Genetic studies of *Busseola fusca* (Lepidoptera: Noctuidae)

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“No road is too long for him who advances slowly and does not hurry, and no attainment is beyond his reach who equips himself with patience to achieve it.”

Jean de La Bruyere

Preface

I declare that the thesis hereby submitted for the degree of *Philosophiae Doctor* at the North-West University (Potchefstroom Campus) has not been submitted by me for a degree at this or another University, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

The North-West University Harvard language and referencing style was used in this thesis. NCBI taxonomy of species from the order Lepidoptera that were mentioned or discussed in this thesis is provided in Annexure A and also include their common names. In terms of scientific names of organisms, the instructions to authors provided by the Journal of Economic Entomology were considered. These instructions state that the unabbreviated name and authority of each organism should be given at first mention in the abstract and again in the text. The only exceptions are Fabricius and Linnaeus, which are abbreviated as F. and L., respectively. Abbreviated scientific names are subsequently used in text, although full scientific names are used in table headings and figure captions. The same rules were applied to all other abbreviations used in this thesis. Names and abbreviations of genes were italicized. However, wherever reference was made to the sequence or protein of a gene, it was not italicized.

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Abstract

Genetically modified (GM) *Bacillus thuringiensis* (*Bt*) Berliner (Bacillales: Bacillaceae) crops have been adopted widely in many countries around the world due to its success in reducing pest damage to crops and the associated insecticides used to control certain pests. However, evolution of resistance by pests threatens the continued sustainable use of these crops. *Busseola fusca* Fuller (Lepidoptera: Noctuidae), a major insect pest in sub-Saharan Africa, has developed high levels of non-recessive resistance to the Cry1Ab toxin expressed in *Bt* maize within eight years after the initial cultivation. Limited population genetic and phylogeography data are available for *B. fusca* and African insects in general. To address this issue, *B. fusca* larvae were sampled from maize across the maize production area of South Africa (SA) and statistical and network analyses of mitochondrial gene sequences were performed. Statistical results and haplotype networks of partial cytochrome c oxidase I (COI) and cytochrome b (cyt b) sequences indicated that South African *B. fusca* populations have low genetic diversity. This low degree of diversity together with non-recessive inheritance and high dispersal capabilities suggest that resistance might evolve rapidly in susceptible *B. fusca* populations if they are subjected to the same selection pressure as their resistant counterparts. On the other hand, the biology of *B. fusca* is also poorly understood, which, in combination with larval movement and feeding behaviour, also contributes to development and spread of resistant populations of this pest. A repertoire of resistance mechanisms to various Cry toxins has been identified from laboratory, greenhouse and field studies in Lepidoptera. However, no study has yet been done to determine the molecular mechanism of Cry1Ab resistance in *B. fusca.* As part of on-going research into resistance evolution, the abovementioned mechanisms of resistance were reviewed in this current study in addition to the transcriptome of *B. fusca* that was sequenced and *de novo* assembled. Several genes that have been associated with Cry toxin resistance in lepidopteran pests were detected in *B. fusca.* These genes include Cry toxin receptors (alkaline phosphatase (ALP), aminopeptidase N (APN) and cadherin (CDH)), ATP-binding cassette (ABC) transporters and mitogen-activated protein kinases (MAPKs). In order to investigate potential mechanisms of resistance in *B. fusca*, differential expression (DE) analysis was performed on larvae that fed on *Bt* and non-*Bt* maize, respectively. The DE results suggest that differential expression of metabolic and immune-related genes might explain resistance to the Cry1Ab toxin in this pest. Further studies are recommended to establish if there is a direct correlation between these differentially expressed genes and the observed resistance. Elucidation of such resistance mechanisms is crucial for developing insect resistance management (IRM) strategies to ensure sustainable use of GM crops. Nevertheless, the transcriptome characterized in this study provides a significant resource base for future studies

on *B. fusca* and contributes to understanding some of the gene regulation and signalling networks involved in *Bt* resistance in this pest.

Key words: Lepidoptera, insect resistance management, next-generation sequencing, stem borer, transcriptome

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List of general abbreviations

ABC	ATP (adenosine triphosphate)-binding cassette
AC	Adenylyl cyclase
ALP	Alkaline phosphatase
AMP	Antimicrobial peptide
ANNEX	Annotation Expander
APN	Aminopeptidase N
APP	Aminopeptidase P
BLAST	Basic Local Alignment Search Tool
CaLP	Cadherin-like protein
cAMP	Cyclic adenosine monophosphate
CDH	Cadherin
COI	Cytochrome c oxidase I
CTL	C-type lectin
cyt b	Cytochrome b
DAMP	Damage associated molecular patterns
DE	Differential expression
DEG	Differentially expressed gene
dsRNA	Double-stranded RNA
FC	Fold change
G protein	guanine nucleotide-binding protein
GalNAc	N-acetylgalactosamine
GM	Genetically modified
GO	Gene ontology
GPI	Glycosylphosphatidylinositol
HDR	High-dose refuge
HMM	Hidden Markov Model
IPM	Integrated pest management
IRM	Insect resistance management
KEGG	Kyoto Encyclopaedia of Genes and Genomes
MAPK	Mitogen-activated protein kinase
MEGA	Molecular Evolutionary Genetics Analysis
mRNA	Messenger RNA
NB	Negative binomial
NBD	Nucleotide-binding domain

NCBI	National Center for Biotechnology Information
NTC	No-template control
OPVs	Open pollinated varieties
ORFs	Open-reading frames
PAMP	Pathogen associated molecular patterns
PKA	Protein kinase A
PO	Phenoloxidase
PPA	proPO-activating protein
proPO	Prophenoloxidase
PRR	Peptidoglycan recognition receptors
qPCR	Quantitative polymerase chain reaction
RNAi	RNA interference
RPKM	Reads per kilobase of transcript per million reads
RSEM	RNA-Seq by expectation maximization
serpin	Serine proteinase inhibitor
SNP	Single-nucleotide polymorphism
SA	South Africa
SRA	Short read archive
USEPA	United States Environmental Protection Agency

List of abbreviations of organism names

Achaea janata	*A. Janata*
Amyelois transitella	*A. transitella*
Bacillus thuringiensis	*B. thuringiensis*
Bombyx mori	*B. mori*
Busseola fusca	*B. fusca*
Busseola phaia	*B. phaia*
Diatraea saccharalis	*D. saccharalis*
Drosophila melanogaster	*D. melanogaster*
Eldana saccharina	*E. saccharina*
Ephestia kuehniella	*E. kuehniella*
Epiphyas postvittana	*E. postvittana*
Helicoverpa armigera	*H. armigera*
Helicoverpa punctigera	*H. punctigera*
Helicoverpa zea	*H. zea*
Heliothis subflexa	*H. subflexa*
Heliothis virescens	*H. virescens*
Lymantria dispar	*L. dispar*
Manduca sexta	*M. sexta*
Melitaea cinxia	*M. cinxia*
Ostrinia furnacalis	*O. furnacalis*
Ostrinia nubilalis	*O. nubilalis*
Papilio machaon	*P. machaon*
Pectinophora gossypiella	*P. gossypiella*
Plodia interpunctella	*P. interpunctella*
Plutella xylostella	*P. xylostella*
Sesamia inferens	*S. inferens*
Spodoptera exigua	*S. exigua*
Spodoptera frugiperda	*S. frugiperda*
Spodoptera littoralis	*S. littoralis*
Spodoptera litura	*S. litura*
Trichoplusia ni	*T. ni*

Chapter 1
Introduction and literature overview

1.1 General introduction

Busseola fusca Fuller (Lepidoptera: Noctuidae), the African maize stem borer, is a major pest of maize in sub-Saharan Africa (Kruger *et al.*, 2009). This pest also feeds on crops such as pearl millet and sorghum and is in many cases responsible for economic losses of these crops (Kfir *et al.*, 2002; Gouse *et al.*, 2005). To combat losses caused by lepidopteran pests, genetically modified (GM) crops with insecticidal traits from *Bacillus thuringiensis* (*Bt*) Berliner (Bacillales: Bacillaceae) were developed (Zhang *et al.*, 2009). From the onset, development of resistance in target pests to toxins produced by the GM plants was a concern (Roush, 1997). To prevent this from happening, a high-dose refuge (HDR) strategy was proposed and introduced (Gould, 2000). This strategy sustains large numbers of susceptible individuals of the pest population, which is expected to mate with individuals that survive on *Bt* maize. If inheritance of resistance is recessive (Tabashnik *et al.*, 2013), resistant alleles in the pest population will be present in low frequencies.

Several studies have previously been conducted under laboratory and field conditions with target pests that developed resistance to *Bt* toxins (Akhurst *et al.*, 2003; Tabashnik *et al.*, 2003; Gahan *et al.*, 2010). To date, eight of them have displayed field resistance to *Bt* crops (Luttrell *et al.*, 2004; Downes *et al.*, 2007; Kruger *et al.*, 2009; Downes *et al.*, 2010b; Storer *et al.*, 2010; Alcantara *et al.*, 2011; Dhurua & Gujar, 2011; Huang *et al.*, 2012). The first report of a resistant stem borer species (*B. fusca*) in the field was mentioned in 2004 (Van den Berg, 2010). In 2007, the first official report was published and since then more has followed (Van Rensburg, 2007; Kruger *et al.*, 2009). Explanations provided for resistance development were initially confined to agronomical reasons (Van Rensburg, 2007; Kruger *et al.*, 2009; 2012) and non-compliance to refuge requirements (Kruger *et al.*, 2009). However, in the case of *B. fusca*, the HDR strategy is undermined by several factors that could have accelerated the development of its resistance to Cry1Ab toxins expressed by *Bt* maize (Van den Berg *et al.*, 2013). Despite the economic importance of this pest and its resistance status (Kfir *et al.*, 2002; Gouse *et al.*, 2005; Kruger *et al.*, 2009), no study has yet been done to determine the Cry1Ab toxin mode of action or the mechanism of resistance in *B. fusca.* There is also no sequencing data, with regard to resistance development, available for this species. The lack in molecular data for *B. fusca* is a great hindrance for the development of sustainable insect resistance management strategies for this pest.

Development of resistance by pests to GM crops with insecticidal traits is highly complex and threatens the continued efficacy of these crops (Yang *et al.*, 2007). A repertoire of resistance mechanisms has been identified in several lepidopteran species (Chapter 3). Several factors determine the genetic predisposition of a species for resistance evolution, including pest population dynamics (Gould, 1998), genetic mode of resistance and gene flow among different geographical populations (Wu & Guo, 2005). Sezonlin *et al.* (2012) determined that *B. fusca* populations in Cameroon have low genetic differentiation (i.e. high genetic similarity), which suggests that this pest is genetically predisposed to display similar behaviour and cause the same damaging effects on cereal crops throughout the country. Knowledge of the ecological and genetic diversity of insect pests will aid in development and improvement of monitoring and management strategies (Sezonlin *et al.*, 2006b). Essentially, the molecular basis of resistance, as well as development, growth and reproduction of insect pests needs to be understood and used in the development of insect resistance management (IRM) strategies to ensure sustainable crop production (Li *et al.*, 2012). Furthermore, non-molecular factors that may contribute to resistance (Chapter 3) should also be taken into account when designing IRM strategies.

All things considered, it is evident that detection, monitoring and management of *B. fusca* resistance will rely on elucidation of how certain factors contribute to resistance development in this pest. The present study therefore intended to investigate some molecular markers to determine whether other susceptible populations of *B. fusca* might independently procure the same mechanism of resistance as existing resistant populations. It is probable that this pest possesses certain genes or factors that only require activation in order to give rise to the resistant phenotype. In an endeavour to potentially elucidate a mechanism of resistance in *B. fusca*, molecular changes resulting from exposure to the Cry1Ab toxin were investigated using high-throughput sequencing technologies. Generation of this fundamental molecular data will be indispensable for developing management strategies for *B. fusca.*

1.2 Research aims and objectives

The aim of this research was to conduct a genetic study of South African *B. fusca* to determine the genetic diversity of this population and attempt to elucidate a potential mechanism of Cry1Ab toxin resistance through DE and comparative analyses.

The specific objectives were to:

(i) collect *B. fusca* larvae from different geographic regions across the maize production area of SA and sequence the cytochrome *c* oxidase I (*COI*) and cytochrome *b* (*cyt b*) mitochondrial genes for statistical and network analyses to determine the genetic diversity within this population;

(ii) review all Cry toxin resistance mechanisms that have been reported for lepidopteran pests to identify the most common mechanism to be investigated for *B. fusca*;

(iii) sequence, assemble and annotate the transcriptome of *B. fusca* to detect potential Cry toxin-receptor genes; and

(iv) challenge neonate *B. fusca* larvae (collected from non-*Bt* maize) with Cry1Ab toxin and perform Next-Generation Sequencing to determine if the potential Cry toxin-receptor genes are differentially expressed in the *Bt*-challenged group, which may indicate a probable mechanism of *Bt* resistance in *B. fusca.*

1.3 Outline of thesis chapters

Chapter 1 provides an introduction to the problem statement, followed by a brief overview of available published research regarding this study, as well as the identification of knowledge gaps. The importance of the study is stated, and the rationale and motivation, research aim and objectives, and outline of thesis chapters are provided. A comprehensive literature overview follows with a focus on the genetic diversity and Cry1Ab resistance of *B. fusca.* Mitochondrial genes and transcriptomics are explored to set the stage for subsequent chapters. An overview of GM crops and the HDR strategy is provided, followed by discussions of different aspects regarding Cry toxins. Cry toxin-binding proteins involved in resistance are then discussed, after which pest management strategies and the future of GM crops are considered. This chapter will serve as a framework for findings in this research.

Chapter 2 discusses the genetic diversity of South African *B. fusca* populations collected from maize. This chapter provides a data set that is novel for this insect and is in line with DNA barcoding approaches used for other species. The data set provides the basis for a DNA barcode database of *B. fusca* sequences. Genetic diversity of this species and the implications thereof are also discussed. This chapter was published as a short communication in the *African Entomology* journal. It is titled “Cytochrome *c* oxidase I and cytochrome *b* gene sequences indicate low genetic diversity in South African *Busseola fusca* (Lepidoptera: Noctuidae) from

maize". The chapter was extended to include results that could not be included in the published short communication due to length restrictions.

Chapter 3 is a mini review published in *Journal of Economic Entomology*, entitled "An overview of mechanisms of Cry toxin resistance in lepidopteran insects". This chapter provides a comprehensive overview of resistance mechanisms that have been reported for lepidopteran pests. The most common resistance mechanisms are identified and candidate genes involved in these mechanisms are investigated in *B. fusca* in the following chapter.

Chapter 4 is a manuscript that will be submitted for publication and is entitled "Transcriptome and differentially expressed genes of *Busseola fusca* larvae challenged with Cry1Ab toxin". Transcriptomic data generated in this study provides a novel resource base for *B. fusca* and allows speculation of the potential mechanism of Cry1Ab toxin resistance in this pest. The postulated hypothesis is that resistance might be controlled by a mitogen-activated protein kinase (MAPK) signalling pathway.

Chapter 5 draws on the conclusions from the individual chapters to summarize the overall novel insights of this study. Recommendations for future studies are also provided.

1.4 Literature overview

1.4.1 *Busseola fusca* resistance to Cry1Ab

Since 1998, *Bt* maize has been planted in SA to target, amongst others, the stem borer *B. fusca* (Gouse *et al.*, 2005; Kruger *et al.*, 2009). These plants produce the Cry1Ab *Bt* toxin to control *B. fusca* and other stem borers (Van Rensburg, 2007; Tabashnik *et al.*, 2009). However, in 2004 initial reports of resistance among this insect to *Bt* maize were made (Van den Berg, 2010). In the 2005-2006 growing season, more damage to *Bt* maize due to stem borer activity was reported, which then increased drastically in 2007-2008. The first official report of *B. fusca* resistance to Cry1Ab *Bt* maize (MON810) was from the Christiana and Vaalharts areas in SA during 2006 (Van Rensburg, 2007), eight years after commencement of *Bt* maize cultivation in that area. This necessitated the application of insecticides to control the stem borers feeding on *Bt* maize (Van Rensburg, 2007; Kruger *et al.*, 2009; Tabashnik *et al.*, 2009).

According to Kruger *et al.* (2009), initial refuge compliance was very low and many farmers in the Vaalharts area in SA did not allow any spatial separation between *Bt* maize fields and adjacent non-*Bt* maize (refugia). This enabled *B. fusca* larvae to move to adjacent plants approximately 14 days after egg hatch. Since *Bt* toxin concentrations can be influenced by various factors (discussed in Section 1.4.4), it is suggested that larger larvae may have been

exposed to sub-lethal doses of Cry1Ab toxin (Siegfried *et al.*, 2001). These larvae might have survived on *Bt* maize after they have developed on conventional refugia to the third or fourth instar, which ultimately contributed to resistance development (Kruger *et al.*, 2009). Interestingly, Kruger *et al.* (2011) demonstrated that larvae found in refugia were also resistant to *Bt* maize. This has led to the Vaalharts irrigation scheme and adjacent areas being regarded as a hot spot for field-developed resistance in *B. fusca* to *Bt* maize. Although resistant populations occur throughout the maize production area of SA, crop failure has only been reported on certain farms in specific geographical regions (Van den Berg *et al.*, 2013).

Evidence from field data suggested that Cry1Ab maize in SA does not kill 99.99% of *B. fusca* larvae (Van Rensburg, 2007). This is below the United States Environmental Protection Agency's (USEPA) standard for a high-dose event (USEPA, 1998). Furthermore, it was demonstrated that inheritance of resistance in *B. fusca* is non-recessive (Campagne *et al.*, 2013). The rapid development of resistance to Cry1Ab by *B. fusca* may therefore be attributed to non-recessive inheritance, failure to achieve the high-dose standard and inadequate refuges (Van Rensburg, 2007; Tabashnik, 2008). Several other ecological factors, such as larval feeding behaviour, migration behaviour, habitat preference and agronomic approaches, could also have promoted resistance development by causing increased selection pressure (Van den Berg *et al.*, 2013). Resistant *B. fusca* larvae have previously been found on non-*Bt* maize in refugia (Kruger *et al.*, 2011). Additionally, it was established that resistance in this pest was not associated with any fitness costs (Kruger *et al.*, 2014). These observations suggest that the efficacy of the HDR strategy to prevent, control and manage resistance development in *B. fusca* is compromised.

Despite the economic importance of controlling this pest, no study has yet been done to determine the Cry1Ab toxin mode of action or the mechanism of resistance in *B. fusca.* In order to hypothesize about the mechanism of resistance, the molecular changes that are associated with resistance should be determined (Gahan *et al.*, 2010). However, limited molecular data is available for *B. fusca*, which complicates any molecular studies on this species. Studies to determine the mechanisms by which resistance is procured are crucial and will allow for the development of an IRM plan. Many factors determine an insect's genetic predisposition to develop resistance. Some of these factors include pest population dynamics (Gould, 1998), genetic mode of resistance and gene flow among different geographical populations (Wu & Guo, 2005). Generally, mitochondrial genes are employed in population (and population migration) studies.

