the aligned sequences.

A



B

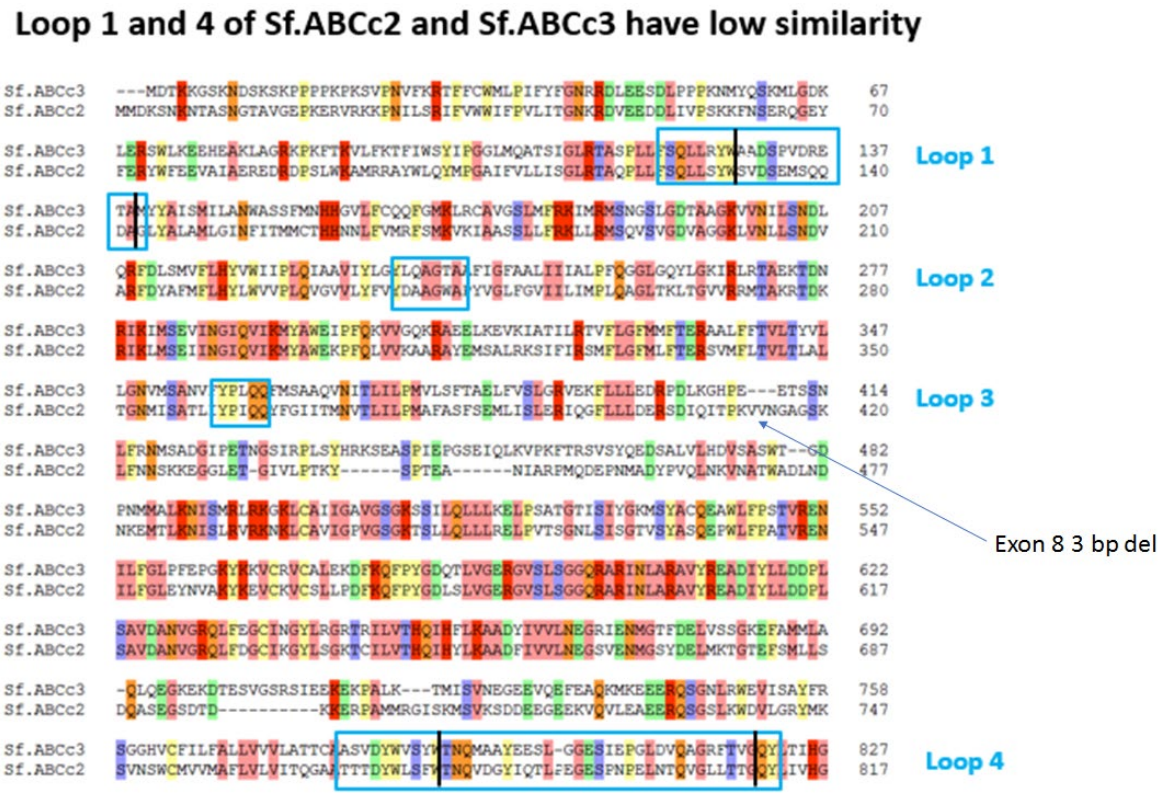


Figure S7. (A) Analyses of indel polymorphism in *ABCC2* of field-collected populations of *Spodoptera frugiperda* (Table S1) mapped on GenBank contig MKQC01018747.1, a complete copy of *ABCC2* containing 24 exons from the *S. frugiperda* Sf9 cell genome. Frame shift indels (i.e. not multiples of 3 nucleotides) are highlighted by red and inframe mutations by black dots. Each dot gives the estimated allele frequency in one location in one pooled population. The highest indel frequency is noticed on exon 14, which corresponds closely to the ABCC2 EC4 region carrying the GY deletion. **(B)** A number of inframe mutations detected in exon 8 delete one of the two valine residues in ABCC2 and missing in ABCC3. The amino acid alignment revealed that the affected region is poorly conserved.

Appendix C supporting information (Chapter 4)

Toxicological and molecular profiling of insecticide resistance in a Brazilian strain of fall armyworm resistant to Bt Cry1 proteins

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Table S1 Overview on insecticides and insecticidal proteins used in the bioassays, including their mode of action according to the IRAC (Insecticide Resistance Action Committee) classification scheme and their respective dose range used in diet bioassays.

IRAC group	Mode of action	Chemical class	Active ingredient	Manufactory (Purity %)	Insecticide range (ng cm^{-2})
IRAC 1A	AChE[2] inhibitor	Carbamate	Thiodicarb	Sigma-Aldrich (99.2)	0.05 - 722
IRAC 1B		Organophosphate	Chlorpyrifos	Sigma-Aldrich (97.5)	0.05 - 722
IRAC 3A	VGSC[3] modulator	Pyrethroids	Deltamethrin	Sigma-Aldrich (99.8)	0.02 - 722
IRAC 5	nAChR[4] allosteric modulators - site I	Spinosyns	Spinosad	Sigma-Aldrich (95.0)	0.02 - 144
IRAC 6	GluCl[5] allosteric modulator	Avermectins	Emamectin	Sigma-Aldrich (99.6)	0.02 - 5.70
			Abamectin	Sigma-Aldrich (97.9)	0.23 - 722
IRAC 11	Disruptors of insect midgut	*Bacillus thuringiensis*	Cry1Ab	Bayer AG (91.0)	7.5 - 40,000
			Cry1Ac	Bayer AG (28.3)	7.5 - 40,000
			Vip3Aa	Bayer AG (100)	0.03 - 200
IRAC 13	Uncoupler of oxidative phosphorylation	Pyrroles	Chlorfenapyr	Dr. Ehrenstorfer (99.6)	0.23 - 722
IRAC 15	Inhibitors of chitin biosynthesis affecting CHS1[6]	Benzoylureas	Triflumuron	Bayer AG (99.6)	0.05 - 722
IRAC 22	VGSC[3] blockers	Oxadiazines	Indoxacarb	Sigma-Aldrich (99.5)	0.23 - 722
IRAC 28	RyR[7] modulators	Diamides	Tetraniliprole	Bayer AG (98.0)	0.23 - 28
			Flubendiamide	Sigma-Aldrich (98.0)	0.23 - 144
			Chlorantraniliprole	Sigma-Aldrich (98.0)	0.01 - 28

Table S2 List of primers used for validation of differentially expressed genes by RT-qPCR analysis of Sf_Des and Sf_Bra *Spodoptera frugiperda* strains.