1.4.2 Mitochondrial genes

In order to study population genetics, reliable molecular markers are needed (Li *et al.*, 2013b). Several studies have employed mitochondrial genes, such as *COI* (DNA barcoding gene) and *cyt b*, to determine the evolutionary relationship of different species (Li *et al.*, 2005a; 2011) and genetic diversity within populations of a single species from different geographical regions (Assefa *et al.*, 2006c; Ong'amo *et al.*, 2008). However, there are shortcomings that have to be considered when the *cyt b* gene is used for phylogenetic analyses. These involve the rate of nucleotide substitutions in the conservative and variable domains (Farias *et al.*, 2001), as well as the recurrence of ancestral polymorphisms and multiple substitutions at single nucleotide sites (Simon *et al.*, 1994). Even so, these sequences have been used in various studies to demonstrate genetic relatedness (Farias *et al.*, 2001; Simmons & Weller, 2001; Sezonlin *et al.*, 2012). For example, Sezonlin *et al.* (2012) used *cyt b* sequences to clarify the genetic relationship between *B. fusca* populations from the Guineo-Congolian rain forest and Afromontane vegetation mosaics in Cameroon. Their study concluded that *B. fusca* populations in Cameroon recently colonized that region. This conclusion was based on low genetic differentiation observed within these populations according to a comparison of the *cyt b* mitochondrial gene. This low genetic diversity also suggests that this pest might exhibit similar damaging effects on cereal crops, like its African counterpart, if the same selection pressure is present.

A study done by Min and Hickey (2007) concluded that the average properties of genomes are significantly reflected by DNA barcodes (i.e. *COI* genes), making these short sequences of DNA usable when variations in species-specific sequences are being determined. Specifically, their results showed that the barcoding region accurately predicts the entire mitochondrial genome composition. The barcoding gene was utilized to determine the genetic diversity of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) populations from West, East and southern Africa (Assefa *et al.*, 2006c). Results from the latter study showed considerable genetic differentiation which correlated with behavioural and geographical variations. The *COI* gene also proved to be useful for distinguishing sister species that are morphologically very similar (Li et al., 2011).

It is suggested that knowledge of the ecological and genetic diversity of insect pests may aid in development and improvement of monitoring and control strategies (Sezonlin et al., 2006b). Although sequence data of molecular markers has accumulated rapidly over the past years (Patwardhan *et al.*, 2014), limited population genetic and phylogeography data are available for *B. fusca* (Sezonlin *et al.*, 2006b). Molecular data for this insect are mostly limited to some COI (Assefa *et al.*, 2006b; Toussaint *et al.*, 2012; Assefa *et al.*, 2015; Le Rü *et al.*, 2015; Peterson *et*

al., 2016), cyt b (Sezonlin *et al.*, 2006a; 2006b; 2012; Peterson *et al.*, 2016) and microsatellite data (Faure & Silvain, 2005; Faure, 2006).

By utilizing the mitochondrial *cyt b* gene, Sezonlin *et al.* (2006a) found that three clades of *B. fusca* exist, and that these could be ascribed to geographic differences and ecological preferences. These clades included a homogeneous and geographically isolated population from West Africa (*W*), and populations from Central, South and East Africa (*KI* and *KII*) with overlapping distributions. Additionally, it was elucidated that the genetic differentiation of *B. fusca* and other herbivorous vertebrate populations was subjective to biogeographic barriers, as well as climatic and geological processes (Sezonlin *et al.*, 2006a; 2006b). In view of the results of studies conducted by Sezonlin and colleagues, it was noted that South African *B. fusca* larvae were only collected from a few localities. To address this issue, larvae were collected from different geographic regions across the maize production area of SA, after which the *COI* and *cyt b* mitochondrial genes were amplified and sequenced for statistical and network analyses (Chapter 2). The latter analyses investigated the genetic diversity of these populations. Additionally, phylogenetic analysis was performed to validate the clade segregation observed by Sezonlin *et al.* (2006a).

1.4.3 Transcriptomics

Molecular information made available by transcriptome sequencing provides direct information about functional and protein coding RNAs (Wang *et al.*, 2009). This data can be employed in several studies, including gene mapping (Wang *et al.*, 2009), gene expression level quantification (Shelby & Popham, 2012; Li *et al.*, 2013b) and gene targeting for insect pest control (Wang *et al.*, 2011; Zhao *et al.*, 2013). Some advantages of transcriptomic analyses include the capacity to resolve single-nucleotide polymorphisms (i.e. SNP discovery) (Schlötterer *et al.*, 2014), quantify gene expression levels (Marioni *et al.*, 2008), distinguish rare or alternatively spliced transcripts (Hiller *et al.*, 2009) and analyse transcriptional immune response to specific compounds, such as toxins (Crava *et al.*, 2015). With regard to insect resistance, several studies have been particularly interested in the differences in gene expression in different strains (Tiewsiri & Wang, 2011; Vellichirammal *et al.*, 2015). The latter studies reported that resistance correlated with differences in gene expression.

It has been determined that several insect species developed resistance to Cry toxins due to differential gene expression or mutations in certain genes (Kumar & Kumari, 2015). Transcriptomics provide the tools to detect these occurrences and thus aid in elucidation of such resistance mechanisms as well as resistance detection and monitoring. Furthermore, it aids in developing tailored resistance monitoring and management approaches for target pests.

In the interest of managing *B. fusca* resistance evolution, all Cry toxin resistance mechanisms that have been reported for lepidopteran pests were investigated (Chapter 3). Another objective of this research involved the elucidation and assessment of its transcriptome to elucidate a potential mechanism of Cry1Ab toxin resistance (Chapter 4). Comprehensively, results from this research would provide key insights into *B. fusca* resistance, which can be employed in crop enhancement and improvement of resistance management strategies.

1.4.4 Overview of GM crops and the HDR strategy

GM crops have been altered with genes that confer certain properties, such as insecticidal properties, herbicide- or drought-tolerance, which make these crops extremely important in agriculture (Yang *et al.*, 2007). These transgenic crops were first commercialized in 1996 with only 6 countries growing these crops then (James, 2010). This number increased to 28 countries in 2015, of which 20 were developing and 8 industrial countries (James, 2015a). Increase in the global hectarage of these crops was over 100-fold, from 1.7 million hectares in 1996 to 179.7 million hectares in 2015 (James, 2015a). This demonstrates that GM technology is rapidly adopted where it has been introduced.

SA was the first country in Africa to produce transgenic crops (*Bt* cotton) commercially in 1997 (Gouse *et al.*, 2005; Van Wyk *et al.*, 2008). In 2015, a total of 2.3 million hectares was been planted to GM maize, soybean and cotton, ranking SA as the ninth biggest producer of transgenic crops in the world (James, 2015a). Burkina Faso and Sudan are now also producing transgenic crops (*Bt* cotton) commercially, while Cameroon, Egypt, Ghana, Kenya, Malawi, Nigeria, Swaziland and Uganda are conducting field trials (James, 2015a).

When GM crops were introduced into main stream agriculture, USEPA mandated a resistance management plan (Gould, 2000). This HDR strategy required farmers to plant refuges (conventional non-*Bt* cultivars) when transgenic *Bt* crops are grown (Gahan *et al.*, 2007). The principle of this strategy is that nearly all individuals of the target pest will be killed by the high dose of toxin expressed by the transgenic crop, while many individuals will survive on the refugia (Gould, 2000; Tabashnik *et al.*, 2003; Van Rensburg, 2007). Individuals that are resistant to the *Bt* crop will ultimately mate with susceptible individuals that survived on the refugia (Gould, 2000). If inheritance of resistance is recessive (Tabashnik *et al.*, 2013), the progeny will have lower resistance to the transgenic crops (Kruger *et al.*, 2009) and thus not be able to survive on the transgenic crops with the high dosage of toxin (Gould, 2000). In this manner, resistance alleles are diluted and development of resistant populations will be inhibited, or at least delayed. Theoretically this strategy will be effective only if inheritance of resistance is recessive, initial allele frequency of resistance is low, and ample refuges are planted along with

Bt crops expressing high doses of toxin (Gould, 1998; 2000). The success of this strategy will furthermore be enhanced if resistance is incomplete and associated with fitness costs (Carriére & Tabashnik, 2001; Tabashnik *et al.*, 2003).

Although this HDR strategy seems realistic in theory, it is undermined by several factors. These include variable toxin production in different plant parts (Siegfried *et al.*, 2001) and under different climatic conditions (Trtikova *et al.*, 2015). Similarly, different crop varieties express different amounts of toxin (Then & Lorch, 2007) and toxin concentrations decrease as plants age (Dong & Li, 2007). Toxin production is also influenced by soil moisture, nutrients, herbivory and topography (Onstad, 2013). These intermediate toxin levels may allow heterozygotes to survive, thus increasing the functional dominance of resistance (Gould, 1998; Chilcutt & Tabashnik, 2004).

Some pests, including *B. fusca*, have the ability to move to adjacent plants (Kruger et al., 2009), thus exposing larger larvae (that developed on susceptible non-*Bt* plants) to sub-lethal doses of toxin (Roush, 1997). Pollen-mediated gene flow from transgenic crops to refuge plants is another weakness in the HDR strategy. This was observed in the U.S. where DNA sequences from transgenic maize, soybean and canola were found in the seed supply of the same, respective conventional crops (Chilcutt & Tabashnik, 2004; Mellon & Rissler, 2004). As a result, refuges produce toxins, thus killing and reducing the number of susceptible individuals. All these problems may reduce the benefits and success of GM crops.

1.4.5 Cry toxins as biopesticides

B. thuringiensis produces parasporal insecticidal crystals, namely *Bt* toxins (Schnepf *et al.*, 1998). There are a number of different protoxins, of which Cry proteins are one type. Biopesticides were developed using these toxins, and later agricultural biotechnology genetically transformed crop plants (*Bt* crops) with modified *cry* genes to express these insecticidal toxins (Tabashnik, 2008; Zhang *et al.*, 2009). Insects in the orders Coleoptera (beetles and weevils), Diptera (flies and mosquitoes), Hymenoptera (wasps and bees), Lepidoptera (butterflies and moths) and nematodes are primarily targeted by these Cry toxins (Gómez *et al.*, 2007; Zúñiga-Navarrete *et al.*, 2012). *Bt* crops thus proved to be an effective control strategy for pests while also providing commercial advantages and environmentally friendly alternatives to conventional insecticides (Morin *et al.*, 2003; Bravo *et al.*, 2007). While these toxins are expected to be innocuous to most other organisms (humans, non-target pests, vertebrates and plants) (Luo *et al.*, 2006; Bravo *et al.*, 2007), viewpoints on the safety of GM crops are still controversial (Adenle, 2011). In order to perform meaningful safety assessments

and promote effective use of GM crops, the diversity, structure and function of these Cry toxins need to be considered.

1.4.6 Cry toxin diversity, structure and function

Cry toxins are classified into 74 types (Cry1 - Cry74) and many sub-types (e.g. Cry1Aa, Cry1Ab or Cry1Ba) according to their primary sequence similarity (Bravo & Soberón, 2008; Crickmore *et al.*, 2014). Based on amino acid sequences and insecticidal activity, the most commonly used Cry toxins comprise five groups: (i) Cry1, toxic to Lepidoptera only; (ii) Cry2, toxic to Lepidoptera and Diptera; (iii) Cry3, toxic to Coleoptera only; (iv) Cry4, toxic to Diptera only; and (v) Cry5, toxic to Lepidoptera and Coleoptera (Crickmore *et al.*, 1998). A single group of Cry toxins can thus target species in more than one phylogenetic order. An exception to these toxin classes is Cry1B, which showed high sequence similarity to Cry3 toxins and was also toxic to two coleopteran pests (Bradley *et al.*, 1995). However, this occurrence could have been due to certain factors, such as host midgut pH or proteases, which influenced the toxin's effectiveness and specificity.

Even though Cry toxins differ considerably in their amino acid sequences and insect specificity, highly similar three domain structures are present in all these toxins (Pigott & Ellar, 2007). Genetic and electrophysiological studies illustrated that domain I is involved in toxin oligomerization, membrane insertion and pore formation (Zúñiga-Navarrete *et al.*, 2012). On the other hand, domain II is mainly involved in receptor recognition and -binding (Karim & Dean, 2000; Zúñiga-Navarrete *et al.*, 2012), whereas domain III has a role in structural integrity (Masson *et al.*, 2002), ion conductance (Wolfersberger *et al.*, 1996), toxin activity (Wolfersberger *et al.*, 1996) and receptor binding (De Maagd *et al.*, 1996). This high degree of structural conservation suggests that they possess a fundamental mechanism of action (Bravo *et al.*, 2007). Cry toxins are secreted as water-soluble proteins that undergo conformational changes to facilitate insertion into, or translocation across, cell membranes of their host (Bravo *et al.*, 2007). These Cry toxins induce changes in the physiological status of the intestines of larvae (Vázquez-Padrón *et al.*, 2000; Xu *et al.*, 2009), which results in the death of these insects. The mode of action of Cry toxins is, however, very complex and is thus discussed in detail in the following section.

1.4.7 Cry1A toxin mode of action

Initially, a two-phase mechanism of Cry toxin action was proposed, namely (i) crystal solubilization and proteolytic activation of protoxins in the midgut; and (ii) toxin-receptor-binding, toxin insertion and pore formation (Schnepf *et al.*, 1998). Later, Pigott and Ellar (2007) proposed

three contrasting models of Cry1A toxin mode of action (Figure 1.1), namely the Bravo, Zhang and Jurat-Fuentes models. The initial steps of all the models are identical. According to these models, a crystalline protein contains the toxin (Heckel *et al.*, 2007). When susceptible insect larvae ingest these Cry toxins, the protein crystal is solubilized in the lumen of the midgut and the protoxin is released (Karim & Dean, 2000). GM plants do not produce these crystalline proteins, but rather produce preactivated toxins that still need further proteolytic activation (Van der Hoeven, 2014). Host digestive proteases then cleave several amino acid residues from the C-terminus of the protoxin (Schnepf *et al.*, 1998) to give rise to an active protease-resistant toxin (Heckel *et al.*, 2007). The monomeric toxin is then translocated through the peritrophic matrix to the brush border membrane (Krishnamoorthy *et al.*, 2007) where protein-receptors on the surface of the midgut epithelial cells bind this activated toxin monomer (Bravo *et al.*, 2004; Soberón *et al.*, 2009) (Figure 1.1).

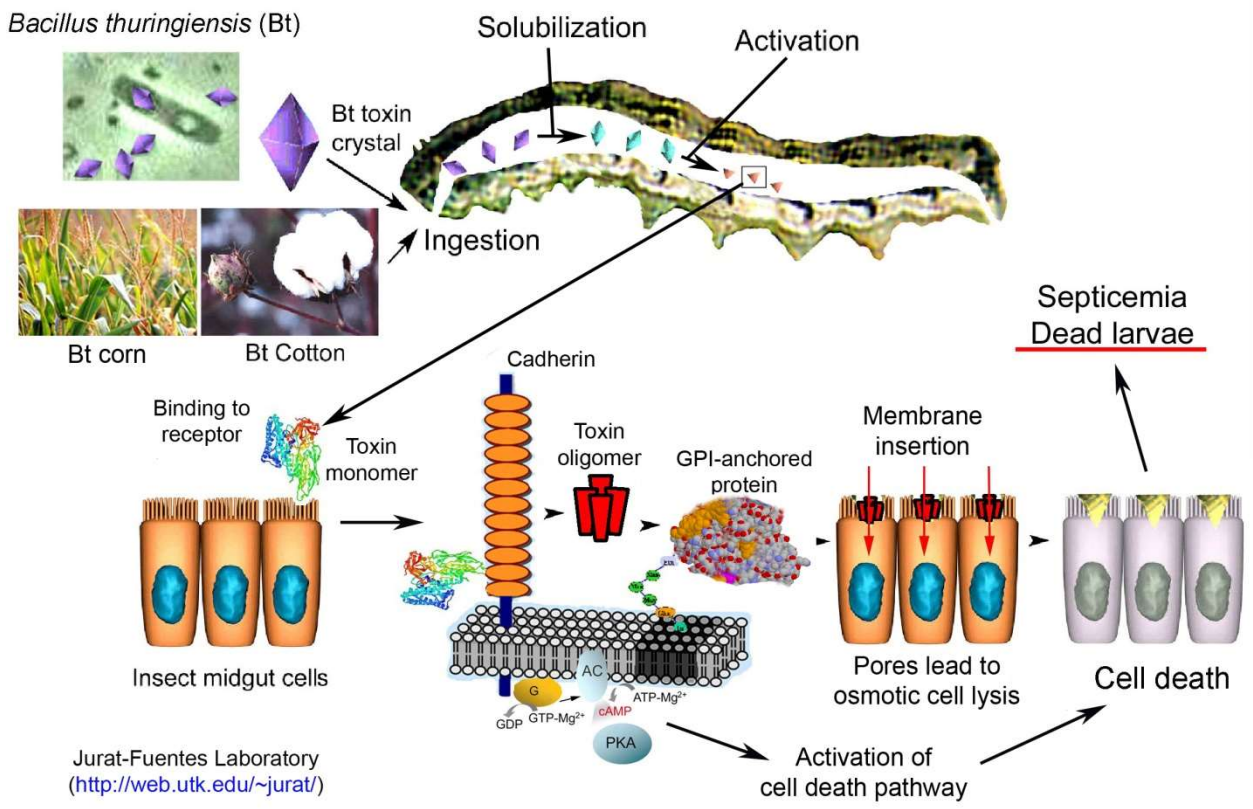


Figure 1.1: Schematic outline that illustrates the Bravo, Zhang and Jurat-Fuentes models of Cry1A toxin mode of action in susceptible larvae (Jurat-Fuentes, 2010). G: guanine nucleotide-binding protein; AC: adenylyl cyclase; cAMP: cyclic AMP; PKA: protein kinase A.

1.4.7.1 Bravo (sequential binding) model

The Bravo model proposes that sequential toxin-receptor-binding occurs. Initially it was said that toxin monomers first bind to the cadherin (CDH) receptor (Bravo *et al.*, 2004). However, a modification to this model was proposed where toxin monomers first bind to abundant glycosylphosphatidylinositol (GPI)-anchored receptors, such as alkaline phosphatase (ALP) and aminopeptidase N (APN). This binding occurs with low affinity to localize the toxin monomers in the brush border microvilli. Subsequently, binding of toxins with high affinity to the less abundant CDH receptors occurs (Pacheco *et al.*, 2009a). Toxin-receptor-binding then induces additional proteolytic activation where helix α-1 (Domain 1) from the N-terminus is cleaved to facilitate the formation of pre-pore oligomer structures that is insertion-competent (Gómez *et al.*, 2002; Bravo *et al.*, 2007). The oligomeric Cry toxin subsequently binds to secondary GPI-anchored receptors, such as APN or ALP, with high affinity (Khajuria *et al.*, 2011; Upadhyay & Singh, 2011; Pardo-López *et al.*, 2013) and are then irreversibly inserted into the bilayer lipid membrane to form pores (Heckel *et al.*, 2007; Pigott & Ellar, 2007). It was demonstrated that only a specific region of domain I is inserted into the membrane, while domain II and III remain exposed on the surface (Zavala *et al.*, 2011). It has been suggested that there is an additional binding step in the Cry toxin mode of action, which entails binding of Cry toxins to ATP-binding cassette (ABC) transporter proteins (Khajuria *et al.*, 2011). According to Heckel (2015), this step is hypothetically critical for Cry pore formation.

Formation of pores subsequently disrupts the membrane integrity (Gill & Ellar, 2002; Heckel *et al.*, 2007). These pores also interchange between an open and closed state, which is influenced by environmental conditions such as pH (Van der Hoeven, 2014). This causes changes in the membrane potential leading to equilibration of ions across the membrane, influx of water, cell swelling and lysis of the midgut epithelial cells (Schnepf *et al.*, 1998). Insect mortality thus results due to starvation or septicemia (Graf, 2011). This lytic pore-formation model has, however, been challenged by the Zhang (signalling pathway) model and involvement of midgut bacteria in Cry toxicity (Broderick *et al.*, 2006; Zhang *et al.*, 2006; Pigott & Ellar, 2007). Also, Vachon *et al.* (2012) reported that toxin monomers can insert into the membrane and thus only assemble as functional oligomeric pores after membrane insertion. Contrarily, several studies argue that oligomer formation is needed to provide for monomer insertion into membranes (Rausell *et al.*, 2004; Muñoz-Garay *et al.*, 2006; Bravo *et al.*, 2007). It is evident that, among other things, the pore structure and pore-formation/assembly mechanism into the membrane still need to be fully elucidated.