Primers	GenBank Accession number	Forward primer (5' to 3')	Reverse primer (5' to 3')
Sf_RPS3A[1]	AF429977	CAACTCTGAACTTCGTGAG	CTACCACCCTCTCCATGAA
Sf_L10[2]	MN044628	GTCGTGCCAAGTTCAAGTTC	GTCCTCACGCAGCTTCTC
Sf_L17[2]	MN044629	GTGACGGAAGCTATCAAGAC	ACTTGTTGCCGAGGACAC
Sf_CYP321A1-like[4]	PRJNA299878[5]	CAAACCAGCCTGCACCTGTA	GGGCAACAGGACGTGTATAGG
Sf_CYP321B1[3]	KC789754	CGTACGATGCAGTCTTGGAA	CATTGCCTACAGGCAGAACA
Sf_CYP321A7[3]	KC789750	TCCAGACCCAGAAGTTTTCG	CGGCCTGGACTTGTAATTTG
Sf_CYP321A9[3]	KC789752	GCGTGGTGTAGCCTTCTACG	CGGGTCAATGACAAACAGTG
Sf_CYP9333B4[3]	FP340412.1	GAATTATGCCGGTGGTGTCT	TAGCGACATGTCTCGGTGAG
Sf_CYP332A1[3]	FP340417.1	GCATGCATGAAACGCTAAGA	CCACGTTCACGTAGACTGGA
Sf_CYP6B39[3]	FP340416.1	AAGTTCCAAGTGGAGCCATCGAGG	CCTCCTTTGGGCCCGACGAGAAG
Sf_CYP6B50[3]	KC789749	CAATCCAGCACGATGAGAAA	GTGCGAATTTTGACCAAGG
Sf_CYP9A9-like[4]	PRJNA299878[6]	CAATGCAATTCCTTGGACCAA	GCACACCATCGATCCAATGA
Sf_CYP9A28[3]	FP340410.1	TCAAGCACATCAAGCCAGTC	CCGTTGTGAGTCCATCACTGAC
Sf_CYP9A59[3]	KJ671578	GGATACCCACGTATGCCATC	TCCTAGGACCAGTGCCAAAT

Primer pairs described by[1] Li et al. (2017); [2] Boaventura et al. (2020); [3] Giraudo et al. (2015);[4]Nascimento et al. (2015);[5] Under the SRA accession number PRJNA299878 in Nascimento et al. (2015) named as transcript L_669_T_9/12; [6]Under the SRA accession number PRJNA299878 in Nascimento et al. (2015) named as transcript L_464_T_3/3.

Table S3 Log-dose mortality data obtained for 12 different insecticides against 3rd instar larvae of Sf_Des and Sf_Bra in diet spray bioassays. The assessment for affected larvae was made three days after treatment.

Compound	Strain	n	EC_{50} (ng ai cm^{-2})	95 % CI[a]	Slope (±SE)	RR[b]
Deltamethrin	Sf_Bra	324	0.20	0.18 - 0.22	2.47 (0.52)	
	Sf_Des	324	3.49	2.37 - 5.14	2.74 (0.87)	17.44
Chlorpyrifos	Sf_Bra	288	10.68	7.99 - 14.27	4.62 (1.00)	
	Sf_Des	288	80.22	62.69 - 102.70	2.78 (0.48)	7.51
Triflumuron	Sf_Bra	252	1.39	0.61 - 3.16	2.50 (0.63)	
	Sf_Des	336	6.28	4.88 - 8.13	0.77 (0.06)	4.53
Emamectin Benzoate	Sf_Bra	401	0.06	0.05 - 0.07	2.79 (1.42)	
	Sf_Des	401	0.19	0.05 - 0.69	0.95 (0.45)	3.28
Thiodicarb	Sf_Bra	540	44.69	39.73 - 50.28	7.38 (0.93)	
	Sf_Des	288	123.10	111.20 - 136.20	3.89 (0.54)	2.75
Spinosad	Sf_Bra	396	5.51	4.64 - 6.53	2.36 (0.85)	
	Sf_Des	396	10.16	8.87 - 11.63	5.18 (0.81)	1.84
Flubendiamide	Sf_Bra	401	1.94	1.77 - 2.12	4.06 (0.39)	
	Sf_Des	401	3.47	3.14 - 3.83	2.69 (0.30)	1.79
Abamectin	Sf_Bra	288	72.06	62.61 - 82.94	2.63 (0.34)	
	Sf_Des	252	102.30	89.86 - 116.50	4.32 (0.50)	1.42
Chlorfenapyr	Sf_Bra	401	36.91	29.52 - 46.15	3.37 (1.35)	
	Sf_Des	401	52.07	41.15 - 65.89	2.15 (0.38)	1.41
Tetraniliprole	Sf_Bra	252	2.01	1.34 - 3.04	4.33 (1.50)	
	Sf_Des	252	2.15	1.28 - 3.61	4.60 (1.78)	1.06
Indoxacarb	Sf_Bra	252	4.65	4.44 - 4.86	4.38 (0.33)	
	Sf_Des	252	4.68	4.21 - 5.19	5.90 (1.21)	1.01
Chlorantraniliprole	Sf_Bra	252	0.82	0.58 - 1.15	2.75 (1.14)	
	Sf_Des	252	0.38	0.33 - 0.43	3.20 (0.36)	0.47

[a] 95 % confidence interval; [b] Resistance ratio (EC_{50} of Sf_Des strain divided by EC_{50} of Sf_Bra).

Table S4 Summary of RNA-Seq analysis obtained from 3rd instar larvae of *Spodoptera frugiperda* strains Sf_Des and Sf_Bra (n=5 per strain).

	All	Sf_Bra	Sf_Des
Total trinity 'genes'	118,013	59,502	74,448
Total trinity transcripts	209,969	112,964	157,228
	Based on all 'transcripts'		
Contig N50	2,163	2,462	1,849
Median contig length	424	506	485
Average contig	993	1,180	992
Total assembled bases	208,581,896	133,268,072	155,966,890
TransDecoder CDS	200,590		

Table S5 Summary of transcript quantification obtained from 3rd instar larvae of *Spodoptera frugiperda* strains Sf_Des and Sf_Bra by pseudoalignment with kallisto v0.45.0 [44] and summarized on gene level using tximport v1.12.3.

Sample	Sequences	Pseudoaligned	Estimated average fragment length	Pseudoaligned (%)	Minimum one read	Minimum one read (%)
Sf-Bra1	19655136	17653881	160	89.82	40855	0.71010185
Sf-Bra2	19627680	17763461	150	90.50	41611	0.72324191
Sf-Bra3	19435796	17559102	149	90.34	41356	0.71880975
Sf-Bra4	21115281	19138106	151	90.64	40873	0.71041471
Sf-Bra5	19937286	18014753	153	90.36	38733	0.67321931
Sf-Des1	19649711	17518417	154	89.15	48975	0.85123579
Sf-Des2	20379104	18267552	143	89.64	49189	0.85495533
Sf-Des3	19228811	17085079	143	88.85	48532	0.843536
Sf-Des4	19106471	16822902	152	88.05	46888	0.81496159
Sf-Des5	21070255	18591915	155	88.24	48034	0.83488024

Table S6 Gene Ontology (GO) term enrichment analyses of differentially expressed genes (DEG) that are up-regulated in Cry1F-resistant strain Sf_Des (P*adjust* < 0.01 and DEG ≥ 5 in each category).

Category	Term	Ontology[a]	P-value	Up-regulated in category	Genes in category
GO:0055114	oxidation-reduction process	BP	2.83E-09	277	1053
GO:0008152	metabolic process	BP	8.50E-04	141	618
GO:0006629	lipid metabolic process	BP	7.00E-05	70	299
GO:0009725	response to hormone	BP	3.31E-03	58	273
GO:0044237	cellular metabolic process	BP	9.37E-04	49	223
GO:0006259	DNA metabolic process	BP	1.44E-03	49	233
GO:0015074	DNA integration	BP	8.87E-06	48	183
GO:0040003	chitin-based cuticle development	BP	2.35E-08	43	108
GO:1901564	organonitrogen compound metabolic process	BP	2.95E-03	43	177
GO:0035220	wing disc development	BP	5.20E-05	39	130
GO:0046680	response to DDT	BP	8.62E-08	38	76
GO:0006030	chitin metabolic process	BP	5.73E-06	33	89
GO:0035149	lumen formation, open tracheal system	BP	6.03E-06	33	79
GO:0031000	response to caffeine	BP	3.08E-04	33	97
GO:0006805	xenobiotic metabolic process	BP	1.10E-03	33	111
GO:0071704	organic substance metabolic process	BP	5.92E-03	33	148
GO:0007391	dorsal closure	BP	8.60E-03	28	104
GO:0042572	retinol metabolic process	BP	3.39E-03	27	81
GO:0008210	estrogen metabolic process	BP	1.94E-04	26	66
GO:0044248	cellular catabolic process	BP	5.22E-03	26	107
GO:0017143	insecticide metabolic process	BP	2.24E-05	24	54
GO:0048252	lauric acid metabolic process	BP	1.74E-04	24	54
GO:0030199	collagen fibril organization	BP	2.70E-03	21	59
GO:0002118	aggressive behavior	BP	7.06E-03	21	75
GO:0040040	thermosensory behavior	BP	8.46E-03	20	62
GO:0042759	long-chain fatty acid biosynthetic process	BP	6.43E-04	19	50
GO:0035002	liquid clearance, open tracheal system	BP	3.50E-03	19	52
GO:0042573	retinoic acid metabolic process	BP	1.63E-03	18	44
GO:0046949	fatty-acyl-CoA biosynthetic process	BP	9.56E-03	18	49
GO:0015879	carnitine transport	BP	2.52E-04	17	39
GO:0006706	steroid catabolic process	BP	1.05E-03	17	42
GO:0035336	long-chain fatty-acyl-CoA metabolic process	BP	2.43E-03	17	45
GO:0010025	wax biosynthetic process	BP	3.29E-03	17	47
GO:0070989	oxidative demethylation	BP	3.33E-03	16	41
GO:0030708	germarium-derived female germ-line cyst encapsulation	BP	1.23E-03	15	31
GO:0042445	hormone metabolic process	BP	4.66E-03	14	48
GO:1901362	organic cyclic compound biosynthetic process	BP	8.11E-04	13	33
GO:0042738	exogenous drug catabolic process	BP	1.01E-03	13	28
GO:0002933	lipid hydroxylation	BP	3.04E-03	13	30
GO:0035204	negative regulation of lamellocyte differentiation	BP	5.62E-03	13	30
GO:0019438	aromatic compound biosynthetic process	BP	1.39E-04	12	26
GO:0072382	minus-end-directed vesicle transport along microtubule	BP	2.16E-03	12	21
GO:0042074	cell migration involved in gastrulation	BP	4.91E-03	12	32

Table S6_ cont.

Category	Term	Ontology[a]	P-value	Up-regulated in category	Genes in category
GO:0007282	cystoblast division	BP	8.57E-03	12	25
GO:0048803	imaginal disc-derived male genitalia morphogenesis	BP	8.74E-03	12	31
GO:0007440	foregut morphogenesis	BP	9.89E-03	12	30
GO:0016098	monoterpenoid metabolic process	BP	5.07E-04	11	21
GO:0006189	'de novo' IMP biosynthetic process	BP	3.55E-03	11	21
GO:0090235	regulation of metaphase plate congression	BP	4.30E-03	11	19
GO:1990048	anterograde neuronal dense core vesicle transport	BP	5.50E-03	11	21
GO:0070647	protein modification by small protein conjugation or removal	BP	6.28E-03	11	28
GO:0051296	establishment of meiotic spindle orientation	BP	9.29E-03	11	23
GO:0009822	alkaloid catabolic process	BP	9.56E-03	11	27
GO:0070789	metula development	BP	3.95E-03	10	17
GO:0098657	import into cell	BP	4.24E-03	10	32
GO:0012501	programmed cell death	BP	8.34E-03	10	31
GO:0051237	maintenance of RNA location	BP	9.95E-03	10	18
GO:0000393	spliceosomal conformational changes to generate catalytic conformation	BP	6.89E-04	9	13
GO:0021682	nerve maturation	BP	1.03E-03	9	13
GO:0010838	positive regulation of keratinocyte proliferation	BP	2.10E-03	9	15
GO:0009395	phospholipid catabolic process	BP	3.06E-03	9	18
GO:1901563	response to camptothecin	BP	4.07E-03	9	17
GO:0006145	purine nucleobase catabolic process	BP	6.79E-03	9	19
GO:0002213	defense response to insect	BP	8.50E-03	9	18
GO:0018130	heterocycle biosynthetic process	BP	5.77E-03	8	18
GO:0042335	cuticle development	BP	6.36E-03	8	16
GO:0021943	formation of radial glial scaffolds	BP	9.15E-03	8	16
GO:0051608	histamine transport	BP	6.25E-03	7	15
GO:0006029	proteoglycan metabolic process	BP	9.21E-03	7	12
GO:0042435	indole-containing compound biosynthetic process	BP	1.74E-03	6	9
GO:1905000	regulation of membrane repolarization during atrial cardiac muscle cell action potential	BP	1.77E-03	6	7
GO:0018212	peptidyl-tyrosine modification	BP	1.96E-03	5	6
GO:0042743	hydrogen peroxide metabolic process	BP	3.93E-03	5	10
GO:0036150	phosphatidylserine acyl-chain remodeling	BP	6.38E-03	5	6
GO:0043227	membrane-bounded organelle	CC	8.75E-04	95	484
GO:0005903	brush border	CC	1.50E-03	58	213
GO:0031012	extracellular matrix	CC	2.06E-06	56	174
GO:0005588	collagen type V trimer	CC	9.92E-03	5	6
GO:0005778	peroxisomal membrane	CC	1.61E-03	32	102
GO:0000974	Prp19 complex	CC	1.53E-03	15	36
GO:0036186	early phagosome membrane	CC	1.61E-03	12	21
GO:0035182	female germline ring canal outer rim	CC	3.26E-03	12	23
GO:0061474	phagolysosome membrane	CC	3.91E-03	12	24
GO:0001411	hyphal tip	CC	4.42E-03	11	21
GO:0032992	protein-carbohydrate complex	CC	8.19E-04	7	12
GO:0005592	collagen type XI trimer	CC	2.20E-03	6	7
GO:0016491	oxidoreductase activity	MF	1.77E-03	146	628
GO:0020037	heme binding	MF	3.39E-08	107	341
GO:0005506	iron ion binding	MF	3.36E-07	103	339

Figure S6_cont.