1.4.7.2 Zhang (signalling pathway) model

The Zhang model proposes an alternative mode of action where cell death (apoptosis) is promoted by a Mg^{2+}-dependent signalling cascade (Zhang *et al.*, 2006). This is supposedly induced when monomeric Cry toxins bind to CDH receptors that are located on midgut epithelial cells (Chen *et al.*, 2005) (Figure 1.1). A guanine nucleotide-binding protein (G protein) is activated, followed by the activation of adenylyl cyclase (AC) which promotes cyclic AMP (cAMP) production (Soberón *et al.*, 2009). This increase in cAMP leads to the activation of protein kinase A (PKA), which elicits apoptosis by activating an intracellular pathway (Zhang *et al.*, 2006). Additionally it was suggested that the cytoskeleton and ion channels are destabilized when G protein and AC causes cAMP levels to increase, thus weakening the cell membrane (Zhang *et al.*, 2006). However, Knowles and Farndale (1988) argued that this increase was due to a secondary effect of the toxin's interaction with the membrane. Nonetheless, this model ignores the involvement of secondary receptors and states that apoptosis is not attributable to pore formation (Zhang *et al.*, 2005; 2006).

According to Fabrick and Wu (2015), no data regarding the direct involvement of signal transduction in cell death has yet been published. Furthermore, several studies suggest that such an *in vivo* intracellular response may be elicited by oligomerization, secondary toxin-receptor-binding (i.e. binding to GPI-anchored receptors) or pore formation, rather than when toxin monomers bind to CDH (Zhuang *et al.*, 2002; Gómez *et al.*, 2007; Zúñiga-Navarrete *et al.*, 2012). This is plausible, since receptors cluster during toxin oligomerization and GPI-anchored receptors are located in lipid rafts involved in signal transduction (Schroeder *et al.*, 1998). Nonetheless, the Zhang model is considerably challenged by various experimental approaches that demonstrate pore formation unambiguously and repeatedly (see Pardo-López *et al.,* 2013 for a review). Moreover, several studies demonstrated that modified toxins are able to kill resistant insects without interacting with CDH (Soberón *et al.*, 2007; Franklin *et al.*, 2009; Tabashnik *et al.*, 2011). Even so, earlier studies (Monette *et al.*, 1997; Potvin *et al.*, 1998) support the intracellular signalling model, but these studies have been largely ignored. More recently, studies observed cellular defence mechanisms which implicate a variety of intracellular pathways against Cry toxins (Cancino-Rodezno *et al.*, 2010; Tanaka *et al.*, 2012; Guo *et al.*, 2015a). Also, a mutation in the cytoplasmic domain of CDH, which is important in the intracellular pathway of the cell signalling model, confers non-recessive resistance of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) (Zhang *et al.*, 2012c), thus supporting the Zhang model. Undoubtedly, more studies are needed to support this model's assumptions.

1.4.7.3 Jurat-Fuentes model

The Jurat-Fuentes model is a combination of the Bravo and Zhang models and proposes that the combined effect of osmotic lysis caused by toxin pore formation and cell signalling leads to cytotoxicity (Pigott & Ellar, 2007). This model suggests that an intracellular pathway is activated after active monomeric Cry toxins have bound to receptors (Zhang *et al.*, 2006; Heckel *et al.*, 2007; Bravo & Soberón, 2008). Toxin oligomerization then proceeds followed by binding to GPI-anchored APN and ALP receptors (Jurat-Fuentes & Adang, 2004). This pathway is apparently regulated by phosphatases (Pigott & Ellar, 2007), which is supported by studies that found differential phosphatase levels in susceptible and resistant insect strains (Jurat-Fuentes & Adang, 2004; 2007; Jurat-Fuentes *et al.*, 2011; Yang *et al.*, 2012). Toxin-receptor-binding leads to toxin insertion and pore formation, which again activates pathways that result in cell death (Pigott & Ellar, 2007). A recent study from Guo *et al.* (2015a) linked the downregulation of certain Cry toxin-receptor genes with a MAPK signalling pathway in a resistant strain of *Plutella xylostella* L. (Lepidoptera: Plutellidae). These authors proposed the following coordinated response model: MAPK activation upregulates genes involved in epithelial healing, whilst downregulating Cry toxin-receptor genes. Nevertheless, further studies are needed to support these findings.

1.4.7.4 Involvement of midgut bacteria in Cry toxicity

Starvation was the assumed mechanism of insect killing for many years, until a study by Broderick *et al.* (2006) showed that larvae of *Lymantria dispar* L. (Lepidoptera: Erebidae) are not killed by *Bt* toxins in the absence of indigenous midgut bacteria. In that study, *Bt* insecticidal activity was abolished when the gut microbial community was eliminated by antibiotics. *Bt*-mediated killing was restored after the midgut microbial community was re-established. This theory is highly controversial and several studies have been done to prove and disprove it (Broderick *et al.*, 2009; Johnston & Crickmore, 2009; Raymond *et al.*, 2009; Paramasiva *et al.*, 2014; Caccia *et al.*, 2016). According to Broderick *et al.* (2006), *Enterobacter* sp. seemed to be mostly responsible for causing septicemia in *L. dispar* larvae when *Bt* toxins were ingested by these larvae. According to this septicemia model, mortality is not induced by the enteric bacteria alone. After the *Bt* toxins permeabilize the gut epithelium, the bacteria and spores are able to reach the hemocoel (Broderick *et al.*, 2006). Then, in the more favourable environment, the spores germinate and reproduce. The vegetative cells cause septicemia and this leads to insect mortality (Schnepf *et al.*, 1998; Broderick *et al.*, 2006).

This alternative mechanism of killing has been proposed due to inconsistent experimental observations found with the starvation model, where it takes larvae 7-10 days to die from

starvation, compared to only 2-5 days when *Bt* toxins are consumed. The septicemia model has also been challenged when mortality of larvae was still induced by the toxin in the absence of bacterial cells (Schnepf *et al.*, 1998; Broderick *et al.*, 2006). Broderick *et al.* (2009) concluded that *Bt*-induced mortality due to contributions of gut bacteria vary across a range of Lepidoptera. Paramasiva *et al.* (2014) also reported that the microbiota diversity not only differs in different insect species, but also varies according to the preferred host plants and geographical regions where these insects occur and feed.

Although various models are used to describe the mode of action of Cry toxins, they do not describe the collective response and behaviour to the presence of the toxin, which can vary between species and be influenced by geospatial feeding factors. Therefore, studies to determine the mechanism of cascading pathways that are activated during toxin exposure, may be helpful in elucidation of resistance mechanisms in insect pests.

1.4.8 Cry toxin-binding site models in Lepidoptera

The mechanism through which the toxins bind to the receptors in brush border membranes is not yet fully understood. Hence, different toxin binding models have been proposed, some of which were recently reviewed by Jakka *et al.* (2015). These authors observed that a shared binding site for Cry1A toxins have been identified in many species, except *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae). It seems that Cry1B and Cry1C toxins do not share binding sites with Cry1A toxins, except in *Spodoptera* spp. Likewise, Cry1E, Cry2 and Cry9 toxins do not share binding sites with Cry1A toxins. Thus, more elaborate analyses of toxin binding sites will aid the understanding of resistance mechanisms associated with altered receptors.

There have been some cases where a resistance allele resulted in resistance to more than one Cry1A toxin, although binding of some of the toxins to one or more receptors still occurred, but incorrectly (Griffitts & Aroian, 2005). This incorrect binding does not result in the conformational changes that usually occur prior to pore-formation. Thus the ability of the toxin to recognize and bind to receptors on the membrane is different from its ability to insert into the membrane and form functional pores (Griffitts & Aroian, 2005). It has been suggested that not all binding sites are equally effective in mediating toxin function (Lee *et al.*, 1995; Luo *et al.*, 1997a). An alternative co-receptor model has been proposed, wherein both receptor and co-receptor are required for toxicity, otherwise incorrect pore formation will occur if either counterpart is lost (Lee *et al.*, 1995; Luo *et al.*, 1997a).

It has been shown by Smedley *et al.* (1997) that activated Cry toxins, in the absence of protein-receptors, can insert and form pores in bilayer lipid membranes. Similarly, several studies have

demonstrated that modified toxins lacking helix α-1 (i.e. CryMod toxins) were able to form oligomers, whether the CHD receptor was present or not (Soberón *et al.*, 2007; Franklin *et al.*, 2009; Tabashnik *et al.*, 2011). CryMod toxins were also reported to be insecticidal against *Diatraea saccharalis* F. (Lepidoptera: Crambidae)*, H. armigera, Manduca sexta* L. (Lepidoptera: Sphingidae)*, Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae)*, Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) and *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) strains with several different mechanisms of resistance to Cry toxins. Further detailed descriptions of these resistance mechanisms are provided in Chapter 3. All these observations thus support the co-receptor model, wherein CHD is not the only receptor involved in cytotoxicity (Soberón *et al.*, 2009).

An understanding of toxin-receptor-binding and toxin mechanisms of action is essential for pest resistance management and subsequent sustainable use of GM crops. Although several models exist to describe these concepts, none of these describe the events observed in all sensitive species. Specific experimental data are required to explain the effects of Cry toxins in a specific target pest, thus demanding development of Cry toxin binding models in a case-by-case manner. Even though these models do not provide information regarding resistance mechanisms unrelated to binding site alterations (Ballester *et al.*, 1999), they demonstrate the association of specific receptors involved in toxin-receptor binding. Such information is valuable in the development of pest management strategies and in the long run, will specifically prevent selection of toxin combinations that might promote cross-resistance (Jurat-Fuentes & Adang, 2001). It is thus important to identify these Cry toxin-binding molecules in order to investigate the toxin-binding site interactions, and consequently, the molecular mechanisms of insect resistance to Cry toxins.

1.4.9 Cry toxin-binding proteins

CDH, ALP and APN are the main Cry1A toxin-binding proteins that have been described for lepidopteran insects (Pigott & Ellar, 2007). The CDH receptor is a transmembrane protein in the brush border membrane (Bravo *et al.*, 2007), whereas APN and ALP are GPI-anchored glycosylated proteins that have been identified in lipid rafts associated with the epithelial membrane in insect midguts (Gahan *et al.*, 2010).

1.4.9.1 Cadherin (CDH)

CDHs are a large family of glycoproteins and have been identified as *Bt* toxin-binding proteins in midguts of several lepidopteran insects: *P. gossypiella* (Fabrick & Tabashnik, 2007); *O. nubilalis* (Flannagan *et al.*, 2005); *Heliothis virescens* F. (Lepidoptera: Noctuidae) (Gahan *et al.*, 2001);

Bombyx mori L. (Lepidoptera: Bombycidae) (Hara *et al.*, 2003); *M. sexta* (Hua *et al.*, 2004); *Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae) (Jin *et al.*, 2014); *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) (Ren *et al.*, 2013); *P. xylostella* (Yang *et al.*, 2012); *H. armigera* (Zhang *et al.*, 2012b). CDH is a transmembrane protein with signal peptide, intracellular (cytoplasmic), transmembrane and extracellular domains in which several cadherin repeats occur (Bel & Escriche, 2006). It has been observed that intron-exon patterns and intron insertion positions are highly conserved in *cdh* genes (Bel & Escriche, 2006). Bel and Escriche (2006) suggested that all the *Bt* related CDHs are from a single origin, although this has not yet been proven. CDH proteins are involved in several cellular processes such as recognition, signalling and communication between cells, morphogenesis and maintenance of cell structure (Angst *et al.*, 2001). All these functions support the Zhang (signalling pathway) model for Cry1A toxin mode of action (Section 1.4.7.2). For lepidopteran species, the physiological functions of CDH proteins are, however, not yet clear (Bel & Escriche, 2006). According to Bel and Escriche (2006), recognition between CHDs and Cry1A toxins is highly specific only in Lepidoptera.

1.4.9.2 Alkaline phosphatase (ALP)

ALP has been identified as a *Bt* toxin-binding protein in midguts of several lepidopteran insects: *H. virescens* (Jurat-Fuentes & Adang, 2004); *M. sexta* (McNall & Adang, 2003); *H. armigera* (Upadhyay & Singh, 2011) and *P. xylostella* (Yang *et al.*, 2012). It is a secondary receptor that is also GPI-anchored (Jurat-Fuentes & Adang, 2006; Bravo *et al.*, 2007), glycosylated and enriched in lipid rafts (Arenas *et al.*, 2010; Gahan *et al.*, 2010). Multiple ALP isoforms exist, and some protein regions (GFFLFVEGGR) are conserved among insect membrane-bound ALPs (Perera *et al.*, 2009). It has been proposed that these ALPs are involved in metabolite absorption, transportation, cell adhesion and differentiation (Chang *et al.*, 1993; Eguchi, 1995). This family of phosphatases are also known to activate intracellular pathways via lipid rafts in response to extracellular stimuli (Eyster, 2007), which supports the Zhang model for Cry1A toxin mode of action (Section 1.4.7.2). ALP also contains GalNAc moieties necessary for binding of Cry1Ac toxins, and reduced levels of ALP were correlated with Cry1Ac resistance in several lepidopteran species. Further detailed descriptions of these resistance mechanisms are discussed in Chapter 3. Alkaline phosphatase thus has a functional role in Cry toxin action.

1.4.9.3 Aminopeptidase N (APN)

APN is an exopeptidase (Banks *et al.*, 2001) that has been identified as a *Bt* toxin-binding protein in midguts of several lepidopteran insects: *H. virescens* (Banks *et al.*, 2001); *O. nubilalis* (Crava *et al.*, 2010); *S. exigua* (Herrero *et al.*, 2005); *M. sexta* (Knight *et al.*, 1995); *L. dispar*

(Lee *et al.*, 1996); *P. xylostella* and *B. mori* (Nakanishi *et al.*, 2002); *Achaea janata* L. (Lepidoptera: Erebidae) (Ningshen *et al.*, 2013); *Spodoptera litura* F. (Lepidoptera: Noctuidae) (Rajagopal *et al.*, 2002); *Epiphyas postvittana* Walker (Lepidoptera: Tortricidae) (Simpson & Newcomb, 2000); *T. ni* (Tiewsiri & Wang, 2011); *D.saccharalis* (Yang *et al.*, 2010); *H. armigera* (Zhang *et al.*, 2009) and *Plodia interpunctella* Guenée (Lepidoptera: Pyralidae) (Zhu *et al.*, 2000). Common motifs have been identified in homologous positions in APN proteins of lepidopteran species by *in vitro* and *in silico* analyses. These include a signal peptide (N-terminus), GPI-anchor sequence (C-terminus), zinc-binding motif $HEXXH(X)_{18}E$ (Hooper, 1994) and the GAMENWG gluzincin aminopeptidase sequence (Banks *et al.*, 2003), which are essential for their enzymatic activity. Previously it was demonstrated by Lee *et al.* (1996) that all APNs contain N-acetylgalactosamine (GalNAc) residues to which Cry1Ac toxins bound.

1.4.9.4 ATP-binding cassette (ABC) transporters

ABC transporters are proteins integrated with the membrane and play a role in the export of toxic molecules from the cell (Heckel, 2012). ABC transporters cycle between closed and open configurations during transportation of molecules, and involve interactions of the oligomeric toxin pre-pore structure in the final binding step (Gahan *et al.*, 2010). This binding supposedly facilitates membrane insertion. Several studies recently demonstrated that mutations in or downregulation of the genes encoding ABC transporter proteins were linked to Cry toxin resistance in lepidopteran species. Further detailed descriptions of these resistance mechanisms are discussed in Chapter 3. A recent study from Tanaka *et al.* (2013) demonstrated that this protein is indeed a functional receptor for Cry1A, Cry1Fa and Cry8Ca toxins.

1.4.9.5 Other Cry toxin-binding proteins

Other Cry toxin receptors that have also been reported in lepidopteran insects include glycolipids (*M. sexta*: Griffitts *et al.,* 2005), vacuolar-ATP synthase subunits (*H. armigera*: Chen *et al.,* 2010; *H. virescens*: Krishnamoorthy *et al.,* 2007), heat shock proteins (*H. armigera*: Chen *et al.,* 2010), actin (*H. armigera*: Chen *et al.,* 2010; *H. virescens*: Krishnamoorthy *et al.,* 2007; *M. sexta*: McNall & Adang 2003), glycoconjugate (namely BTR-270) (*L. dispar:* Valaitis *et al.,* 2001) and P252 protein (*B. mori:* Hossain *et al.,* 2004). Griffitts *et al.* (2005) suggested that glycolipid receptors possibly modulate Cry toxin activity because of the conservation of both structures, and are therefore functional to the *in vivo* Cry toxin function. Then again, BTR-270 and P252 are glycosylated GPI-anchored glycoproteins (Pandian *et al.*, 2008) that are enriched in lipid

rafts associated with the membrane, and have also been identified as Cry toxin receptors (Pigott & Ellar, 2007).

More recently, a metalloprotease glycoprotein with a predicted GPI-anchor signal peptide, namely aminopeptidase P (APP), was identified in *O. nubilalis* (Khajuria *et al.*, 2011). This gene has not yet been identified in any other lepidopteran species. In mammals, this gene plays a role in protein turnover of collagen and peptide hydrolysis (Cunningham & O'Connor, 1997). The physiological role of APP in insects is, however, still unclear. It has been suggested that this gene has a possible role in development and might also be part of the intracellular signal processing system in insects, like *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Kulkarni & Deobagkar, 2002). Since this gene is poorly characterized, further studies are needed to establish whether it plays a role in *Bt* toxicity or resistance.

Griffitts *et al.* (2005) proposed that glycolipid and protein receptors are sequentially or simultaneously involved in toxin positioning or insertion. It is not yet clear whether CDHs, APNs, ALPs, ABC transporters, glycolipids or an unknown receptor mediates specificity for these Cry toxins. Nonetheless, even though toxins may bind to any (or several) of these receptors, it does not necessarily implicate that these receptors have a functional insecticidal role (Xu & Wu, 2008). Toxin binding also does not necessarily implicate that an organism is susceptible (Banks *et al.*, 2003).

In order to investigate the mode of toxin action and molecular mechanisms of insect resistance to Cry toxins, it is crucial to identify toxin-binding molecules that mediate toxicity in insects. Jurat-Fuentes and Adang (2006) suggested that by comparing midgut epithelium proteins from susceptible larvae to those from resistant larvae, one can reveal receptor alterations involved in resistance. The latter approach was exploited in the present study (Chapter 4). By identifying receptor alterations, different binding proteins can be targeted by novel or multiple toxins in order to maintain pest susceptibility (Peferoen, 1997; Gómez *et al.*, 2007). This will aid in developing management strategies to prevent or delay resistance development. However, resistance may arise via mechanisms other than receptor alterations (these resistance mechanisms are discussed in detail in Chapter 3) and therefore IRM strategies are required to delay and manage resistance manifestations.

1.4.10 Pest management and the future of GM crops

Long-term, sustainable transgenic crop use relies on understanding the mode of Cry toxin action and mechanisms of resistance (Banks *et al.*, 2003). Consequently, Cry toxin-binding proteins involved in *Bt* toxin interactions need to be identified. An understanding of the

biochemical and genetic basis of resistance is crucial, but will be facilitated by the availability of sequence data (Candas *et al.*, 2002). A DNA-based method of detecting resistant alleles directly would be more efficient than conventional bioassay-based monitoring methods (Xu *et al.*, 2005; Zhang *et al.*, 2009), especially when resistance is rare and recessive. The ideal method would apply the development of resistance biomarkers that could efficiently differentiate between susceptible and tolerant/resistant insects, regardless of the resistance mechanism, mode of inheritance, *Bt* crop or Cry toxin being expressed (Jurat-Fuentes *et al.*, 2011). Such biomarkers can then be used for resistance/susceptibility monitoring to facilitate early detection of field evolved resistance.