Category	Term	Ontology[a]	P-value	Up-regulated in category	Genes in category
GO:0042302	structural constituent of cuticle	MF	9.25E-32	93	159
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	MF	2.44E-08	86	242
GO:0052689	carboxylic ester hydrolase activity	MF	3.66E-03	52	211
GO:0004518	nuclease activity	MF	2.72E-04	51	210
GO:0005215	transporter activity	MF	8.39E-03	43	211
GO:0008061	chitin binding	MF	2.78E-06	42	116
GO:0008201	heparin binding	MF	9.71E-03	42	153
GO:0004497	monooxygenase activity	MF	2.35E-04	39	125
GO:0030246	carbohydrate binding	MF	6.27E-04	39	126
GO:0008010	structural constituent of chitin-based larval cuticle	MF	5.78E-17	38	53
GO:0008011	structural constituent of pupal chitin-based cuticle	MF	1.24E-16	35	47
GO:0016758	transferase activity, transferring hexosyl groups	MF	2.45E-03	33	103
GO:0018685	alkane 1-monooxygenase activity	MF	5.07E-05	27	61
GO:0080019	fatty-acyl-CoA reductase (alcohol-forming) activity	MF	2.82E-04	23	56
GO:0016627	oxidoreductase activity, acting on the CH-CH group of donors	MF	2.30E-03	22	57
GO:0005496	steroid binding	MF	5.56E-04	19	47
GO:0005504	fatty acid binding	MF	7.73E-03	19	51
GO:0005214	structural constituent of chitin-based cuticle	MF	2.29E-08	18	28
GO:0004177	aminopeptidase activity	MF	1.86E-03	18	51
GO:0015651	quaternary ammonium group transmembrane transporter activity	MF	8.43E-06	16	32
GO:0050062	long-chain-fatty-acyl-CoA reductase activity	MF	8.91E-04	16	38
GO:0050649	testosterone 6-beta-hydroxylase activity	MF	2.16E-03	15	36
GO:0015293	symporter activity	MF	7.48E-03	15	55
GO:0101020	estrogen 16-alpha-hydroxylase activity	MF	6.58E-04	14	29
GO:0000386	second spliceosomal transesterification activity	MF	1.06E-03	14	28
GO:0008186	RNA-dependent ATPase activity	MF	1.98E-03	14	33
GO:0008401	retinoic acid 4-hydroxylase activity	MF	1.06E-03	13	27
GO:0030343	vitamin D3 25-hydroxylase activity	MF	1.95E-03	13	28
GO:0070006	metalloaminopeptidase activity	MF	9.31E-03	13	34
GO:0017070	U6 snRNA binding	MF	3.29E-04	12	19
GO:0008395	steroid hydroxylase activity	MF	1.79E-03	12	32
GO:0034875	caffeine oxidase activity	MF	2.23E-04	11	19
GO:0030619	U1 snRNA binding	MF	5.19E-04	11	17
GO:0101021	estrogen 2-hydroxylase activity	MF	2.92E-03	11	23
GO:0070330	aromatase activity	MF	1.18E-03	10	17
GO:0015103	inorganic anion transmembrane transporter activity	MF	2.04E-03	10	28
GO:0070576	vitamin D 24-hydroxylase activity	MF	8.47E-03	10	26
GO:0033791	3alpha,7alpha,12alpha-trihydroxy-5beta-cholestanoyl-CoA 24-hydroxylase activity	MF	9.76E-03	10	19
GO:0030623	U5 snRNA binding	MF	1.34E-03	9	13
GO:0030620	U2 snRNA binding	MF	1.61E-03	9	14
GO:0004558	alpha-1,4-glucosidase activity	MF	4.92E-03	8	16
GO:0030023	extracellular matrix constituent conferring elasticity	MF	9.42E-03	8	17
GO:0004167	dopachrome isomerase activity	MF	2.05E-03	7	12

Figure S6_cont.

Category	Term	Ontology[a]	P-value	Up-regulated in category	Genes in category
GO:0004641	phosphoribosylformylglycinamidine cyclo-ligase activity	MF	5.20E-03	6	8
GO:0004644	phosphoribosylglycinamide formyltransferase activity	MF	5.20E-03	6	8
GO:1904399	heparan sulfate binding	MF	2.23E-03	5	5
GO:0034988	Fc-gamma receptor I complex binding	MF	5.41E-03	5	6
GO:0003823	antigen binding	MF	9.89E-03	5	7

[a]BP: Biological Process; CC: Cellular Component; MF: Molecular Function

Table S7 Gene Ontology (GO) term enrichment analyses of differentially expressed genes (DEG) that are down-regulated in Cry1F-resistant strain Sf_Des (P*adjust* < 0.01 and DEG ≥ 5 in each category).

Category	Term	Ontology[a]	P-value	Down-regulated in category	Genes in category
GO:0055114	oxidation-reduction process	BP	6.55E-05	113	1053
GO:0008152	metabolic process	BP	2.12E-05	72	618
GO:0006508	proteolysis	BP	4.77E-03	65	666
GO:0015074	DNA integration	BP	1.36E-14	42	183
GO:0005975	carbohydrate metabolic process	BP	9.42E-05	40	292
GO:0006259	DNA metabolic process	BP	1.45E-07	36	233
GO:0042742	defense response to bacterium	BP	4.99E-03	30	272
GO:0007584	response to nutrient	BP	3.91E-04	29	199
GO:0032196	transposition	BP	3.96E-11	28	113
GO:0009617	response to bacterium	BP	1.84E-03	27	217
GO:0046686	response to cadmium ion	BP	4.15E-04	26	180
GO:0043627	response to estrogen	BP	1.45E-04	25	162
GO:0044249	cellular biosynthetic process	BP	5.57E-03	23	197
GO:0008202	steroid metabolic process	BP	1.08E-04	20	120
GO:0044248	cellular catabolic process	BP	9.35E-04	16	107
GO:0006633	fatty acid biosynthetic process	BP	3.83E-03	16	100
GO:0017085	response to insecticide	BP	1.25E-04	14	66
GO:0042572	retinol metabolic process	BP	3.55E-03	14	81
GO:0009744	response to sucrose	BP	4.23E-03	13	79
GO:0008610	lipid biosynthetic process	BP	5.27E-03	13	87
GO:1901575	organic substance catabolic process	BP	5.94E-03	13	94
GO:0010288	response to lead ion	BP	9.87E-03	12	81
GO:0010040	response to iron(II) ion	BP	2.47E-03	11	53
GO:0071385	cellular response to glucocorticoid stimulus	BP	1.17E-03	10	47
GO:0017143	insecticide metabolic process	BP	6.92E-03	10	54
GO:0031288	sorocarp morphogenesis	BP	8.50E-03	10	55
GO:0042574	retinal metabolic process	BP	8.96E-03	10	56
GO:0007021	tubulin complex assembly	BP	3.42E-05	9	24
GO:0006066	alcohol metabolic process	BP	5.51E-05	9	27
GO:0001507	acetylcholine catabolic process in synaptic cleft	BP	1.10E-03	9	32
GO:0090304	nucleic acid metabolic process	BP	4.55E-03	9	52
GO:0009410	response to xenobiotic stimulus	BP	5.21E-03	9	59
GO:0006707	cholesterol catabolic process	BP	6.14E-03	9	42
GO:0042573	retinoic acid metabolic process	BP	6.70E-03	9	44
GO:0010045	response to nickel cation	BP	7.90E-03	9	46
GO:0052695	cellular glucuronidation	BP	6.47E-03	8	40
GO:0044241	lipid digestion	BP	7.00E-03	8	41
GO:0044245	polysaccharide digestion	BP	8.69E-03	8	34
GO:0071294	cellular response to zinc ion	BP	1.82E-03	7	31
GO:0009809	lignin biosynthetic process	BP	2.06E-03	7	22
GO:0034754	cellular hormone metabolic process	BP	9.83E-03	7	38
GO:0007023	post-chaperonin tubulin folding pathway	BP	1.17E-05	6	8
GO:0071378	cellular response to growth hormone stimulus	BP	3.71E-03	6	20
GO:0030422	production of siRNA involved in RNA interference	BP	4.03E-03	6	20
GO:0070980	biphenyl catabolic process	BP	4.07E-03	6	21
GO:0045071	negative regulation of viral genome replication	BP	4.33E-03	6	21
GO:0007304	chorion-containing eggshell formation	BP	4.56E-03	6	23
GO:0071236	cellular response to antibiotic	BP	5.08E-03	6	25
GO:0042760	very long-chain fatty acid catabolic process	BP	6.29E-03	6	20
GO:0006069	ethanol oxidation	BP	9.04E-03	6	22