Monitoring of resistance plays a key role in its management (Wu, 2007; Zhang *et al.*, 2009) – see Tabashnik *et al.* (2009) and Wu (2014) for reviews of resistance detection and monitoring methods. Andow and Hilbeck (2004) emphasized the importance of performing pre-release risk assessments, which is useful in proactive resistance management (i.e. preventing damage before it occurs). It is crucial to consider that susceptible populations may develop resistance mechanisms similar to their resistant counterparts if the same selection pressure is present (McKenzie & Batterham, 1994). Selection pressure may be produced by means of either independent evolution or gene flow, by which adaptive mutations can spread (Raymond *et al.*, 1991; Ffrench-Constant *et al.*, 1993). Ultimately, resistance management strategies should aim to prevent or delay resistance development in other susceptible pest populations (Khajuria *et al.*, 2011), whilst mitigating non-target effects.

In addition to the HDR strategy, crop rotation can be used as a complementary strategy, which entails planting crops expressing different *Bt* toxins (that bind to different receptors) in consecutive growing seasons (Schnepf *et al.*, 1998). Crop rotation will place resistant pests at a disadvantage, especially if resistance is associated with fitness costs, thus decreasing the frequency of resistant alleles. Concerningly, discontinuation of *Bt* treatments did not rapidly restore susceptibility in a resistant *P. xylostella* strain, suggesting that crop rotations may not be as effective (Tabashnik *et al.*, 1991). Nonetheless, the ultimate goal is to reduce selection pressure (Siqueira *et al.*, 2006). This could also be achieved by temporally restricted expression, which entails restricting gene expression to plant tissues that are most susceptible to damage. The parts of the plant that do not express these genes thus serve as a form of spatial refuge (Schnepf *et al.*, 1998). Some concerns have been raised by Mallet and Porter (1992) regarding the latter strategy. Alternatively, gene expression could be induced when insect feeding causes plant damage (Gould, 1988) or when a specific chemical is applied (Williams *et al.*, 1992). Management strategies for cereal stem borers, specifically *B. fusca*, were reviewed by Kfir *et al.* (2002), and include cultural control, habitat management,

management of crop residues, biological control and manipulation of planting dates and plant densities.

The latest commercial strategy in *Bt* crops is toxin stacking, whereby multiple toxins are co-expressed in the same crop (a pyramid crop) to target single or multiple pest species (Zhao *et al.*, 2005). For instance, the MON89034 pyramid crop expresses the Cry1Ab.105 and Cry2Ab2 proteins (Monsanto, 2009) and has been shown to effectively control *B. fusca* that has developed resistance to the Cry1Ab *Bt* toxin (Huyser, 2015). This strategy is based on the understanding that certain combinations of Cry toxins are unlikely to be resisted simultaneously (Griffitts & Aroian, 2005). However, Tabashnik *et al.* (1996) developed a model which predicts that cross-resistance will arise when Cry1 toxins have similar binding sites and domain II amino acid similarity. It is also predicted that pests may actually evolve resistance to pyramids faster if they are grown concurrently in the same area with crops producing similar single toxins (Zhao *et al.*, 2005). In such cases, cross-resistance studies are instrumental in the selection of suitable toxins to be used in pyramid crops (Anilkumar *et al.*, 2008). Expression of fusion proteins is yet another alternative to control pests, since these proteins have different host ranges and bind to distinct receptors compared with either of the parent toxins (Sanahuja *et al.*, 2011).

Additionally, identification of gene silencing targets will be useful in disrupting the basic mechanisms underlying resistance. One such example is RNA interference (RNAi) which entails the inhibition of endogenous gene expression in certain species (Li *et al.*, 2012). RNAi results when expressed double-stranded RNA (dsRNA) inhibits gene expression by degrading homologous target messenger RNA (mRNA) molecules in species (Schumann *et al.*, 2010). Target genes typically include genes that are essential for survival, such as insect development, hormone biosynthesis, hormone signalling, growth and detoxification (Li *et al.*, 2012).

This mechanism of RNAi was exploited as a means to control *H. armigera* by expressing dsRNA of insect endogenous genes in plants, which were subsequently ingested by these pests (Li *et al.*, 2012). Several other studies obtained similar results, where RNAi led to larval mortality or developmental delay (e.g. Baum *et al.* (2007); Mao *et al.* (2007) and Wang *et al.* (2011)). Furthermore, a study by Wang *et al.* (2011) demonstrated that direct delivery of dsRNAs of larval stage specific genes via the exoskeleton were lethal to *O. furnacalis.* In order to modify crops so that only the target pest is affected, the molecular basis of resistance, development, growth and reproduction of insect pests needs to be understood (Li *et al.*, 2012; Li *et al.*, 2013a). Sufficient transcriptomic information and species-specific target gene screening are indispensable for the elucidation of RNAi mechanisms (Li *et al.*, 2012).

Recent studies investigated the synergistic effects of coexpressing certain compounds with Cry toxins (Pacheco *et al.*, 2009b; Soberón *et al.*, 2009; Chen *et al.*, 2015). Some synergistic compounds (such as Cyt toxins) are proposedly capable of inserting into the epithelium membrane in the midguts of insects where they expose protein regions that facilitate Cry toxin recognition, oligomerization and pore formation (Soberón *et al.*, 2009). Recently, it was demonstrated for the first time that an ALP fragment synergized with Cry1Ac against susceptible and resistant strains of *H. armigera* (Chen *et al.*, 2015). Similar studies have demonstrated synergistic effects with CDH fragments and *Bt* toxins against lepidopteran pests (e.g. Abdullah *et al.,* 2009; Pacheco *et al.,* 2009b and Peng *et al.,* 2010). Transgenic plants containing genes coding for protease inhibitors have also resulted in synergism with *Bt* toxins by enhancing their insecticidal activity against *H. virescens, Helicoverpa zea* Boddie (Lepidoptera: Noctuidae)*, T. ni* and *M. sexta* (MacIntosh *et al.*, 1990). It is hypothesized that these inhibitors prevent proteases from inactivating *Bt* toxins or degrading membrane-bound receptors. It has also been demonstrated that amino acid substitution in Cry toxins can enhance their toxicity by increasing their binding affinity to receptors (Rajamohan *et al.*, 1996).

Recently, Deist *et al.* (2014) reviewed several Cry toxin modification strategies in order to developed improved strategies against target pests. These strategies include truncation of toxins, cleavage site modifications and binding modifications to promote proteolytic activation, enhance binding affinity, specificity and activity, as well as protect toxins from degradation by host proteases. Some of these strategies have proven to be successful. Cry1AbMod were found to be toxic to *M. sexta* (with silenced CDH) and Cry1AcMod toxins were able to overcome Cry1Ac CDH-based resistance in *P. gossypiella* (Soberón *et al.*, 2007). Moreover, these modified toxins were also toxic to other pest insects (*O. nubilalis, D. saccharalis, H. armigera* and *T. ni*) with alternate mechanisms of resistance that are not related to CDH (Franklin *et al.*, 2009; Tabashnik *et al.*, 2011). Genes producing these modified toxins can thus be introduced into crops to counter insect resistance, but an understanding of these toxins' mechanisms of action and the target pests' gut physiology is crucial. It is unclear whether results from all these laboratory bioassays will translate to field conditions. Corrective action strategies should therefore be in place prior to *Bt* maize product failure (Shelton *et al.*, 2002).

Several important statements were made by James (2014) regarding aspects that should be considered when resistance management and stewardship programmes are implemented. These include timely detection and reporting of resistance and regular replacement of transgenic crops with improved *Bt* hybrids. The latter should be accompanied by assurance of pure, high quality seeds that adequately express the expected traits. Preferably, these traits should have multiple modes of action with respect to the insect's mechanism of resistance

(James, 2014). Selection pressure for resistance should be reduced through IRM strategies, while promoting survival of susceptible individuals to reduce the frequency of resistant alleles. Undeniably, the farming community should be educated and trained in managing these crops, whilst compliance with regulatory requirements should be reinforced (James, 2014). Most importantly, the successful implementation of IRM strategies is dependent on the collective responsibility of several participants, including scientists, farmers, policy makers and the private sector (James, 2014; Monnerat *et al.*, 2015). Integrated pest management (IPM) strategies should be employed, where a combination of control measures is implemented to promote sustainable agriculture, whilst limiting pest damage and protecting the environment and non-target species.

1.4.11 Summary of literature overview

The literature referred to above provided an overview of Cry1Ab resistance of *B. fusca* and demonstrated how mitochondrial genes and transcriptomics can be employed in resistance studies, which set the stage for subsequent chapters. An overview of GM crops and the HDR strategy was provided, after which different aspects regarding Cry toxins were discussed. These included the use of Cry toxins as biopesticides, as well as their diversity, structure and function. This chapter also dealt with the Cry1A toxin mode of action and receptors involved herein. A short summary of Cry toxin binding site models in Lepidoptera was also provided. The final section proposed strategies for future GM crop technology with regard to IPM.

The preceding literature provides information relevant to the present study and will serve as a framework for findings of the study. This research was concerned with the genetic diversity of *B. fusca*, as well as its transcriptome and differentially expressed genes which may be involved in resistance. In conclusion, the present study produced much needed molecular data for *B. fusca,* which will be indispensable for developing pest management strategies for this pest.

Chapter 2

Low genetic diversity in South African *Busseola fusca*

This chapter has been published as a short communication in *African Entomology* (title page is provided in Annexure D):

Peterson, B., Bezuidenhout, C.C. & Van den Berg, J. 2016. Cytochrome *c* oxidase I and cytochrome *b* gene sequences indicate low genetic diversity in South African *Busseola fusca* (Lepidoptera: Noctuidae) from maize. *African Entomology*, 24(2):518-523.

It was extended to include results that could not be included in the published short communication due to length restrictions.

2.1 Introduction

The African maize stem borer, *B. fusca*, feeds on a limited number of host plant species (Calatayud *et al.*, 2014), which include crops such as maize, pearl millet and sorghum. This pest occurs throughout sub-Saharan Africa where it causes economic damage to maize and sorghum crops (Kfir *et al.*, 2002). In an attempt to combat lepidopteran stem borers, genetically modified *Bt* maize that express insecticidal Cry toxins were developed to kill larvae that feed on these plants (George *et al.*, 2012). *Bt* maize was introduced into SA during 1998 and within the first decade after release, field-evolved resistance of *B. fusca* to *Bt* maize was reported (Van Rensburg, 2007). Since this first report, resistant populations have been reported from several parts of the maize production region of SA (Kruger *et al.*, 2011; Van den Berg *et al.*, 2013). Explanations provided for this rapid resistance development were confined to agronomical reasons (Van Rensburg, 2007; Kruger *et al.*, 2009; 2012), non-compliance to refuge requirements (Kruger *et al.*, 2009) and non-recessive inheritance of resistance (Campagne *et al.*, 2013). Not much is however understood about the molecular genetics of *B. fusca*. Previously, Sezonlin *et al.* (2006b) noted that limited population genetic and phylogeography data are available for *B. fusca* and African insects in general.

Sequences available for *B. fusca* are limited to some COI (Assefa *et al.*, 2006b; Toussaint *et al.*, 2012; Assefa *et al.*, 2015; Le Rü *et al.*, 2015) cyt b (Sezonlin *et al.*, 2006a; 2006b; 2012) and some microsatellite data (Faure & Silvain, 2005; Faure, 2006). Through the use of DNA-sequence data from the mitochondrial genome, phylogenetic relationships among species and/or populations can be estimated (Simon *et al.*, 1994; Assefa *et al.*, 2006a). Sezonlin *et al.*

(2006a) used cyt b sequences of *B. fusca* from Western, Central, Southern and East Africa to demonstrate that three clades (*W*, *KI* and *KII*) were dominant, and that these were associated with geographic differences and ecological preferences. Based on genetic differentiation in cyt b sequences they concluded that *B. fusca* populations could be divided into three major groups, comprising 108 haplotypes. This included a homogeneous and geographically isolated population from West Africa (*W*), and two populations from East, Central and Southern Africa (*KI* and *KII*). The latter two had overlapping distributions, although clade *KI* was restricted to East Africa (Sezonlin *et al.*, 2006a). This clade distribution corresponded to the distribution patterns of various plant and animal species and climatic conditions and could be linked to the paleogeography of the region. However, in that study, *B. fusca* individuals were only collected at six localities in SA.

In studies on related species it has been hypothesized that understanding the exact genetic relationship of the pest species as well as the genetic diversity is important in the development of integrated pest management approaches (Zhang *et al.*, 2012a). Thus the aim of this study was to determine the genetic diversity of South African *B. fusca* populations from maize, sampled widely across the maize production region, using COI and cyt b sequences. A secondary aim was to validate the clustering of South African *B. fusca* populations within clade *KII*, which included haplotypes from Central Africa (Kenya and Cameroon).

2.2 Material and Methods

Seventy-two *B. fusca* larvae were collected from maize at 25 localities in SA (Figure 2.1, Table 2.1).

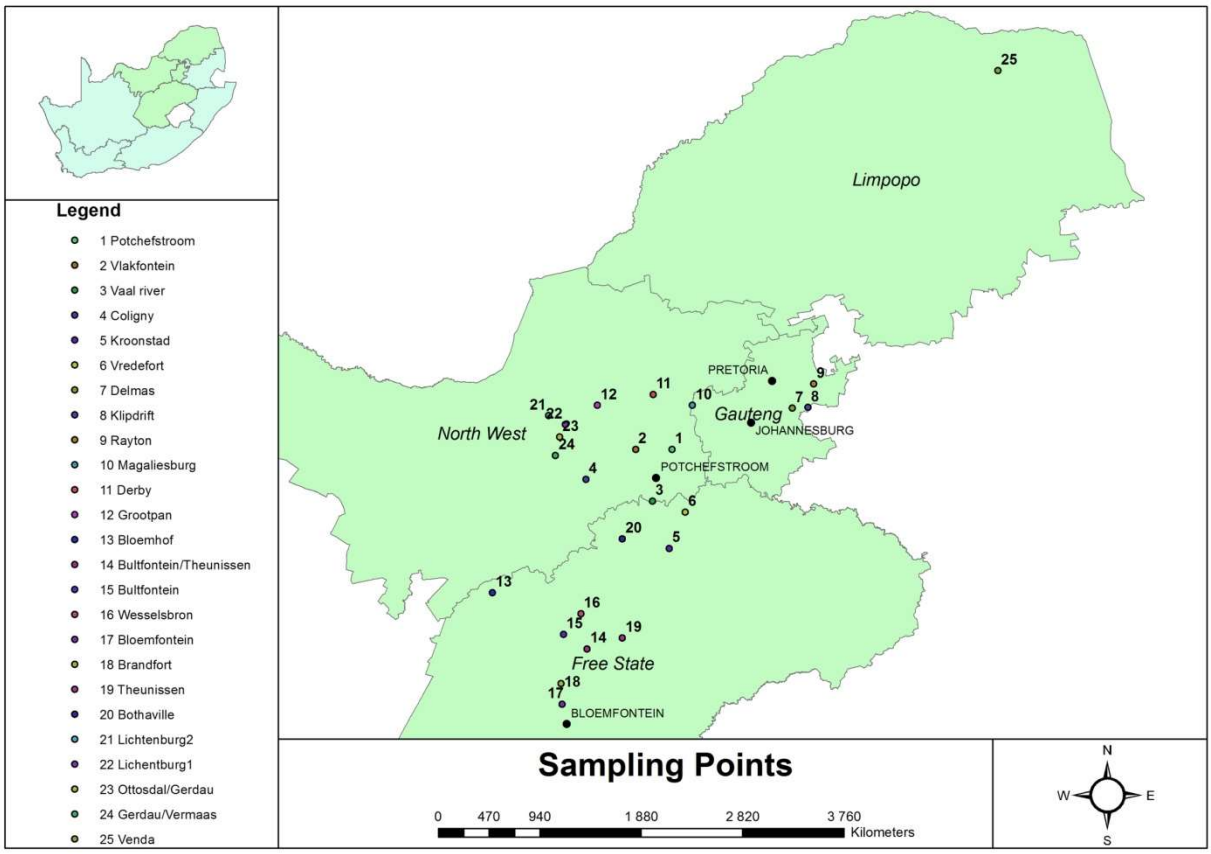


Figure 2.1: Map indicating localities in South Africa where *Busseola fusca* larvae were collected from maize.

Table 2.1: Latitude and longitude coordinates of sites in South Africa where *Busseola fusca* larvae were collected from maize.

	Town	Latitude, Longitude
1	Potchefstroom	S26°25.493, E27°14.929
2	Vlakfontein	S26°25.493, E26°53.484
3	Vaal river	S26°55.994, E27°03.435
4	Coligny	S26°43.119, E26°24.199
5	Kroonstad	S27°23.699, E27°13.291
6	Vredefort	S27°02.432, E27°22.640
7	Delmas	S26°01.237, E28°25.714
8	Klipdrift	S26°00.870, E28°34.947
9	Rayton	S25°46.958, E28°38.290
10	Magaliesburg	S25°59.525, E27°26.955
11	Derby	S25°53.176, E27°03.814
12	Grootpan	S25°59.632, E26°30.989
13	Bloemhof	S27°49.803, E25°29.245
14	Bultfontein/Theunissen	S28°22.784, E26°24.909
15	Bultfontein	S28°14.338, E26°10.950
16	Wesselsbron	S28°02.173, E26°21.247
17	Bloemfontein	S28°55.332, E26°10.164
18	Brandfort	S28°43.199, E26°09.630
19	Theunissen	S28°16.359, E26°45.719
20	Bothaville	S27°18.219, E26°45.719
21	Lichtenburg2	S26°05.605, E26°02.230
22	Lichtenburg1	S26°10.863, E26°12.114
23	Ottosdal/Gerdau	S26°18.252, E26°08.701
24	Gerdau/Vermaas	S26°29.052, E26°06.320
25	Venda	S22°42.834, E30°26.971

DNA was isolated using a NucleoSpin® Tissue Kit (Macherey-Nagel, Germany) following the instructions of the manufacturer. A 710 bp fragment of the *COI* barcoding gene was amplified using the LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') primer set from Folmer *et al.* (1994). The CP1 (5'-GATGATGAAATTTTGGATC-3') (Sezonlin *et al.*, 2006b) and TRs (5'-TATTTCTTTATTATGTTTTCAAAAC-3') (Simon *et al.*, 1994) primer set was used to amplify a 1000 bp fragment of the *cyt b* gene from the same individuals. Each 25 µl PCR mixture contained 1X PCR Master Mix (Fermentas Life Science, US), 0.5 µM of each primer (InqabaBiotec, SA) and 20 ng DNA. A no-template control (NTC) was also included. PCR cycling conditions of Voua Otomo *et al.* (2009) were used to amplify the *COI* barcoding gene. For the amplification of the *cyt b* gene, PCR cycling conditions of Sezonlin *et al.* (2006b) were adjusted and included an initial denaturation of 300 seconds at 94 ºC, followed by 40 cycles of 60 seconds at 94 ºC, 90 seconds at 55 ºC and 120 seconds at 72 ºC. The final extension step consisted of 600 seconds at 72 ºC. PCR products were resolved by agarose gel electrophoresis and visualised using a ChemiDoc™ MP Imaging System (Bio-Rad, USA) and Image Lab™ v4.0.1 software.

PCR products were sequenced in both directions by Inqaba Biotec (SA) and the individual sequences were viewed and edited using Geospiza FinchTV (v1.4). Consensus sequences were prepared using the BioEdit Sequence Alignment Editor v7.2.5 (Hall, 1999). BLAST (Basic Local Alignment Search Tool) was employed to compare the obtained sequences to the GenBank database of sequences, using the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST) Internet server. All the new sequences generated in the present study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank; accession numbers KM061880-KM061951 and KM587541-KM587612).

The BioEdit Sequence Alignment Editor v7.2.5 (Hall, 1999) was used to assemble the multiple sequences, after which MAFFT online software (http://mafft.cbrc.jp/alignment/server/) was used to perform the alignment. Sequence alignments were trimmed to produce equal sequence lengths of 651 and 940 bases for the COI and cyt b alignments, respectively. DnaSP v5.10.01 (Librado & Rozas, 2009) was used to calculate the following basic sequence statistics: number of polymorphic (segregating) sites (S), number of parsimony informative sites (PI), number of haplotypes (h), haplotype diversity (Hd) and nucleotide diversity (π). Haplotype networks of partial COI and cyt b sequences were constructed independently with NETWORK v4.6.1.2 software (Fluxus Technology, Clare, Suffolk, U.K.). Molecular Evolutionary Genetics Analysis (MEGA) v7.0.21 (Kumar *et al.*, 2016) software was used to find the best DNA model and construct a bootstrap phylogenetic tree with the cytochrome *b* gene sequences from this study (one representative from each haplotype) and the 108 haplotypes from the Sezonlin *et al.*

(2006a) study. GenBank accession numbers of sequences used to construct the phylogenetic tree are as follow: (i) this study: KM587588, KM587543, KM587544, KM587547, KM587551, KM587556, KM587561, KM587565, KM587566, KM587571, KM587576, KM587579, KM587596, KM587597, KM587600, KM587605, KM587609 (ii) the Sezonlin *et al.* (2006a) study: AY769536 to AY769605 and DQ284857 to DQ284895.

2.3 Results

Genomic DNA was successfully isolated from *B. fusca* larvae and was of sufficient quality and quantity for PCR amplification. The expected 710 bp and 1000 bp fragments were obtained for all 72 samples with amplification of the *COI* and *cyt b* genes, respectively. Since amplification results are the same for all the samples, an example of these results are shown for 24 of the 72 samples (Figure 2.2). BLAST results of these sequences confirmed that the obtained sequences were partial *COI* and *cyt b* gene sequences of *B. fusca*. These sequences (GenBank accession numbers KM061880-KM061951 and KM587541-KM587612) were used in basic statistical and network analyses. Basic sequence statistics for each sequence data set are indicated in Table 2.2, while haplotype networks are indicated in Figure 2.3.

According to the sequence statistics, the COI sequence data set presented lower values for all the parameters (Table 2.2). The COI sequence data set contained seven variable (polymorphic) sites (1.08 %), of which three sites (0.46 %) were parsimony-informative. Similarly, the cyt b sequence data set contained 17 variable (polymorphic) sites (1.81 %), of which only eight sites (0.85 %) were parsimony-informative. Based on genetic differentiation in both these mitochondrial sequences, South African *B. fusca* populations show low genetic diversity. For both mitochondrial genes, strong haplotype diversity was associated with very low levels of nucleotide diversity, which concurs with results of Sezonlin *et al.* (2006a; 2006b) and is indicative of accumulation of mutations (Grant & Bowen, 1998). The latter is also demonstrated by the star-like topography of the haplotype networks (Teixeira *et al.*, 2011), where several small haplotypes are diverged from one main central haplotype via one or two mutation steps (Figure 2.3). Haplotype network analyses demonstrated eight and 17 haplotypes in the South African population of *B. fusca* for the *COI* (Figure 2.3a) and *cyt b* (Figure 2.3b) mitochondrial genes, respectively. According to Grant and Bowen (1998), such mutation accumulations suggest that a population underwent major demographic expansion after experiencing bottlenecks at its origin.

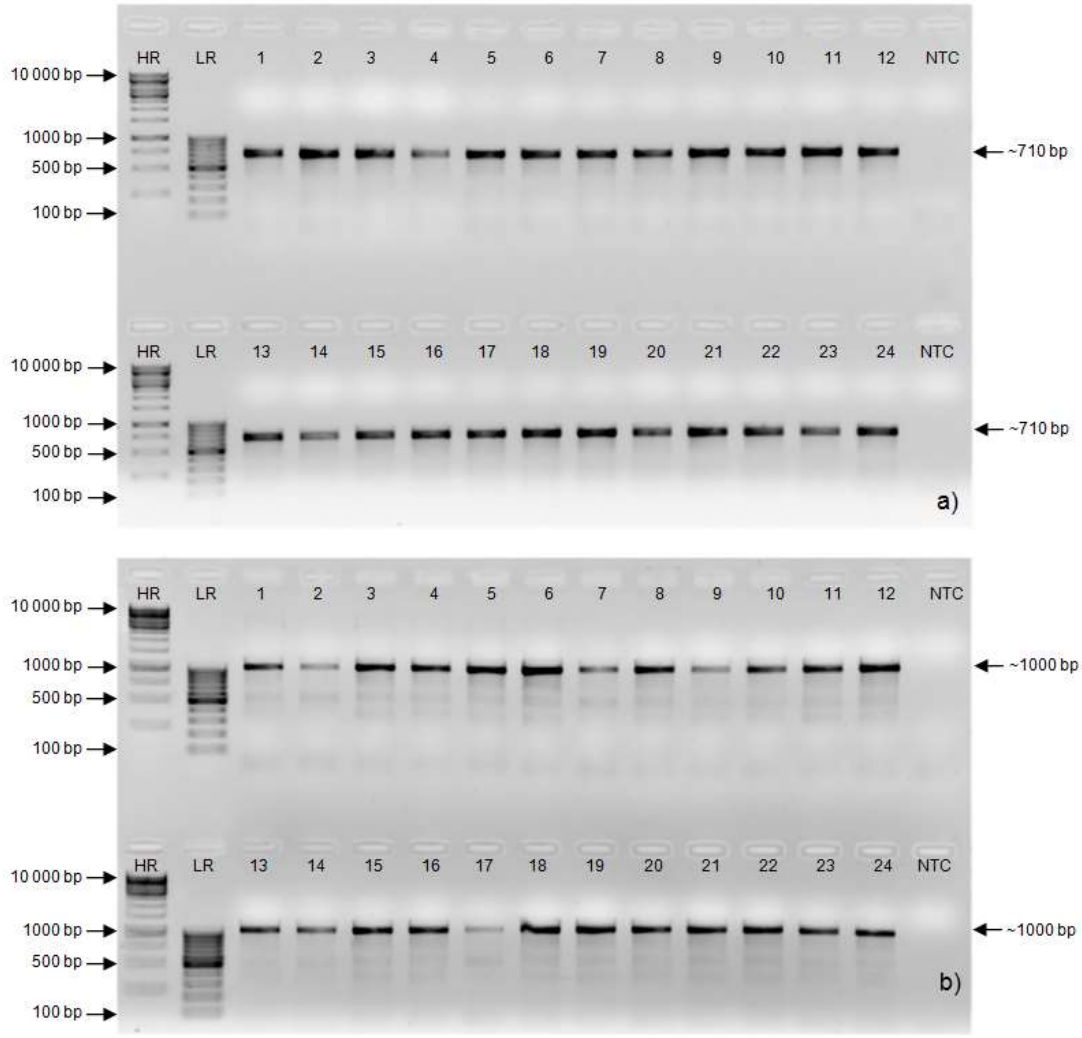


Figure 2.2: Negative electrophoretograms showing some amplification results for (a) cytochrome c oxidase I (COI) and (b) cytochrome b (cyt b). HR = 1 kb High Range molecular weight marker, LR = 100 bp Low Range molecular weight marker, NTC = no-template control.

Table 2.2: Basic sequence statistics of several parameters calculated for each mitochondrial sequence data set.

Parameter	COI	Cyt b
S	7	17
PI	3	8
h	8	17
Hd ± stdev	0.368 ± 0.071	0.694 ± 0.058
π	0.00062	0.00119

S = number of polymorphic (segregating) sites; PI = number of parsimony informative sites; h = number of haplotypes; Hd = haplotype diversity; π = nucleotide diversity

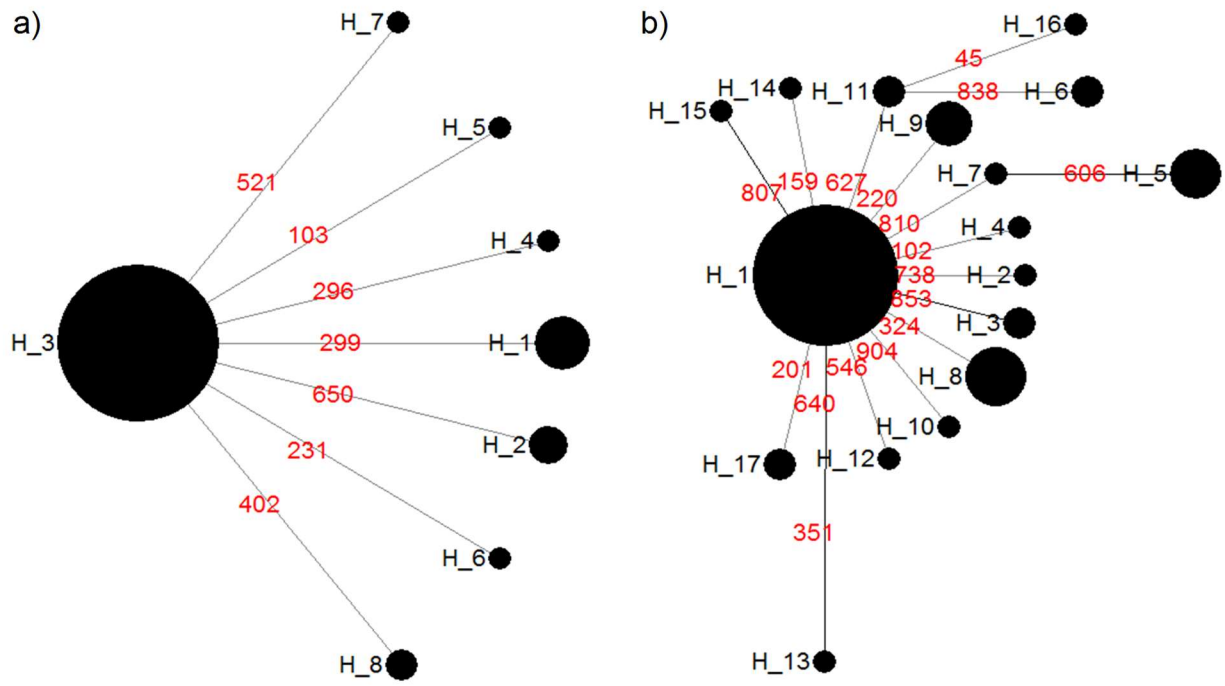


Figure 2.3: Haplotype networks for South African *Busseola fusca* populations based on (a) cytochrome c oxidase I (COI) and (b) cytochrome b (cyt b) sequence data sets. Circle sizes are directly proportional to the number of individuals present in that haplotype. Each red value indicates one base pair change, while mv1 represents the median vector.

In order to validate the clustering of South African *B. fusca* populations within clade *KII*, as described by Sezonlin *et al.* (2006a), and which included haplotypes from Central Africa (Kenya and Cameroon), phylogenetic analysis was performed. According to the DNA model test, the best model for this data set was identified as T92+G. Consequently, the Tamura 3-parameter (T92) method (to compute evolutionary distances) and gamma distribution (G) model (to model the rate variation among sites) were used to construct a Neighbor-Joining bootstrap phylogenetic tree (Figure 2.4). This analysis compared cytochrome *b* gene sequences of *B. fusca* from this study (one representative from each haplotype, i.e. 17 representatives) to that of the 108 haplotypes (GenBank accession numbers AY769536 to AY769605 and DQ284857 to DQ284895) from the Sezonlin *et al.* (2006a) study. The same three clades (*W, KI, KII*) were obtained, with representatives sequenced in this study clustering within clade *KII* (Central to East Africa). There was 86% bootstrap support for the observed clustering pattern.

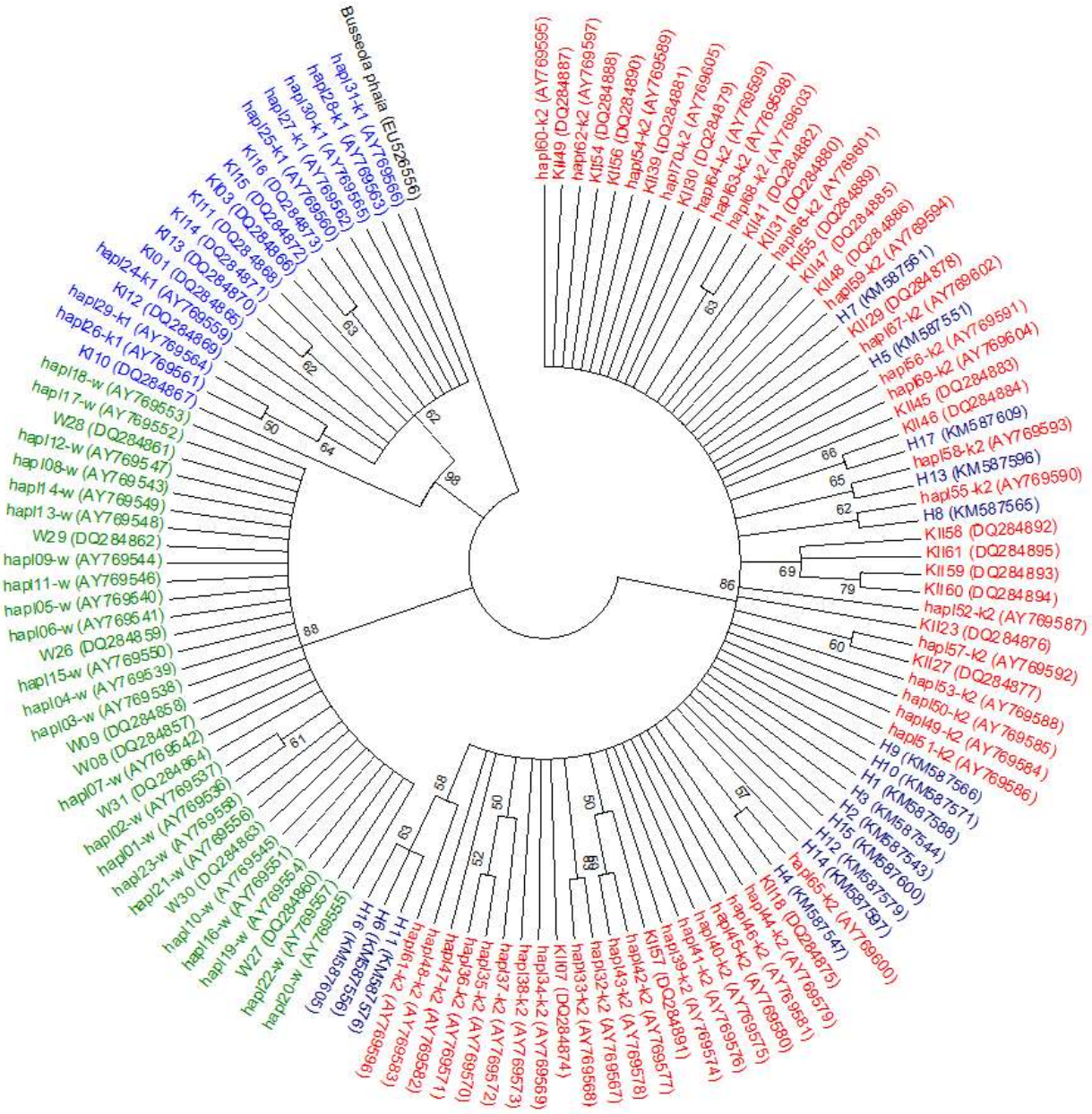


Figure 2.4: A circular neighbor-joining cladogram based on an alignment of South African *Busseola fusca* cytochrome *b* gene sequences obtained after amplification (navy) and sequences of *B. fusca* populations from West Africa (*W*, blue), East Africa (*KI*, green) and East-, Central- and South Africa (*KII*, red). H1-H17 = haplotypes 1 to 17 (from the present study), hapl*number* = haplotypes from the Sezonlin *et al.* (2006b) study, KI/KII/W*number* = haplotypes from the Sezonlin *et al.* (2006a) study. *Busseola phaia* (black) was chosen as an outgroup taxon.

2.4 Discussion

Compared to other mitochondrial genes, the *COI* barcoding gene has a greater range of phylogenetic signal with a rapid evolution rate (Cox & Hebert, 2001). This makes this gene a taxonomic tool that allows discrimination of phylogeographic groups within a single species (Hebert *et al.*, 2003). A study by Behere *et al.* (2007) also demonstrated the suitability of the *COI* mitochondrial gene for resolving phylogeny of two closely related lepidopteran pests. Even though the power of COI as a taxonomic tool has been criticised (Will *et al.*, 2005; Roe & Sperling, 2007; Jacquet *et al.*, 2012; Havird & Santos, 2014), several studies have demonstrated the successful use of this mitochondrial gene. Likewise, the *cyt b* gene is useful for studying genetic variation between insect populations (Boudabous *et al.*, 2011) or inferring an evolutionary relationship (Li *et al.*, 2005a). Sezonlin *et al.* (2006a; 2006b; 2012) successfully used sequences from this gene to address phylogeographical and species population aspects regarding *B. fusca.* The clustering of South African *B. fusca* populations within clade *KII*, as described by Sezonlin *et al.* (2006a; 2006b; 2012) was validated and the same clustering was observed (Figure 2.4).

The increased number of informative sites, and consequently nucleotide and haplotype diversity obtained with the *cyt b* gene is attributable to the faster rate at which amino acid changes occur in this gene (Table 2.2) (Simmons & Weller, 2001). The limited number of individuals used in this study was reflected in the low haplotype diversity (Figure 2.3), which is in accordance with observations of Behere *et al.* (2007). Haplotypes were not restricted to particular geographic regions, but instead had a wide geographic distribution. According to Avise *et al.* (1987) this pattern of distribution of haplotypes is common in organisms that are capable of long-range movement. Results from Dupas *et al.* (2014) indicated that *B. fusca* has great dispersal capabilities.

Results from this study contribute to future studies on the evolution of *B. fusca* within diverse sub-Saharan environments. This low genetic diversity and high dispersal capabilities (Dupas *et al.*, 2014) suggest that the geographic expansion of a *B. fusca* strain with competitive traits may be rapid. Low genetic diversity together with any pre-existent trait that is selected for (e.g. resistance to Cry1Ab proteins) that provides a competitive advantage to a new strain of this species may lead to rapid expansion in the distribution of this trait in the population, especially since it was reported that resistance was non-recessively inherited in *B. fusca* (Campagne *et al.*, 2013). This may have implications for IRM strategies in *Bt* maize in SA and elsewhere.

2.5 Conclusion

The phenomenon of resistance evolution may not be reversed in the near future and may escalate. A follow-up study with a much greater sample size, as well as inclusion of other host crops, is recommended. Subsequently, the genetic structure of South African *B. fusca* populations can be characterised and its evolutionary history inferred, as was done for Cameroonian *B. fusca* populations by Sezonlin *et al.* (2012). Results can then also be correlated with specific geographic regions and the associated host crops. These results may be useful in future studies on the evolution of *B. fusca* within diverse sub-Saharan environments. Moreover, the diversity trend can then be studied through time, which will be invaluable for IRM purposes. In conclusion, IRM strategies must be adapted to delay evolution of resistance to Cry proteins in other *B. fusca* populations, since these populations will be predisposed to evolve the same mechanism of resistance.

Chapter 3
Mechanisms of cry toxin resistance in Lepidoptera

This chapter has been published in *Journal of Economic Entomology* (title page is provided in Annexure D):

Peterson, B., Bezuidenhout, C.C. & Van den Berg, J. 2016. An overview of mechanisms of Cry toxin resistance in lepidopteran insects. *Journal of Economic Entomology,* 1-16. doi: 10.1093/jee/tow310.