Table S7_cont.

Category	Term	Ontology[a]	P-value	Down-regulated in category	Genes in category
GO:0051901	positive regulation of mitochondrial depolarization	BP	9.06E-05	5	9
GO:0036267	invasive filamentous growth	BP	2.67E-03	5	12
GO:0097054	L-glutamate biosynthetic process	BP	2.67E-03	5	12
GO:0048194	Golgi vesicle budding	BP	2.77E-03	5	14
GO:0006751	glutathione catabolic process	BP	3.76E-03	5	14
GO:0032223	negative regulation of synaptic transmission, cholinergic	BP	5.01E-03	5	16
GO:0002175	protein localization to paranode region of axon	BP	5.02E-03	5	17
GO:0030245	cellulose catabolic process	BP	6.44E-03	5	16
GO:0000467	exonucleolytic trimming to generate mature 3'-end of 5.8S rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	BP	7.91E-03	5	17
GO:0019676	ammonia assimilation cycle	BP	9.45E-03	5	16
GO:0043030	regulation of macrophage activation	BP	9.89E-03	5	19
GO:0005777	peroxisome	CC	1.07E-03	28	215
GO:0070578	RISC-loading complex	CC	2.03E-03	6	17
GO:0042600	chorion	CC	5.97E-03	5	16
GO:0005615	extracellular space	CC	7.14E-03	89	969
GO:0005604	basement membrane	CC	9.06E-03	16	112
GO:0016740	transferase activity	MF	6.43E-09	69	542
GO:0004518	nuclease activity	MF	3.81E-06	32	210
GO:0004497	monooxygenase activity	MF	8.02E-06	24	125
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	MF	4.28E-05	25	135
GO:0016787	hydrolase activity	MF	9.33E-05	61	570
GO:0001972	retinoic acid binding	MF	1.43E-04	12	43
GO:0004252	serine-type endopeptidase activity	MF	3.07E-04	37	279
GO:0016779	nucleotidyltransferase activity	MF	3.08E-04	23	157
GO:0102799	glucosinolate glucohydrolase activity	MF	5.54E-04	5	9
GO:0008395	steroid hydroxylase activity	MF	7.85E-04	8	32
GO:0047782	coniferin beta-glucosidase activity	MF	9.49E-04	7	19
GO:0017168	5-oxoprolinase (ATP-hydrolyzing) activity	MF	1.19E-03	5	10
GO:0015925	galactosidase activity	MF	1.25E-03	5	11
GO:0052689	carboxylic ester hydrolase activity	MF	1.66E-03	27	211
GO:0000016	lactase activity	MF	1.73E-03	10	42
GO:0005488	binding	MF	1.78E-03	61	712
GO:0140097	catalytic activity, acting on DNA	MF	2.21E-03	7	33
GO:0019137	thioglucosidase activity	MF	2.27E-03	5	12
GO:0016758	transferase activity, transferring hexosyl groups	MF	2.29E-03	17	103
GO:0017171	serine hydrolase activity	MF	2.38E-03	7	26
GO:0015923	mannosidase activity	MF	2.54E-03	7	24
GO:0003824	catalytic activity	MF	2.60E-03	68	699
GO:0004806	triglyceride lipase activity	MF	2.68E-03	16	92
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	MF	2.78E-03	32	242
GO:0015020	glucuronosyltransferase activity	MF	3.23E-03	10	46
GO:0005518	collagen binding	MF	3.38E-03	13	78

Table S7_cont.

Category	Term	Ontology[a]	P-value	Down-regulated in category	Genes in category
GO:0042626	ATPase activity, coupled to transmembrane movement of substances	MF	3.73E-03	12	75
GO:0043878	glyceraldehyde-3-phosphate dehydrogenase (NAD+) (non-phosphorylating) activity	MF	3.84E-03	5	12
GO:0008390	testosterone 16-alpha-hydroxylase activity	MF	4.14E-03	5	16
GO:0030343	vitamin D3 25-hydroxylase activity	MF	5.27E-03	7	28
GO:0003990	acetylcholinesterase activity	MF	5.51E-03	9	41
GO:0005506	iron ion binding	MF	5.67E-03	39	339
GO:0020037	heme binding	MF	5.77E-03	39	341
GO:0004190	aspartic-type endopeptidase activity	MF	5.77E-03	8	44
GO:0015928	fucosidase activity	MF	6.03E-03	5	15
GO:0016162	cellulose 1,4-beta-cellobiosidase activity	MF	6.03E-03	5	15
GO:0097599	xylanase activity	MF	6.03E-03	5	15
GO:0016616	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	MF	6.13E-03	14	93
GO:0016040	glutamate synthase (NADH) activity	MF	6.30E-03	5	14
GO:0016491	oxidoreductase activity	MF	6.52E-03	63	628
GO:0035197	siRNA binding	MF	6.68E-03	5	16
GO:0004028	3-chloroallyl aldehyde dehydrogenase activity	MF	9.24E-03	6	22
GO:0008061	chitin binding	MF	9.30E-03	16	116
GO:0048487	beta-tubulin binding	MF	9.53E-03	10	58
GO:0032451	demethylase activity	MF	9.63E-03	5	19

[a]BP: Biological Process; CC: Cellular Component; MF: Molecular Function

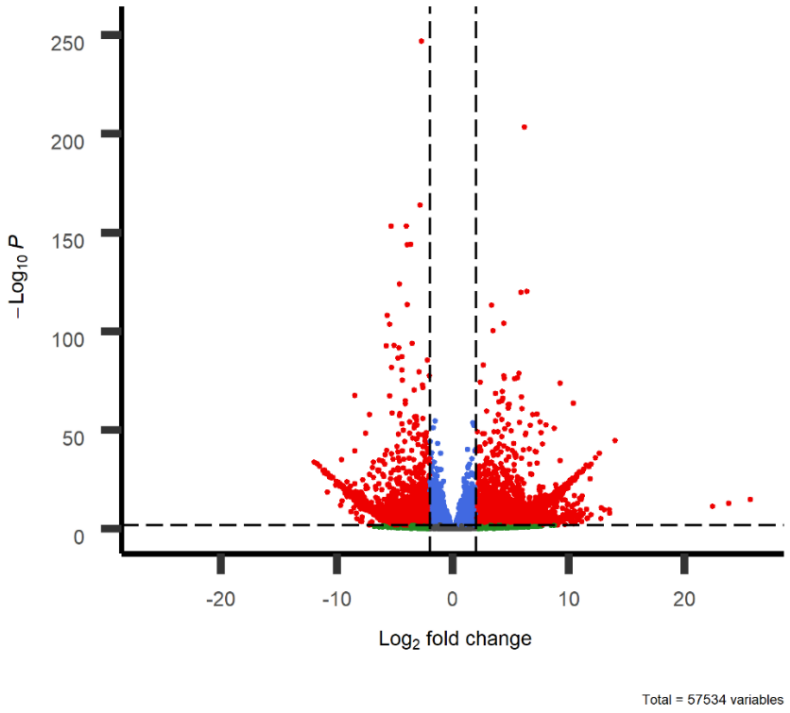


Figure S1 Volcano plot of overall expression level of transcripts obtained for pooled 3rd instar larvae of *Spodoptera frugiperda*.

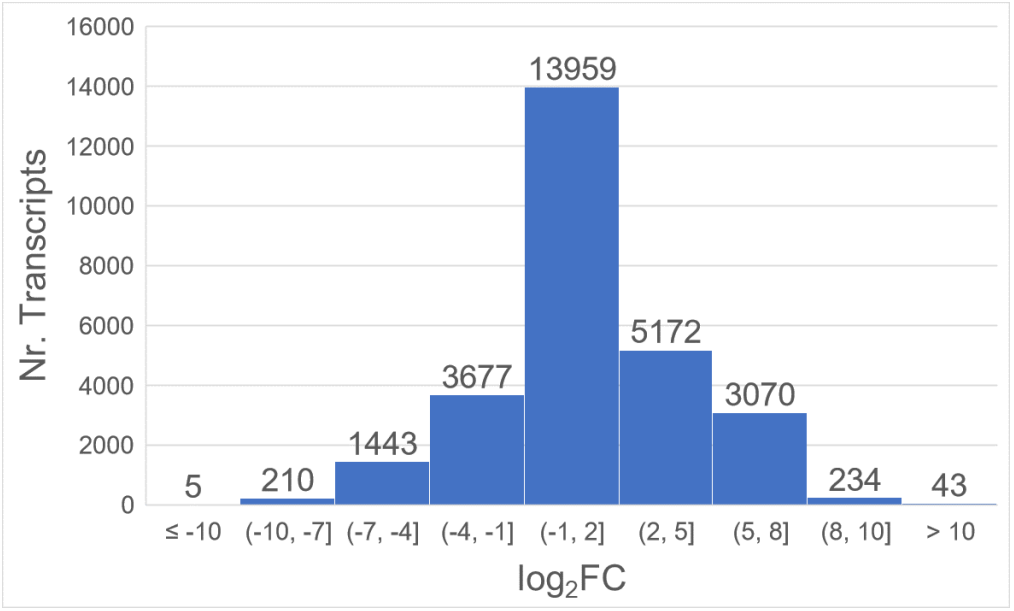
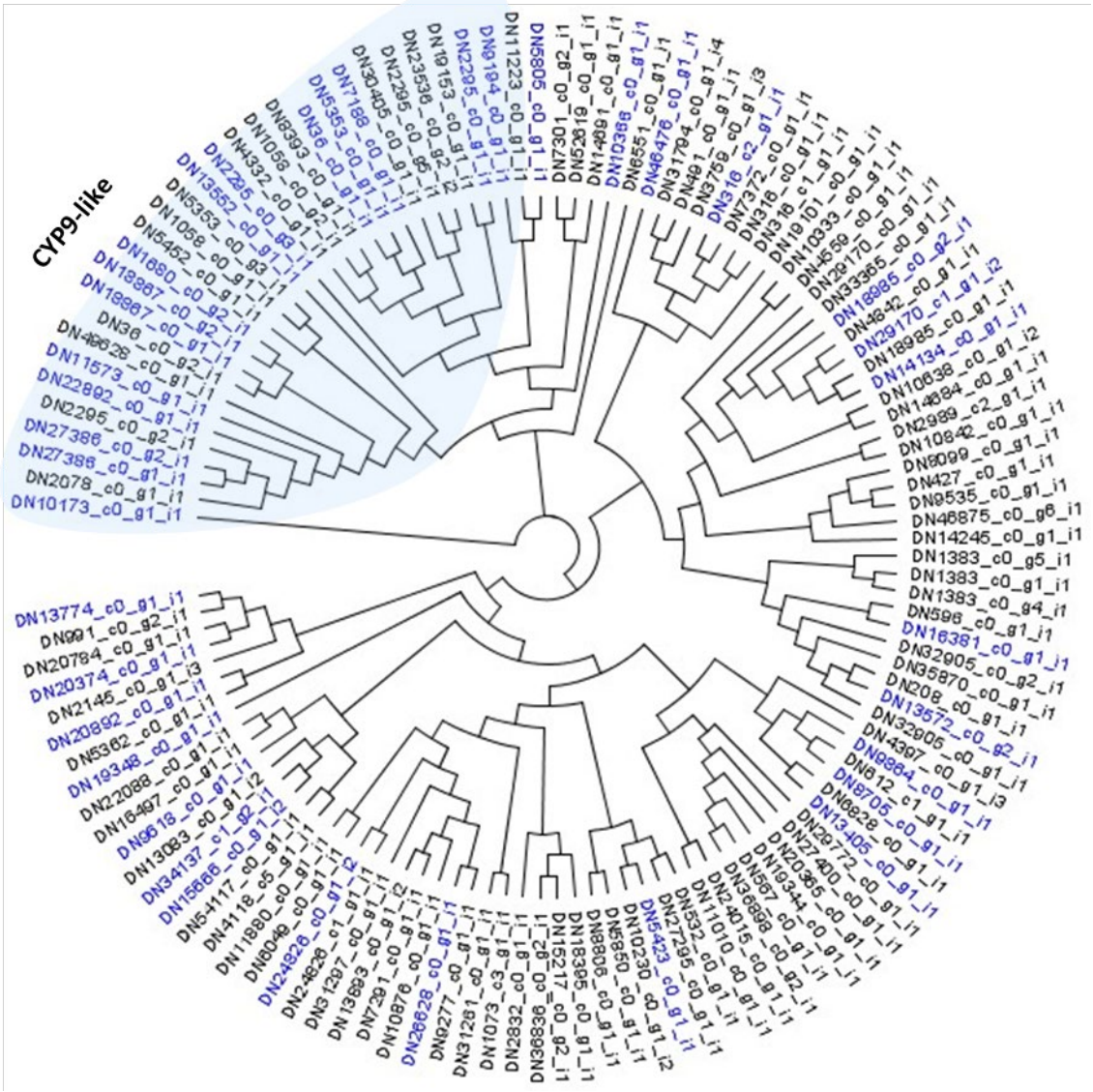


Figure S2 Differentially expressed genes (P*adjust* ≤ 0.01) obtained from *Spodoptera frugiperda* strains Sf_Des and Sf_Bra. Sf_Des is highly resistant to Cry1F insecticidal protein and Sf_Bra is the susceptible reference strain.