3.1 Introduction

GM crops with insecticidal traits from *B. thuringiensis*, known as *Bt* crops, have been cultivated since 1996 for the management of insect pests (James, 2015b). While no resistance to *Bt* crops were reported in target pests during the first few years of cultivation, evolution of target pest resistance to *Bt* crops was a concern since the development of these crops (Roush, 1997). As a result, IRM programs were imposed to prevent or delay resistance evolution (Bates *et al.*, 2005). However, various factors (social, economic, biological and environmental) hampered the efficacy of these programs (Kruger *et al.*, 2009), which enabled several insect species to develop *Bt* resistance.

While *P. interpunctella* was the first lepidopteran pest reported to have developed resistance to a sprayable *Bt* product under laboratory conditions in 1985 (McGaughey, 1985), the first field-evolved resistance to commercial *Bt* formulations was observed in a *P. xylostella* population in 1986 (Tabashnik *et al.*, 1990). According to Tabashnik *et al.* (2013), *H. zea* was the first species reported to have evolved field resistance to *Bt* crops (cotton). This was reported in 2002, a mere six years after the introduction of *Bt* crops. Since then, several reports of field-evolved resistance to *Bt* crops have been published. Some examples are discussed in recent reviews (Pardo-López *et al.*, 2013; Tabashnik *et al.*, 2013; Kumar & Kumari, 2015). The presumption that resistance was unlikely to evolve in the field was ascribed to a lack of resistance reports, as well as inaccurate reflections of potential for resistance evolution in laboratory selection experiments compared to the field (Tabashnik *et al.*, 1990; Wang *et al.*, 2007; Yang *et al.*, 2007).

Field-evolved resistance to *Bt* crops in lepidopterans has been reported for *B. fusca* in SA (Kruger *et al.*, 2009), *D. saccharalis* in the USA (Huang *et al.*, 2012), *H. armigera* in China and Australia (Downes *et al.*, 2007; Liu *et al.*, 2010), *Helicoverpa punctigera* Wallengren

(Lepidoptera: Noctuidae) in Australia (Downes *et al.*, 2010b), *H. zea* in the USA (Luttrell *et al.*, 2004), *O. furnacalis* in the Philippines (Alcantara *et al.*, 2011), *P. gossypiella* in China and India (Dhurua & Gujar, 2011; Wan *et al.*, 2012) and *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) in Brazil and the USA (Storer *et al.*, 2010; Farias *et al.*, 2014). It is evident that evolution of resistance in these pests is a major threat to the sustainable use of these transgenic crops (Yang *et al.*, 2007; Tabashnik *et al.*, 2013).

Studies into resistance mechanisms commenced mainly during the early 2000s. These studies have indicated that resistance can be ascribed to several factors, including variations in any one of the steps of the Cry toxin mode of action (Jurat-Fuentes *et al.*, 2004). A general overview of potential mechanisms of resistance is indicated in Figure 3.1. The most reported mechanism of resistance seems to be altered binding of Cry toxins to receptors (Ferré & Van Rie, 2002). It is also notable that a single species can evolve a repertoire of resistance mechanisms to the same or different Cry toxins (Table 3.1). Numerous studies have been published on the topic of *Bt* toxin resistance in lepidopteran insects, but these studies are invariably restricted to specific species and strains, geographical regions, or certain mechanisms of resistance. The aim of this study was thus to compose an overview on all the mechanisms of Cry toxin resistance that have been reported for lepidopteran pests from laboratory, greenhouse and field studies. Many of these reports did not establish a causal link to resistance, but rather proposed factors that contributed to resistance. Be that as it may, all reports were reviewed during this study.

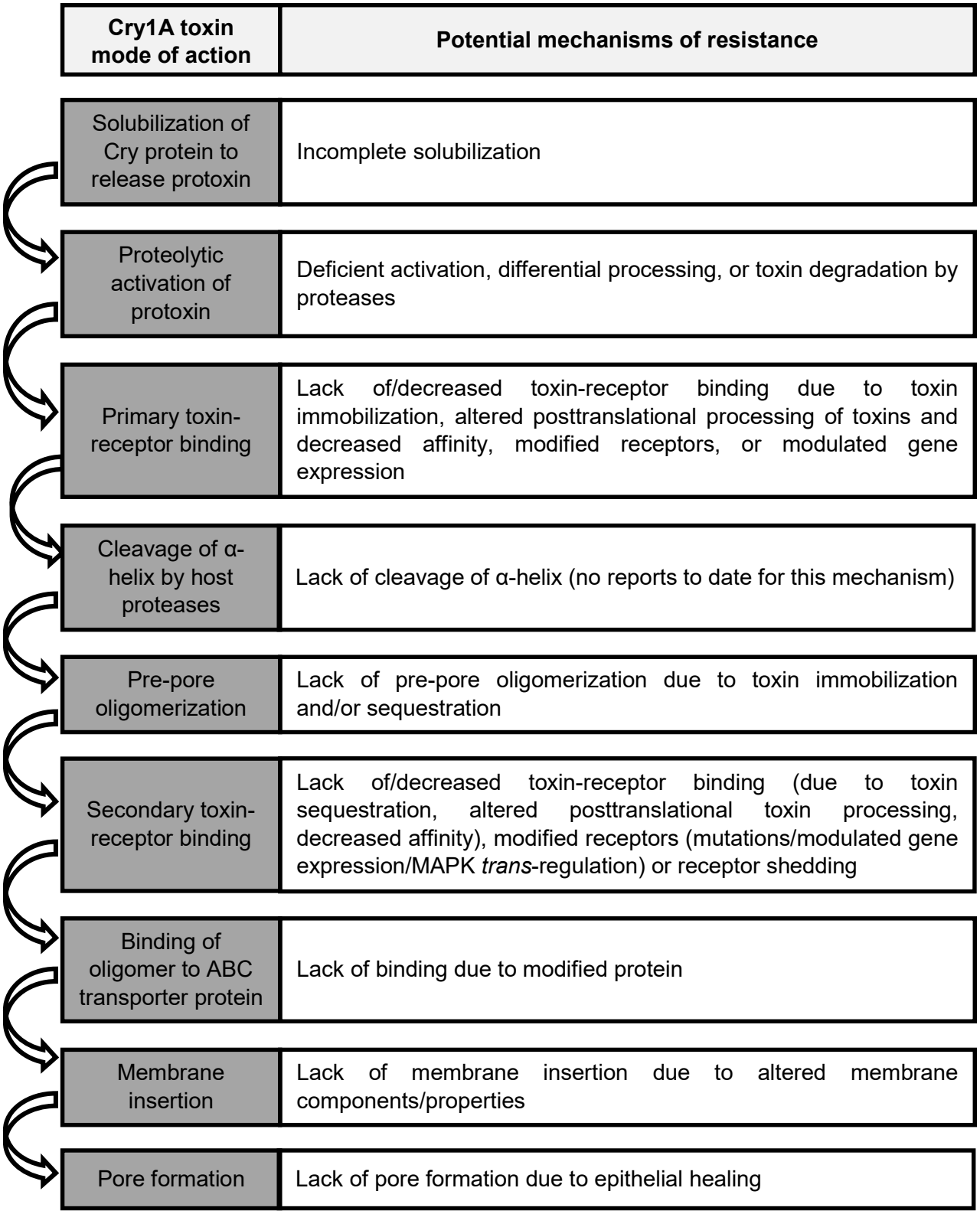


Figure 3.1: General overview of potential mechanisms of resistance (white blocks) for each step in the Cry1A toxin mode of action (grey blocks).

Table 3.1: Mechanisms of Cry toxin resistance in lepidopteran species reported from laboratory, greenhouse and field studies.

Species	Strain	Country	Toxin	Cross-resistance	Mode of inheritance	Mechanism of resistance																	References
						A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	
Bombyx mori	C2	China	Cry1Ab	-	Recessive											X								Atsumi *et al.*, 2012; Tanaka *et al.*, 2013
Diatraea saccharalis	Cry1Ab-RR	USA	Cry1Ab	-	Incompletely recessive						X		X											Wu *et al.*, 2009; Yang *et al.*, 2010; Yang, 2011; Yang *et al.*, 2011
Ephestia kuehniella	No name	Australia	Bt[a]	-	-		X																	Rahman *et al.*, 2007
Helicoverpa armigera	5-405(NA405), 6-364, 6-798, 9-4802, 10-485, 12-2169	Australia	Cry2Ab	Cry2Aa Cry2Ae	-											X								Caccia *et al.*, 2010; Tay *et al.*, 2015
	BX	Australia	Cry1Ac	Cry1Ab	Incompletely recessive				X															Akhurst *et al.*, 2003
	Silver	Australia	Cry1Ac	-	Semi-dominant		X*																	Gunning *et al.*, 2005
	SP15	Australia	Cry2Ab	Cry2Aa Cry2Ae	Recessive											X								Mahon *et al.*, 2007; Caccia *et al.*, 2010; Downes & Mahon, 2012; Tay *et al.*, 2015
	BtR	China	Cry1Ac	-	Incompletely recessive[b]			X				X			X*									Liang *et al.*, 2008; Zhang *et al.*, 2009; Jurat-Fuentes *et al.*, 2011; Cao *et al.*, 2013

Species	Strain	Country	Toxin	Cross-resistance	Mode of inheritance	Mechanism of resistance																References
						A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	
Helicoverpa armigera	GYBT	China	Cry1Ac	Cry1Aa Cry1Ab Btk[c#]	Recessive[d]					X														Xu *et al.*, 2005; Yang *et al.*, 2006; Yang *et al.*, 2007
	LF5	China	Cry1Ac	-	Incompletely recessive			X																Liu *et al.*, 2014
	LF10, LF30, LF120	China	Cry1Ac	-	-										X									Chen *et al.*, 2015
	LF60	China	Cry1Ac	-	Recessive										X	X								Xiao *et al.*, 2014; Chen *et al.*, 2015
	ISOC4	Australia	Cry1Ac	-	-		X												X					Akhurst *et al.*, 2003; Ma *et al.*, 2005
	SCD-r1[e]	Ivory Coast + China	Cry1Ac	Cry1Aa[#] Cry1Ab[#] Cry2Ab[#]	Recessive[f]					X														Yang *et al.*, 2009; Zhao *et al.*, 2010; Zhang *et al.*, 2012a
	XJ-r15	China	Cry1Ac	Cry1Aa[#] Cry1Ab[#] Cry2Ab[#]	Nonrecessive					X														Zhang *et al.*, 2012b; Zhang *et al.*, 2013
	AY-r15	China	Cry1Ac	-	Nonrecessive					X														Zhang *et al.*, 2012b; Zhang *et al.*, 2013
	Akola-R	India	Cry1Ac	-	-			X																Rajagopal *et al.*, 2009
	BH-R	India	Cry1Ac	-	Semi-dominant					X														Nair *et al.*, 2010; Nair *et al.*, 2013

Species	Strain	Country	Toxin	Cross-resistance	Mode of inheritance	Mechanism of resistance																References
						A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	
Helicoverpa armigera	Cry1Ac-SEL	Pakistan	Cry1Ac Conventional insecticides[g]	-	Incompletely dominant		X*																	Alvi *et al.*, 2012
Helicoverpa punctigera	Hp4-13	Australia	Cry2Ab	Cry2Ae	Fully recessive											X								Caccia *et al.*, 2010; Downes *et al.*, 2010a; Downes *et al.*, 2010c; Downes & Mahon, 2012; Tay *et al.*, 2015
Helicoverpa zea	AR1	USA	Cry1Ac	Cry1Ab	-								X		X							X*		Caccia *et al.*, 2012
Heliothis virescens	CP73-3	USA	Cry1Ac	Cry1Aa# Cry1Ab Cry1B# Cry1C# Cry2Aa	Nonrecessive			X									X*							Gould *et al.*, 1992; Forcada *et al.*, 1996; Martínez-Ramírez *et al.*, 1999
	CXC[h]	USA	Cry1Ac	Cry1Aa Cry1Ab Cry1Fa Cry2Aa	-			X							X*		X*							Forcada *et al.*, 1999; Jurat-Fuentes *et al.*, 2003; Jurat-Fuentes *et al.*, 2004; Karumbaiah *et al.*, 2007; Castagnola & Jurat-Fuentes, 2009; Jurat-Fuentes *et al.*, 2011

Species	Strain	Country	Toxin	Cross-resistance	Mode of inheritance	Mechanism of resistance																References
						A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	
Heliothis virescens	KCB	USA	Cry1Ac	Cry1Ab Cry2Aa Cry1Aa Cry1Ab Cry1Fa Cry2Aa	-			X[i]									X							Forcada *et al.*, 1999
	KCBhyb[j]	USA	Cry1Ac		-			X[*]		X[k,l]					X[*]									Jurat-Fuentes *et al.*, 2003; Jurat-Fuentes *et al.*, 2004; Karumbaiah *et al.*, 2007; Jurat-Fuentes *et al.*, 2011
	No name	USA	Cry1Ab	-	Partially recessive				X															MacIntosh *et al.*, 1991
	No name	USA	Cry1Ac	-	-												X							Loeb *et al.*, 2001
	YHD2	USA	Cry1Ac	Cry1Aa Cry1Ab Cry1B[#] Cry1C[#] Cry1Fa Cry2A	Partially recessive					X[k]														Gould *et al.*, 1995; Gahan *et al.*, 2001
	YHD2-B (YHD3)	USA	Cry1Ac	Cry2Aa[#]	Recessive			X			X[k]		X		X	X						X[*]	X[*]	Jurat-Fuentes & Adang, 2004; Jurat-Fuentes & Adang, 2006; Karumbaiah *et al.*, 2007; Gahan *et al.*, 2010; Jurat-Fuentes *et al.*, 2011
	YEE	USA	Cry1Ac	-	-											X								Gahan *et al.*, 2010
Ostrinia furnacalis	ACB-AcR	China	Cry1Ac	-	-					X	X													Jin *et al.*, 2014
	ACB-Ac200	China	Cry1Ac	-	-					X														Jin *et al.*, 2014

Species	Strain	Country	Toxin	Cross-resistance	Mode of inheritance	Mechanism of resistance																References
						A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	
Ostrinia furnacalis	ACB-Ac24	China	Cry1Ac	-	-						X													Jin *et al.*, 2014
Ostrinia nubilalis	Europe-R	Italy	Cry1Ab	Cry1Aa Cry1Ac	Dominant / Recessive[m]					X														Alves *et al.*, 2006; Bel *et al.*, 2009
	RSTT-R[n]	Italy + USA	Cry1Ab	Cry1Ac	Dominant / Recessive[m]									X*										Alves *et al.*, 2006; Siqueira *et al.*, 2006; Khajuria *et al.*, 2011
	Cry1Ab-R	USA	Cry1Ab	-	Partially recessive								X											Coates *et al.*, 2013
	KS-SC	USA	*Btk*[c]	Cry1Ba	Incompletely dominant			X																Huang *et al.*, 1999a; Huang *et al.*, 1999b; Li *et al.*, 2004a; Li *et al.*, 2004b; Li *et al.*, 2005b
	SKY-R	USA	Cry1Ab	Cry1Aa Cry1Ac Cry1F[#]	Incompletely recessive									X*										Crespo *et al.*, 2009; Crespo *et al.*, 2011; Khajuria *et al.*, 2011
Pectinophora gossypiella	AGJ	India	Cry1Ac	-	-					X														Fabrick *et al.*, 2014
	KMP	India	Cry1Ac	-	-					X														Fabrick *et al.*, 2014
	AMH	India	Cry1Ac	-	-					X														Fabrick *et al.*, 2014
	APHIS-98R	USA	Cry1Ac	-	Recessive					X														Tabashnik *et al.*, 2004
	AZP-R	USA	Cry1Ac	Cry1Aa[#] Cry1Ab Cry1Bb[#]	Recessive					X														González-Cabrera *et al.*, 2003; Morin *et al.*, 2003; Tabashnik *et al.*, 2004

Species	Strain	Country	Toxin	Cross-resistance	Mode of inheritance	Mechanism of resistance																References
						A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	
Pectinophora gossypiella	Bt4R	USA	Cry1Ac	-	Recessive					X														Fabrick & Tabashnik, 2012
	MOV97-R	USA	Cry1Ac	-	Recessive					X														Morin *et al.*, 2003; Tabashnik *et al.*, 2005
	r1r1	USA	Cry1Ac	-	-					X														Fabrick *et al.*, 2011
	r2r2	USA	Cry1Ac	-	-					X														Fabrick *et al.*, 2011
	r3r3	USA	Cry1Ac	-	-					X														Fabrick *et al.*, 2011
	SAF97-R	USA	Cry1Ac	-	Recessive					X														Morin *et al.*, 2003; Tabashnik *et al.*, 2005
	TX01-R	USA	Cry1Ac	-	Recessive					X														Morin *et al.*, 2003
Plodia interpunctella	133-r	USA	*Bta*[o]	-	-			X																Oppert *et al.*, 1997
	198-r	USA	*Bte*[p]	-	-	X		X					X					X	X					Oppert *et al.*, 1996; Oppert *et al.*, 1997; Herrero *et al.*, 2001; Candas *et al.*, 2002
	343	USA	*Btk*[c]	Cry1C[q]	-				X															Van Rie *et al.*, 1990
	Dpl-r	USA	*Btk*[c]	Cry1Ac	-				X															Herrero *et al.*, 2001
Plutella xylostella	Multiple	Brazil	*Bta*[o] *Btk*[c#]	-	-													X						Zago *et al.*, 2014
	SZ-R (T2-R)	China	Cry1Ac	-	-											X[r]								Guo *et al.*, 2015b
	SH-R	China	Cry1Ac	-	-											X[r]								Guo *et al.*, 2015b

Species	Strain	Country	Toxin	Cross-resistance	Mode of inheritance	Mechanism of resistance																References
						A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	
Plutella xylostella	*Btk*-Sel	Malaysia	Btk$^{c\#}$	Cry1Ac	Partially recessive / dominant^{m}				X															Sayyed *et al.*, 2000
	Bta-Sel	Malaysia	*Bta*$^{o\#}$	Cry1Ac	Partially recessive / dominant^{m}				X															Sayyed *et al.*, 2000
	Cry1Ab-Sel	Malaysia	Cry1Ab	Cry1Ac	Partially recessive / dominant^{m}				X															Sayyed *et al.*, 2000
	Cry1Ac-Sel	Malaysia	Cry1Ac	*Bta*^{o} *Btk*^{c} Cry1Ab$^{\#}$	Partially recessive^{s}	X*	X*		X															Sayyed *et al.*, 2000; Sayyed *et al.*, 2001
	No name	Philippines	Cry1Ab	-					X															Ferré *et al.*, 1991
	PHI	Philippines	Cry1Aa Cry1Ab Cry1Ac	-	Various^{t}				X															Tabashnik *et al.*, 1997b; Ballester *et al.*, 1999
	Cry1Ac-R (DBM1Ac)	USA	Cry1Ac	-	Incompletely recessive				X	X*			X		X	X^{u}								Tang *et al.*, 1996; Tang *et al.*, 1997; Tang *et al.*, 1999; Yang *et al.*, 2012; Guo *et al.*, 2015b
	NIL-R (BC6F4)	USA	Cry1Ac	Cry1Ab Cry1Ah	Recessive				X*															Zhu *et al.*, 2015
	NO-95C	USA	Cry1C	-	Incompletely dominant^{m}	X*		X*																Liu & Tabashnik, 1997; Liu *et al.*, 2000

Species	Strain	Country	Toxin	Cross-resistance	Mode of inheritance	Mechanism of resistance																References
						A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	
Plutella xylostella	NO-P	USA	Cry1Ac	*Btk*[c]	Recessive				X															Tabashnik *et al.*, 1994
	NO-Q	USA	Cry1Ac	-	Recessive				X															Tabashnik *et al.*, 1992; Tabashnik *et al.*, 1994
	NO-QA	USA	Cry1Aa Cry1Ab Cry1Ac	Cry1Fa Cry1Ja H04[v]	Partly to completely recessive[w]				X													X[*]		Luo *et al.*, 1997b; Tabashnik *et al.*, 1997a; Tabashnik *et al.*, 1997b; Ballester *et al.*, 1999; Tabashnik *et al.*, 2000
	NO-QAGE	USA	Cry1Ac	-	Recessive											X								Baxter *et al.*, 2011
	NO-R	USA	Cry1Ac	-	-				X															Tabashnik *et al.*, 1994
	NO-U	USA	Cry1Ac	-	-				X															Tabashnik *et al.*, 1994
	PEN	USA	Cry1Aa Cry1Ab Cry1Ac	Cry1F Cry1J	Incompletely recessive[w]				X												X			Tabashnik *et al.*, 1997b; Ballester *et al.*, 1999
	PXR	Japan	Cry1Ac	-	-															X				Kumaraswami *et al.*, 2001
	SZBT	China	Cry1Ac	Cry1Aa Cry1Ab Cry1F	Incompletely recessive				X															Gong *et al.*, 2010
	Cry1C-Sel	USA	Cry1C	-	Recessive[x]				X															Zhao *et al.*, 2000
Sesamia inferens	No name	China	Cry1Ab Cry1Ac	-					X															Han *et al.*, 2014
Spodoptera exigua	No name	USA	Cry1Ca	Cry1Ab Cry1H Cry2A									X[y]								X			Moar *et al.*, 1995; Herrero *et al.*, 2005

Species	Strain	Country	Toxin	Cross-resistance	Mode of inheritance	Mechanism of resistance																References
						A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	
Spodoptera exigua	Xen-R[z]	USA	*Bta*[o]	-	Completely recessive											X								Hernández-Martínez *et al.*, 2010; Park *et al.*, 2014
Spodoptera frugiperda	456, 512	USA	Cry1Fa	456: Cry1Ab Cry1Ac	Recessive										X									Jurat-Fuentes *et al.*, 2011; Jakka *et al.*, 2015
Spodoptera littoralis	No name	Israel	Cry1C	-				X																Sneh *et al.*, 1981; Keller *et al.*, 1996
Spodoptera litura	No name	India	Cry1C	-	-			X																Barkhade & Thakare, 2010
Trichoplusia ni	GLEN-Cry1Ac-BCS	Canada	Cry1Ac	Multiple[r]	Recessive								X			X								Baxter *et al.*, 2011; Tiewsiri & Wang, 2011

A: toxin activation defects; B: toxin sequestration/coagulation; C: altered proteinase activity; D: altered/loss of binding site (not yet identified); E: mutation in CDH; F: altered transcription of CDH; G: mutation in APN; H: altered transcription of APN; I: mutation in APP; J: altered transcription of ALP; K: mutation in ABC transporter protein; L: enhanced epithelial regeneration/healing; M: physiological adaptation; N: enhanced immune status; O: reduced glycolipid levels; P: increased nonspecific toxin-binding; Q: toxin-binding receptor shedding; R: altered lipid rafts

- Unknown;
* Only putative or speculative at this stage or no direct correlation with resistance established yet;
[#] Only low or moderate level of cross-resistance;
[a] Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa;
[b] Degree of dominance decreased as resistance increased;
[c] *kurstaki* (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Cry2Ab, Cry2B);
[d] Incompletely recessive for the r1 allele, but completely recessive for the r2 and r3 alleles;
[e] SCD (susceptible) x GYBT (resistant);
[f] Conflicting results: completely recessive (Yang *et al.*, 2009); incompletely recessive (Zhao *et al.*, 2010);
[g] Deltamethrin, chlorpyrifos, profenofos, cypermethrin, spinosad, indoxacarb, abamectin;
[h] CPN (susceptible) x CP73-3 (resistant);
[i] Induction of different active proteolytic enzymes instead of altered proteinase activity;
[j] CPN (susceptible) x KCB (resistant);
[k] Cadherin-like protein (CaLP);
[l] Total absence of CaLP and not mutation in CDH;
[m] Dominant at low toxin concentrations; recessive at high toxin concentrations;
[n] Europe-R x Nebraska-R (not noted in table – no mechanism identified for this strain);
[o] *aizawai* (Cry1Aa, Cry1Ab, Cry1Ca, Cry1Da);
[p] *entomocidus* (Cry1C);
[q] Negative cross-resistance, i.e. resistance is associated with increased susceptibility to Cry1C and increased number of Cry1C receptors;
[r] Cry1Aa[#], Cry1Ab, Cry1Bb[#], Cry1C[#], Cry1D[#], Cry1E[#], Cry1F[#], Cry1J[#], Cry2Ab[#], Cry9C[#];
[s] Partially recessive at lowest toxin concentration;
[t] Dominance varies for different toxins: recessive for Cry1Ab, semi-dominant for Cry1Ac and dominant for Cry1Aa;
[u] Not mutation – down regulation of the ABCG transporter gene, *Pxwhite*;
[v] H04 (hybrid with domains I and II of Cry1Ab and domain III of Cry1C);
[w] For Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa toxins;
[x] Incompletely recessive (also more recessive at higher toxin concentrations) when using the leaf dip assay; recessive when using Cry1C transgenic broccoli;
[y] Total lack of expression of APN1;
[z] No name resistant colony selected every two generations with Xentari™ to maintain selection pressure.

3.2 Systematic approach to finding reported mechanisms of resistance

Firstly, a combination of appropriate keywords (resistance mechanism Lepidoptera cry toxin) was used, which yielded ~ 62,000 results. Of these, several of the most recent publications were selected and reviewed regarding resistance mechanisms. Wherever a resistance mechanism was reported, information regarding the publication, species, strain, location, toxin, cross-resistance, mode of inheritance and mechanism associated with resistance were recorded in a spreadsheet. In-text/secondary references (in case the reported mechanism was not original), were also recorded. Secondary references were then searched, read, and information recorded. This step was reiterated until all references regarding resistance to Cry toxins in Lepidoptera were recorded. If a publication only reported on resistance and no mechanism was identified, such an article was excluded from the final table. In total, 351 publications were consulted. However, 228 publications were excluded based on the following reasons: no mechanism of resistance was identified (219), resistance was reported on coleopteran insects (4) and some assays only used gut contents (5). The remaining 123 publications that reported on mechanisms of resistance to Cry toxins in lepidopteran insects were included in the study.

3.3 Resistance mechanisms in Lepidoptera

Table 3.1 provides a summary of all the mechanisms of Cry toxin resistance that have been reported for lepidopteran pests from laboratory, greenhouse and field studies. For the purpose of conciseness, several mechanisms were grouped under one sub-header and selected examples are discussed below.

3.3.1 Solubilization and incomplete toxin processing

In order for the Cry toxins to become biologically active, the crystalline inclusions need to be solubilized in the midgut of the insect larva (Schnepf *et al.*, 1998). Reduced solubilization of protoxin crystals may result in reduced conversion of protoxin to toxin and reduced enzymatic activation by proteases and ultimately lead to resistance (Bradley *et al.*, 1995). Resistance due to incomplete crystal solubilization is, however, only of concern when sprayable *Bt* formulations are used. This resistance mechanism is not a concern in *Bt* transgenic crops, since these crops produce Cry protoxins or partially activated toxins (Van der Hoeven, 2014). Still, these Cry protoxins or partially activated toxins require further proteolytic activation once they are ingested by insects (Van der Hoeven, 2014).

Resistance due to incomplete toxin activation and processing has been proposed for the Cry1C-resistant *P. xylostella* strain NO-95C from the USA (Liu *et al.*, 2000), as well as the CrylAc-resistant strain (Cry1Ac-Sel) of *P. xylostella* from Malaysia (Sayyed *et al.*, 2001). An alternative mechanism has also been suggested, whereby protoxin activation is prevented by degradation or precipitation of the solubilized toxin (Sayyed *et al.*, 2001).

Lightwood *et al.* (2000) established a correlation between the proteolytic cleavage of Cry proteins by specific host proteases and the toxicity of Cry proteins. Changes in protease expression may cause Cry protoxins to be differentially processed, thus affecting their toxicity. The exact mechanism of altered proteinase activities is still unclear, but could be the result of blocked or altered transcription and/or translation of the genes encoding these proteinases or inhibition of enzyme synthesis and/or secretion (Oppert *et al.*, 1996). Even so, Rausell *et al.* (2004) emphasized that the activity of host proteases (also called proteinases) could also be influenced by larval development stage, starvation, dietary changes and the presence of inhibitors or toxic compounds.

Altered protease expression has been identified as a resistance mechanism in several lepidopteran species (Table 3.1). For example, in the case of *O. nubilalis* (strain KS-SC) which is resistant to *Btk* (*Bt* var. *kurstaki*) spray formulations, the expression of the T23 gene encoding a full-length trypsin-like proteinase was reduced (Li *et al.*, 2005b). Similarly, downregulation of protease genes conferred Cry1Ac-resistance to a *H. armigera* strain (Akola-R) from India (Rajagopal *et al.*, 2009) and another strain (LF5) from China (Liu *et al.*, 2014). Studies to determine the proteolytic activation of specific Cry proteins are therefore important to understand how such toxins are completely activated in target pests.

3.3.2 Modified Cry toxin-binding sites and differential gene expression

Normally, activated monomeric toxins move through the peritrophic matrix to where toxin binding receptors are located (Krishnamoorthy *et al.*, 2007; Soberón *et al.*, 2009). In many cases, the target binding sites are still unidentified, but it is known that alterations in these sites are associated with resistance (Sayyed *et al.*, 2000; Zhao *et al.*, 2000; Akhurst *et al.*, 2003; Downes & Mahon, 2012; Han *et al.*, 2014). Selection experiments with species in the Noctuidae, Pyralidae and Plutellidae families (Lepidoptera) under laboratory conditions, as well as greenhouse and field experiments have demonstrated that mutations (insertions or deletions) in certain Cry toxin-receptor genes is the most common mechanism that confers resistance (Tabashnik *et al.*, 2003). This mechanism of resistance has been identified in numerous lepidopteran species (Table 3.1). It was observed that resistance might be accompanied by a loss in toxin-binding in some insect species, while others may evolve resistance with toxin-

binding still taking place (Li *et al.*, 2004a; Pigott & Ellar, 2007). In such cases, it is hypothesized that post-binding events could be altered without inhibiting the binding of toxins (González-Cabrera *et al.*, 2003). This also supports the multistep mode of action (Figure 3.1) where Cry1A toxins interact with multiple receptors in order to form functional pores in midgut membranes.

Resistance to Cry1Ac in nine strains of *P. gossypiella* from the USA was linked with four alleles (*r1*, *r2, r3 and r4*), each carrying a different mutation which codes for an incomplete CDH Cry toxin receptor (Morin *et al.*, 2003; 2004; Tabashnik *et al.*, 2004; 2005; Fabrick *et al.*, 2011; Fabrick & Tabashnik, 2012). These *r1, r2* and *r4* mutations resulted from deletions, whereas the *r3* mutation resulted from an insertion of a large DNA fragment, i.e., transposable element. Additionally, eight resistance alleles (*r5-r12*) associated with three Cry1Ac-resistant *P. gossypiella* strains from western India were recently discovered (Fabrick *et al.*, 2014). Seven of these alleles elicited alternative splicing, resulting in two or more different transcript isoforms. Likewise, 15 CDH resistance alleles have been identified in several strains of *H. armigera* from China and one strain from western India (Xu *et al.*, 2005; Yang *et al.*, 2006; 2007; 2009; Zhao *et al.*, 2010; Zhang *et al.*, 2012a; 2012b; Nair *et al.*, 2013; Zhang *et al.*, 2013).

In the case of two Cry1Ac-resistant *H. virescens* strains (KCBhyb and YHD2) from the USA, resistance was due to the absence (Jurat-Fuentes *et al.*, 2004) and retro-transposon mediated disruption (Gahan *et al.*, 2001) of a cadherin-like protein (CaLP), respectively. Several major mutations in the *CaLP* and *cdh* genes contributed to Cry toxin resistance in strains of *O. furnacalis* and *O. nubilalis*, respectively (Bel *et al.*, 2009; Jin *et al.*, 2014). It was further suggested that the *cdh* mutations in *O. nubilalis* only contributed to resistance additively, since no difference was found in the expression of the *cdh* gene between the resistant and susceptible strains (Siqueira *et al.*, 2006; Bel *et al.*, 2009). According to Zhang *et al.* (2012a) all these results propose that both pore formation and cell signalling play a role in Cry protein toxicity (Pigott & Ellar, 2007).

Carrière *et al.* (2004) proposed that mutations in *cdh* genes increase gut membrane permeability to toxic substances, thus resulting in fitness costs. This is plausible, since CDH proteins contribute to maintaining midgut membrane integrity (Midboe *et al.*, 2003). The only reports of resistance due to downregulation of *cdh* gene expression are for the Cry1Ab-RR strain of Cry1Ab-resistant *D. saccharalis* (Yang *et al.*, 2011), the YHD2-B strain of Cry1Ac-resistant *H. virescens* (Jurat-Fuentes *et al.*, 2011) and the Cry1Ac-R/DBM1Ac strain of Cry1Ac-resistant *P. xylostella* (Yang *et al.*, 2012). It has not yet been determined whether downregulation of this gene is due to mutations.

The aminopeptidase Cry toxin-receptor has also been implicated in resistance. Like the downregulated *cdh* gene, reduced expression of three *apn* genes was also functionally associated with resistance in the Cry1Ab-RR strain of *D. saccharalis* (Yang *et al.*, 2010). According to Yang (2011), the reduced expression of the *cdh* and *apn* genes in this strain of *D. saccharalis* was not due to mutations in these genes, which suggests that expression is regulated by *cis*- or *trans*-acting elements. For *H. armigera*, Zhang *et al.* (2009) demonstrated that Cry1Ac resistance in the BtR strain of this species was due to a mutation in the *apn1* gene, which codes for the Cry1Ac receptor. Furthermore, a mutation of two amino acids in the *app* Cry toxin-receptor gene was reported to be associated with resistance in two Cry1Ab-resistant *O. nubilalis* strains (RSTT-R and SKY-R) from Italy-USA and the USA, respectively (Khajuria *et al.*, 2011). There were, however, no changes in expression levels of this *app* gene. Cry1Ac resistance in the GLEN-Cry1Ac-BCS strain of *T. ni* was associated with differential alteration of two midgut aminopeptidases, APN1 and APN6 (Tiewsiri & Wang, 2011). Only the downregulation of APN1 correlated with resistance. Interestingly, these alterations were not caused by mutations in these genes, but rather differential transcription. These authors proposed that these alterations are conferred by an unidentified *trans*-regulatory mechanism. Similarly, the resistant strains of *H. zea* (AR1), *O. nubilalis* (Cry1Ab^{R}) and *P. xylostella* (Cry1Ac-R/DBM1Ac) had altered *apn* transcription levels (Caccia *et al.*, 2012; Yang *et al.*, 2012; Coates *et al.*, 2013).

Alternatively, some studies hypothesized that reduced receptor activity may be caused by the selective solubilization of GPI-anchored proteins (such as ALP or APN) in resistant species. This results in removal of potential Cry toxin-binding sites (Lu & Adang, 1996; Luo *et al.*, 1997b). It was demonstrated that solubilized APN was still able to bind the toxins, thus simultaneously sequestering these toxins. In the case of a Cry1Ca-resistant strain of *S. exigua*, total lack of APN1 expression was observed. Linkage between resistance and this lack of expression has not yet been determined (Herrero *et al.*, 2005). Also, in the case of the 198-r strain of *P. interpunctella* resistant to *Bte* (*Bt* var. *entomocidus*) spray formulations, it was suggested that the altered transcription of *apn* contributed to an enhanced immune status (Candas *et al.*, 2002).

In other research with *H. virescens,* Jurat-Fuentes *et al.* (2002) initially hypothesized that resistance to Cry1Ac toxins in this species was due to altered glycosylation of microvilli proteins, which resulted in reduced toxin-binding and pore formation. Later, Jurat-Fuentes and Adang (2004) established that this resistance was due to decreased levels of ALP. Strains of Cry1Ac-resistant *H. armigera* and *P. xylostella* and Cry1Fa-resistant *S. frugiperda* also revealed reduced ALP levels, suggesting that this is a common occurrence in lepidopterans (Jurat-

Fuentes *et al.*, 2011). In the case of the BtR strain of *H. armigera*, no direct correlation was found between the reduced ALP levels and resistance (Jurat-Fuentes *et al.*, 2011). It has not yet been determined if reduced expression of ALP in these four species is due to mutations.

In contrast to the above-mentioned decreases in ALP levels, a Cry1Ac-resistant strain of *H. zea* showed significantly reduced ALP transcript levels, but increased ALP activity (Caccia *et al.*, 2012). Soluble ALP was also present in the midgut lumen, suggesting simultaneous toxin sequestration. Previously, a study demonstrated that ALP in *B. mori* could be solubilized by enzymes present in the midgut epithelium (Eguchi *et al.*, 1972). The solubilized ALP was also found to be extremely resistant to protease degradation. It has been suggested that these different intracellular phosphatase levels might be involved in Cry toxin-mediated signalling (Jurat-Fuentes & Adang, 2006). Furthermore, Jurat-Fuentes *et al.* (2011) proposed that the reduced expression of *alp* may be used as a potential biomarker for resistance to diverse Cry proteins. Further research is, however, needed to determine if the reduced ALP levels cause a direct decrease in the number of functional Cry toxin receptors or other beneficial alterations in resistant larvae. Three hypotheses were proposed to explain the reduced ALP levels in the YHD2-B/YHD3 Cry1Ac-resistant strain of *H. virescens,* namely, (i) changes in gene copy number or transcription; (ii) solubilization of GPI-anchored ALP; or (iii) modified lipid rafts that affected the number of ALP receptors (Jurat-Fuentes & Adang, 2004). It is evident that more research is needed to characterize this protein and its physiological role in lepidopteran larvae.

The pore formation model proposes two binding steps, namely, binding to CDH protein and binding to GPI-anchored proteins (ALP or APN). Heckel (2012) suggested that there is an additional binding step where Cry toxins bind to the open configuration of the ABC transporter protein, which facilitates subsequent membrane insertion. Recently, several studies demonstrated that a mutation in the gene encoding the ABCC2 transporter protein was linked to Cry resistance. This resistance mechanism has been observed in *B. mori* (Atsumi *et al.*, 2012; Tanaka *et al.*, 2013), *H. armigera* (Xiao *et al.*, 2014), *H. virescens* (Gahan *et al.*, 2010), *P. xylostella* (Baxter *et al.*, 2011) and *S. exigua* (Park *et al.*, 2014). Resistance in a strain of *T. ni* was genetically linked with the *ABCC2* gene, but no mutation has yet been identified in this strain (Baxter *et al.*, 2011). Nevertheless, it seems that the function of this gene is conserved in lepidopteran species (Atsumi *et al.*, 2012). Additionally, *H. armigera* and *H. punctigera* evolved resistance to Cry2Ab toxin due to mutations in the *ABCA2* gene (Tay *et al.*, 2015). Screening of *ABC* transporters as candidate resistance genes in all resistant lepidopteran strains is thus encouraged. In the case of three other resistant strains of *P. xylostella* (SZ-R, SH-R and Cry1Ac-R/DBM1Ac), resistance was not caused by a mutation in an *ABC* gene, but rather downregulation of an *ABCG* gene named *white* (Guo *et al.*, 2015b). These authors suggested

that the *white* gene in *P. xylostella* might encode a Cry-toxin receptor, thus acting as both transporter and receptor of Cry toxins. Further investigation is necessary to test this hypothesis. The structure and function of this protein in insects are still unknown, but seem to be implicated in Cry toxin resistance in a large number of lepidopteran pests.