Figure S3 Maximum-likelihood tree built by FastTree 2.1.5 (Geneious v.10.2.6) from multiple sequence alignment of 125 protein sequences identified as P450. Transcripts with log_2FC >5 (n=37) are highlighted in blue as well as the monophyletic group encompassing CYP9-like transcripts.



Appendix D supporting information (Chapter 5)

Monitoring of target-site mutations conferring insecticide resistance in *Spodoptera frugiperda*

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Table S1. Populations of *Spodoptera frugiperda* collected in different countries and years used for genotyping of target-site mutations.

Country	Sample ID	City. State	Year	Host plant
Brazil[1]	Sf_Bra	Unknown, São Paulo	2005	corn
	Sf_Cor	Correntina, Bahia	2016	corn
	Sf_Des	São Desidério, Bahia	2016	corn
	PR-PG	Ponta Grossa, Paraná	2018	corn
	SP-IT	Ituverava, São Paulo	2018	corn
	MS-CS	Chapadão do Sul, Mato Grosso do Sul	2018	corn
	MT-SZ	Sapezal, Mato Grosso	2018	corn
	MT-TS	Tangará da Serra, Mato Grosso	2018	corn
	MT-PL1-2	Primavera do Leste, Mato Grosso	2018	corn
	MT-LV	Lucas do Rio Verde, Mato Grosso	2018	corn
	BA-SD	São Desidério, Bahia	2018	corn
	RO-VI	Vilhena, Rondônia	2018	corn
Indonesia	WS-I	Padang Pariaman, Sumatra	2019	corn
	DS-I	Deli Serdang, Sumatra	2019	corn
	S-I	Simalungun, Sumatra	2019	corn
	WC-I	Waled Cirebon, Java	2019	corn
	BC-I	Babakan Cirebon, Java	2019	corn
	JL-I	Jati Agung, Lampung	2019	corn
	SB-I	Saputih Banyak, Lampung	2019	corn
	K-I	Kediri, Java	2019	corn
	B-I	Blitar, Java	2019	corn
Kenya	EP-K	Eldoret	2019	corn
	KV-K	Kisii	2019	corn
	NJ-K	Nakuru	2019	corn
	MJ-K	Muranga	2019	corn
	MD-K	Mombasa	2019	corn
	KF-K	Kajiado	2019	corn
	BA-K	Bungoma	2019	corn
	NW-K	Narok	2019	corn
Puerto Rico	PR60	Unknown	2017	corn
	PR61	Unknown	2017	corn
	PR62	Unknown	2017	corn
	PR63	Unknown	2017	corn
	PR64	Unknown	2017	corn

[1] Samples were described in Boaventura et al. (2020) [28] and Boaventura et al. (2020) [30].

Table S2. List of primers for pyrosequencing and dual fluorescence probe assay used for the identification of different target-site mutations and *Spodoptera frugiperda* strain identification by RFLP-PCR and Sanger sequencing.

Table S2

Target	Mutation	Primers	Sequence (5'- 3')	Annealing Temperature (°C)	Assay
Ryanodine receptor	G4946E[1]	Sf_G4946_F	GTGATGGGCAACTTCAAC	50	Pyrosequencing
		Sf_G4946_R.btn	[btn]TTTTCCGTTATGCGTGAC		
		Sf_G4946_F.Seq	ATTTGCTAGATGTCGCT		
	I4790M[1]	Sf_I4790_F.btn	[btn]CGAGGACTTCTTCTACATGG-	50	
		Sf_taq_I4790_R	CACCTTGAGATGATAGTACC		
		Sf_I4790_R.Seq	ATGGTAGTACCCGATGA		
	I4790M[1]	Sf_taq_I4790_F	ACGACGATGCACTAGAAG	60.6	Probe assay
		Sf_taq_I4790_R	CACCTTGAGATGATAGTACC		
		Sf_I4790_HEX	[HEX]TGTCGCTCGCTATACTCATCG[BHQ1]		
		Sf_I4790_mut_FAM	[6FAM]CTCGCTATGCTCATCGGGT[BHQ1]		
Voltage-gated sodium channel	L1014F	Sf_L1014_F	TCTTCCTGGCTACAGTCG	50	Pyrosequencing
		Sf_L1014_R.btn	[btn]GACAGTAACAGGGCCAAG		
		Sf_L1014_Seq	CAGTCGTCATYGGCA		
	L932F/T929I	Sf_L932_T929_F.btn	[btn]TAATGGGTAGGACAATGG	53	
		Sf_L932_T929_R	AATCCACGTAATTTTTCC		
		Sf_L932_T929_R.Seq	AAATATGAAAATAATGATGC		
Acetylcholinesterase	F290V	Sf_F290_F	GCATCCGATTAGCAGAAG	52	Pyrosequencing
		Sf_F290_R	[btn]TATGATGGGCACAAAAGG		
		Sf_L932_F	GAACTCTTGGTATTTGTGA		
		Sf_taq_F290_F	CCAGATGAACTAGTCAATAATG	60	Probe assay
		Sf_taq_F290_R	GGAACGAACCATCTATGA		
		Sf_F290_FAM	[FAM]TATTTGTGAATTTCCTTTTGTGCCC[BHQ1]		
		Sf_F290_mut_HEX	[6HEX]TATTTGTGAAGTTCCTTTTGTGCCC[BHQ1]		
	A201S / G227A	Sf_A201S_ G227A_F	TTTGATACCCCTGATGTACC	53	Pyrosequencing
		Sf_A201S_ G227A_R	[btn]AATGAAACCGAAACTGCTC		
		Sf_A201S_Seq	TAACATTATTCGGTGAGTC		
		Sf_G227A _Seq	GGCGATAATGCAGTCA		
ATP-binding cassette transporter subfamily C2	GY deletion[2]	Sf_788-Gydel_F	[btn] CCGACTACTGGCTTAGTTT	50	Pyrosequencing
		Sf_788-Gydel_R	GCTCGCATAGTCATCACT		
		Sf_788-GYdel_seq	CTTCGGGTAAAGTTTGT		
	GC insertion[3]	Sf_ABCC2_F	TGGAGGCCGAAGAGAGACA	50	Probe assay
		Sf_ABCC2_R	AGGAGTTGACTGACTTCATGTACCT		
		SfABCC2mut allele	[HEX]AAGCACATCGCCCACTT[BHQ1]		
		SfABCC2	[6FAM]CCAAGCACATCCCACTT [BHQ1]		
Mitochondrial cytochrome oxidase subunit I		JM76[5]	GAGCTGAATTAGGRACTCCAGG	60	PCR-RFLP
		JM77[5]	ATCACCTCCWCCTGCAGGATC		
		891F_COI[5]	TACACGAGCATATTTTACATC	52	
		c1303R_COI[5]	CAGGATAGTCAGAATATCGACG		
Triosephosphate isomerase		TpiE4[6]	CCGGACTGAAGGTTATCGCTTG	56	PCR-Seq
		850R[6]	AATTTTATTACCTGCTGTGG		

Primers described by [1] Boaventura et al. (2020) [30];[2] Boaventura et al. (2020) [28]; [4] Banerjee et al. (2017) [36]; [5] Nagoshi et al. (2017) [66]; [6] Nagoshi et al. (2019) [73].

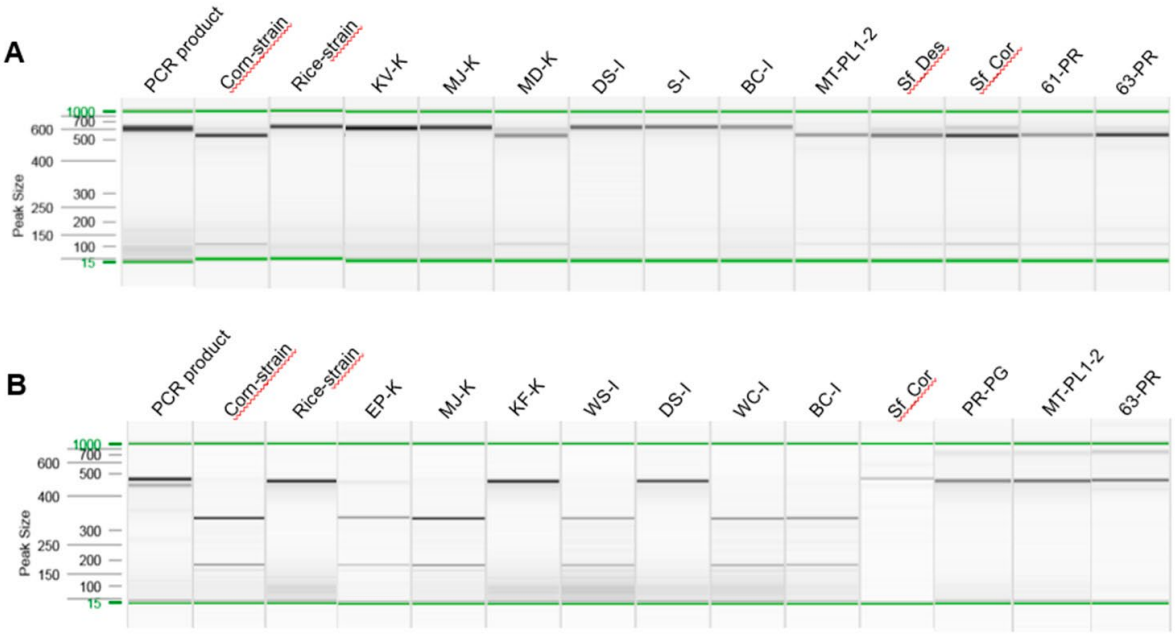

Figure S1 Automated analysis of DNA fragments showing COI polymorphism in *Spodoptera frugiperda*. (A) PCR product containing a strain specific MspI site that was amplified using the JM76 and JM77 primers (Table S2) followed by products obtained after the digestion with FastDigest MspI. Corn-strain is cut and rice-strain remains uncut as it does not have the MspI site. (B) PCR product amplified with the primers 891F_COI and c1303R_COI (Table S2) that contains a EcoRV strain specific site. After digestion with EcoRV the corn-strain amplicon remains uncut whereas it is cut in the rice strain. Details about samples, see Table S1.

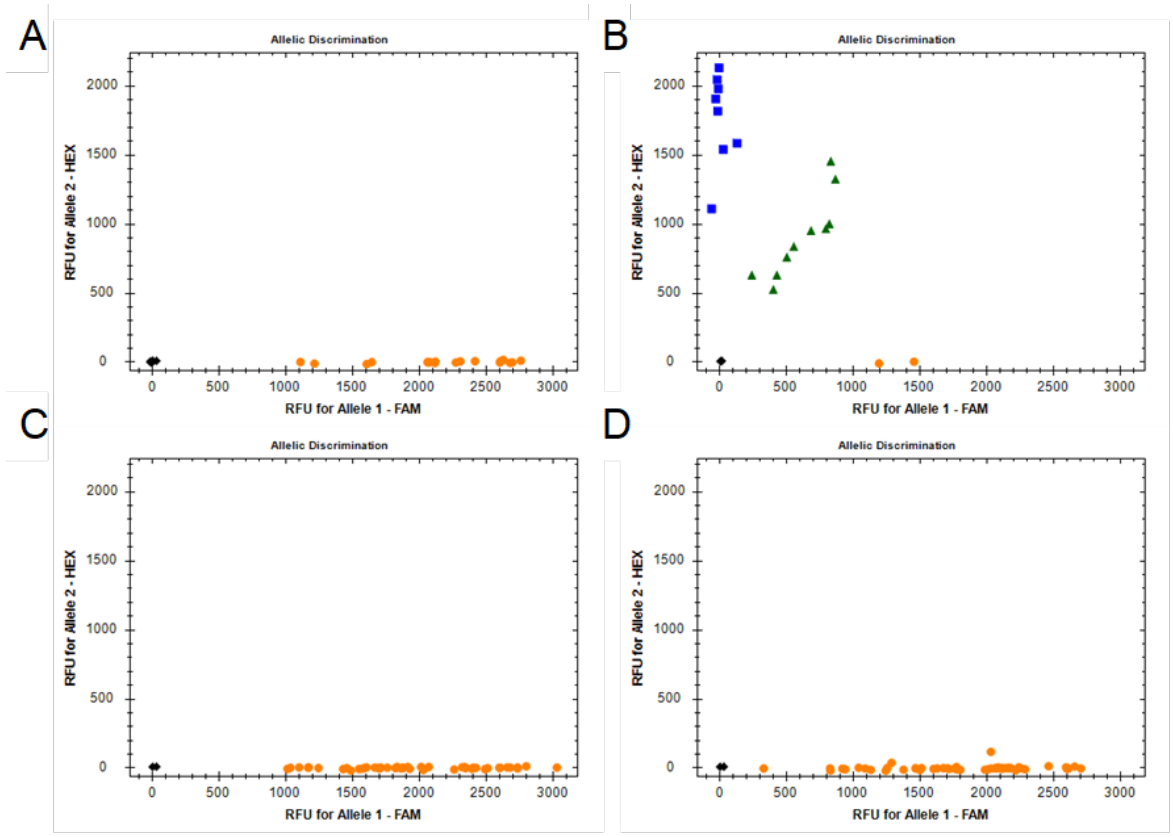

Figure S2 Detection of GC insertion allele at the ATP-binding cassette subfamily C2 (ABCC2) conferring resistance to *Bacillus thuringiensis* Cry1F toxin using PCR fluorescent probe assay described by Banerjee et al. (2017). Blue squares represent mutant ABCC2 homozygotes for the GC insertion, orange circles ABCC2 wildtype SS homozygotes, and green triangles SR representing heterozygotes. Analysis of fall armyworm field samples collected in (A) Brazil, (B) Puerto Rico, (C) Kenya, and (D) Indonesia.

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus Veröffentlichungen entnommen sind, wurden als solche kenntlich gemacht.

Diese Dissertation wurde weder in dieser noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt.

Debora Duarte Boaventura Leverkusen, 15.10.2020