3.3.3 Other mechanisms

When toxins are completely activated, they need to pass through the gut peritrophic membrane to reach the midgut epithelial cells, where the Cry toxin receptors are located, to exert toxic effects. Toxin immobilization has, however, been proposed as a possible mechanism of resistance. Toxin immobilization occurs when sequestration or coagulation prevents toxins to pass through the peritrophic gut membrane (Gunning *et al.*, 2005; Ma *et al.*, 2005). Also, active toxins may get trapped due to binding with sites within the membrane or decreased peritrophic membrane permeability (Milne *et al.*, 1998; Heckel *et al.*, 2007; Bravo *et al.*, 2011). The latter was observed in a *H. armigera* strain, where chitin deacetylase genes, involved in increasing membrane permeability, were downregulated (Han *et al.*, 2015).

When Cry toxins are sequestered by nonspecific carboxylesterases (hydrolysing enzymes) in the insect gut, it renders the toxins harmless before they reach the receptors. Such esterase-based resistance mechanisms in insects are not uncommon (Gunning *et al.*, 2005). This esterase-based resistance mechanism was observed in the CryIAc-resistant strain (silver) of *H. armigera* from Australia, where both Cry1Ac protoxin and activated toxin were bound by esterase, contrary to susceptible *H. armigera* where esterase did not bind to Cry1Ac protein (Gunning *et al.*, 2005). The same mechanism of resistance was suggested for the CryIAc-resistant strain (Cry1Ac-SEL) of *H. armigera* from Pakistan (Alvi *et al.*, 2012). However, Heckel *et al.* (2007) argued that this conclusion was invalid due to lack of evidence linking or co-segregating these enzymes or their level of activity to *Bt* resistance.

In another study, an insoluble coagulum was formed in a Cry1Ac-resistant *H. armigera* strain (ISOC4) from Australia. This was due to an increased level of hexamerin, a larval serum protein. Hexamerin interacted with the toxin and aggregated to form the insoluble coagulum and possibly elevated the immune response of the target pest (Akhurst *et al.*, 2003; Ma *et al.*, 2005).

Although the molecular regulation of epithelial regenerative mechanisms in resistance to Cry proteins are not yet understood, several studies found a direct correlation between midgut stem cell-mediated regeneration and resistance to Cry1Ac in four strains (CP73-3, CXC, KCB and no name) of Cry1Ac-resistant *H. virescens* from the USA (Forcada *et al.*, 1999; Martínez-Ramírez *et al.*, 1999; Loeb *et al.*, 2001; Castagnola & Jurat-Fuentes, 2009). A comparison of the

secretomes of susceptible and resistant *H. virescens* larvae revealed differentially secreted proteins, and the resistant strain showed efficient regeneration of midgut cells in response to Cry toxin treatment (Castagnola & Jurat-Fuentes, 2009). Specifically, in one of the *H. virescens* strains, increased stem cell differentiation was presumably directed by the upregulation of peptidic factor MDF1, which induces stem cell differentiation (Loeb *et al.*, 2001). This suggests that larvae may recover completely after intoxication due to epithelial regeneration and healing. Castagnola and Jurat-Fuentes (2009) suggested that the latter may be ascribed to specific proteins (likely growth factors) that are produced and secreted. Indeed, REPAT and arylphorin have been identified as candidate genes involved in epithelial healing (Park *et al.*, 2014; Guo *et al.*, 2015a). A recent study from Guo *et al.* (2015a) furthermore linked the downregulation of *ALP* and two *ABCC* toxin receptor genes with a MAPK signalling pathway in a Cry1Ac-resistant strain of *P. xylostella*. These authors proposed a coordinated response model where the MAPK signalling pathway upregulates genes involved in epithelial healing (such as REPAT and arylphorin) whilst downregulating Cry toxin-receptor genes (such as ALP and ABCC).

Previous studies demonstrating signalling pathways to be activated in the presence of *Bt* toxins only implicated the involvement of protein kinases and phosphatases (Zhang *et al.*, 2006; Tanaka *et al.*, 2012; Guo *et al.*, 2015a). The role of lipids in signal transduction was reviewed by Eyster (2007), who indicated that lipids also mediate signalling pathways. Kumaraswami *et al.* (2001) observed that a Cry1Ac-resistant *P. xylostella* strain had reduced glycolipid content levels in its brush border membrane vesicles, which might suggest that the physicochemical features of the plasma membrane are altered so that toxin penetration is obscured, or that resistance is caused by internal signalling.

Changes in immune status have previously also been associated with resistance. For example, *P. interpunctella* strain 198-r, resistant to *Bte* (*Bt* var. *entomocidus*) spray formulations, had an enhanced immune status due to altered transcription of APN. Additionally, resistance was associated with reduced levels of alkaline chymotrypsin (proteinase), as well as increased levels of midgut enzymes associated with oxidative metabolism (Candas *et al.*, 2002). It was hypothesized that the latter might be a physiological adaptive response to facilitate detoxification. Similarly, multiple resistant strains of *P. xylostella* evolved physiological resistance by means of altered behavioural responses, indicated by reduced oviposition on surfaces treated with *Bt* formulations (Zago *et al.*, 2014).

Rahman *et al.* (2007) indicated that *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) was able to develop tolerance to a commercial spray formulation of Cry toxins due to toxin coagulation by lipophorin particles. It has also been demonstrated that a Cry1C-resistant strain of *S. exigua* had an increased level of nonspecific toxin binding in comparison to the susceptible

strain (Moar *et al.*, 1995). This occurrence may implicate nonspecific binding in resistance mechanisms, wherein nonspecific binding interferes or competes with specific high-affinity binding, subsequently reducing pore formation in larval midgut membranes (Moar *et al.*, 1995).

Another hypothesis that has been proposed for resistance evolution is bacterial involvement, where indigenous midgut bacteria are allegedly essential for *Bt* toxicity (Broderick *et al.*, 2006). This theory is highly controversial and several studies have been done to prove and disprove it (Broderick *et al.*, 2009; Johnston & Crickmore, 2009; Raymond *et al.*, 2009; Paramasiva *et al.*, 2014; Caccia *et al.*, 2016). These controversial results might be due to the fact that this phenomenon is species specific. Paramasiva *et al.* (2014) indicated that the microbiota diversity not only differs in different insect species, but also varies according to the preferred host plants and geographical regions where these insects occur. More studies are needed to investigate the relationship between bacteria and resistance to Cry proteins.

3.4 Factors contributing to resistance

Numerous resistant strains reported in this manuscript showed cross-resistance to one or more other Cry toxins, i.e., resistance to one toxin also conferred resistance to other toxins (Table 3.1). Only one strain of *P. interpunctella* showed negative cross-resistance: resistance to the Cry1Ab toxin was associated with an increased susceptibility to the Cry1C toxin, as well as an increase in the number of Cry1C toxin-receptors (Van Rie *et al.*, 1990).

Even though fitness costs were not included in this study, it is crucial to also consider this factor when developing IRM strategies. It has been demonstrated that fitness costs and cross-resistance patterns varied in resistant *P. xylostella* strains from different locations (Zhu *et al.*, 2015), laying emphasis on the importance of geographical differences.

A recent study from Deans *et al.* (2016) suggested that nutritionally mediated effects may explain the variable efficacy in *Bt* that has been observed in laboratory and field studies. This is mainly because diets used in laboratory studies are generally artificial, nutritionally suboptimal and not ecologically realistic. These authors caution against overestimation of insect susceptibility to *Bt* toxins and emphasize that phenotypic plasticity (where one genotype can produce multiple phenotypes) may occur under different environmental conditions.

Other factors that also need to be considered for IRM strategies are the biology and physiology of insects, environmental factors (including diet-Cry interactions), Cry toxin mode of action, dominancy and initial frequency of resistance alleles, mode of inheritance and genetic differentiation of populations. The latter is important since susceptible insect populations with

low genetic diversity may be genetically predisposed to develop the same mechanism of resistance as the resistant populations.

3.5 Conclusion

The table in this mini review provides a comprehensive summary of resistance mechanisms – to conventional *Bt* insecticides, as well as *Bt* crops – that have been identified in lepidopteran insects. Selected examples discussed in the text demonstrate that *Bt* resistance evolution is extremely complex and influenced by interactions among various factors. From all these resistance studies, the most common resistance mechanism seems to be altered binding sites (Cry receptors and ABC transporter protein). It is yet to be established whether all these altered binding sites are regulated by a MAPK signalling pathway, which might suggest a universal mechanism of resistance in lepidopterans. However, seeing as various factors contribute to resistance evolution, studies on resistance mechanisms should be done on a case-by-case basis and IRM strategies should be adapted accordingly. An understanding of biological, ecological and genetic data is crucial to devise long-term sustainable IRM strategies.

Chapter 4

Transcriptome and differentially expressed genes of *Busseola fusca* larvae challenged with Cry1Ab toxin

4.1 Introduction

The African maize stem borer, *B. fusca*, occurs throughout sub-Saharan Africa where it causes economic damage to maize and sorghum crops (Kfir *et al.*, 2002). This pest has evolved non-recessive field resistance to *Bt* maize expressing the Cry1Ab toxin to control this pest (Van Rensburg, 2007; Campagne *et al.*, 2013). Agricultural pests are usually non-model organisms, as is the case with *B. fusca*, and thus very little genomic information is available in public databases (Camargo *et al.*, 2015). At present, the only available sequences for this insect are limited to some COI (Peterson *et al.*, 2016), cyt b (Sezonlin *et al.*, 2006a; 2006b; 2012; Peterson *et al.*, 2016) and microsatellite data (Faure & Silvain, 2005). The distribution, diapause and reproductive biology (sex ratio, longevity and fecundity) of *B. fusca* is described in Calatayud *et al.* (2014), Hauptfleisch *et al.* (2014), Kfir *et al.* (2002) and Kruger *et al.* (2012), but is still poorly understood. This, in combination with its larval-specific behaviour, may pose problems for managing the spread of current resistant *B. fusca* populations or resistance development in other susceptible populations of this pest (Van den Berg & Campagne, 2015). Moreover, small-scale farming systems in Africa, farming practices and the cultivation of open pollinated varieties (OPVs) contribute to landscape heterogeneity (Van den Berg & Campagne, 2015). This also complicates management of resistance evolution in this pest.

An understanding of the molecular basis of development, growth, reproduction and mechanisms of resistance to Cry toxins designated to control insect pests is required to develop alternative GM crops (Li *et al.*, 2012). Since only a fraction of the genome is transcribed and mRNA represents only the vital part of the genome, transcriptome sequencing is a perfect cost-saving tool for understudied non-model organisms such as *B. fusca* (Haas *et al.*, 2013). Recently, several studies employed mRNA sequencing and *de novo* transcriptome assemblies for various lepidopterans (Vera *et al.*, 2008; Miller *et al.*, 2012; Camargo *et al.*, 2015; Nascimento *et al.*, 2015; Perera *et al.*, 2015; Liu *et al.*, 2016; Tassone *et al.*, 2016). Transcriptional analyses show the molecular basis of an organism's development or response to certain environmental factors or conditions, thus enabling novel ways to manage pests. For example, gene targets can be identified for gene silencing using the RNAi approach (Camargo *et al.*, 2015). The current study characterized the larval transcriptome of *B. fusca*, and also compared the gene expression

profiles between *Bt*-challenged and unchallenged larvae to enhance our understanding of the molecular basis of the Cry1Ab resistance mechanism of this non-model organism. This is the first study on gene expression profiles of *B. fusca* strains challenged with Cry1Ab toxin. Additionally, amino acid sequences of Cry toxin receptors, ABC transporters and MAPK transcripts, that have previously been implicated in *Bt* resistance, were aligned and compared to other lepidopteran species. The aim of the sequence alignments was to detect mutations that might potentially be contributing to Cry toxin resistance in *B. fusca.* The postulated hypothesis is that Cry1Ab toxin resistance might be mainly controlled by a MAPK signalling pathway, in the same manner that Cry1Ac toxin resistance was conferred to *P. xylostella* strains (Guo *et al.*, 2015a). Together, these results may aid development of sustainable pest and resistance management tools and strategies.

4.2 Material and Methods

An overview of the methods for this chapter is illustrated in the workflow diagram presented in Figure 4.1.

4.2.1 Larval collection, rearing and toxin challenge

B. fusca larvae (4th instar stage) were collected from Cry1Ab expressing *Bt* maize (PAN 5Q749BR, group S0-Bt) in three villages (Nxanxadi, Ndukudeni and Maliwa), as well as non-*Bt* maize (OPVs, group S0-nBt) in Noqhekwana, in the Eastern Cape province of SA. Larvae at these localities have recently been reported to be highly susceptible to Cry1Ab *Bt* maize (Kotey *et al.*, 2017). Larvae collected from *Bt* maize were maintained on tissue of *Bt* plants (NR29Phb30y79B) under laboratory conditions, but failed to complete their lifecycle. On the other hand, larvae collected from non-*Bt* maize were placed in a container with tissue of non-*Bt* maize (NR23Phb30y83) stalks under the same conditions and they completed their life cycle. In order to investigate if genes are differentially expressed when larvae are challenged with the Cry1Ab toxin, neonate larvae from group S0-nBt were placed on *Bt* (group S1-Bt) and non-*Bt* (group S1-nBt) maize plant tissue (Figure 4.1). Larvae were allowed to feed for three days prior to RNA isolation. The direct feeding (*in vivo*) approach was chosen to simulate biologically relevant conditions.

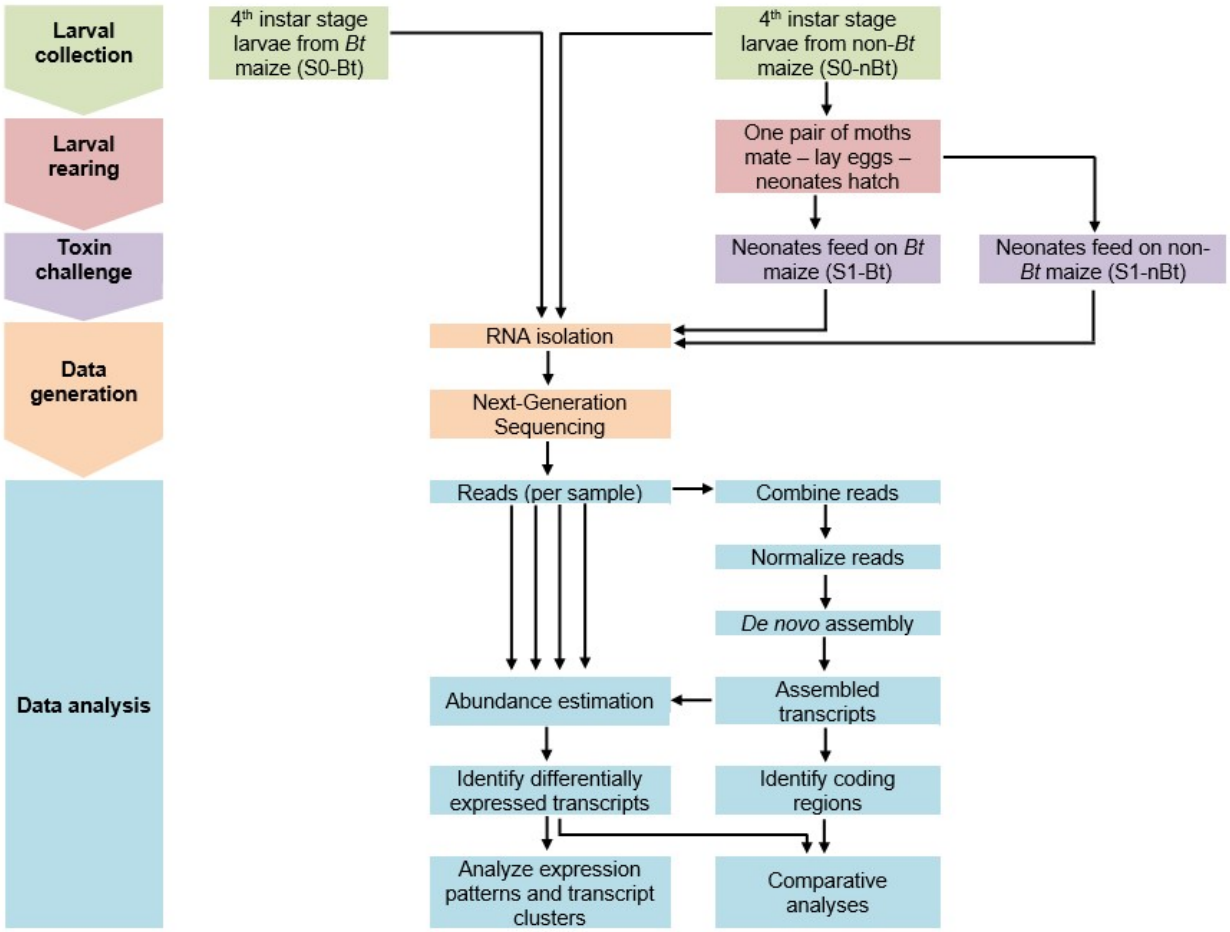


Figure 4.1: Workflow diagram demonstrating sample preparation, data generation, *de novo* transcriptome assembly and downstream analyses. Sample names follow the format Sx-y, where 'S' represent susceptible larvae, 'x' is either 0 (founding generation) or 1 (second generation) and 'y' is either Bt or nBt to indicate the type of maize tissue (*Bt* or non-*Bt*, respectively) on which the larvae were feeding.

4.2.2 RNA isolation and sequencing

RNA was isolated from (i) 9 individual whole larvae: 5 from *Bt* maize (S0-Bt) and 4 from non-*Bt* maize (S0-nBt) and (ii) six groups of pooled neonate larvae: three from *Bt* (S1-Bt) and three from non-*Bt* (S1-nBt) maize (Figure 4.1). Whole larvae were used for RNA isolation to obtain a good quality assembled and annotated transcriptome for *B. fusca*. Even though neonate larvae were challenged with Cry1Ab toxin to determine if the potential Cry toxin-receptor genes in the midgut are differentially expressed in the *Bt*-challenged group, whole neonates were subjected to RNA isolation since they were too small for isolation of midguts only. Nevertheless, normalization of cDNA and altered criteria (discussed in section 4.4) were applied in differential expression (DE) analysis to account for the dilution effect caused by whole-body RNA isolation. A NucleoSpin® RNA II Kit (Macherey-Nagel, Germany) was used, according to the instructions

of the manufacturer, to isolate RNA from all samples, separately. In the case of the neonate larvae, a tissuelyser (Qiagen, Hilden, Germany) was used to lyse the larval tissue at 50 Hz oscillations for 2 min prior to RNA isolation. Integrity of the RNA was verified with the Qubit 3.0 Fluorometer (Invitrogen, Life Technologies).

The TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) was used to prepare paired-end cDNA libraries for transcriptome sequencing. All 15 RNA samples were sequenced on the Illumina HiSeq 2500 platform (San Diego, CA, USA) at the Biotechnology platform at the Agricultural Research Council – Onderstepoort Veterinary Institute (ARC-OVI). The raw sequence reads of *B. fusca* are available at the National Center for Biotechnology Information Short Read Archive (NCBI SRA) (accession numbers SRR5479218-SRR5479345, Bioproject number PRJNA383837).

4.2.3 *De novo* transcriptome assembly and annotation

All the commands and parameters used to analyse the obtained RNA data are provided in Annexure B. The quality of the raw reads was analysed using the FastQC (v0.0.13) tool. All forward and reverse reads of the four groups (S0-Bt, S0-nBt, S1-Bt and S1-nBt) were combined, respectively, to create two large data files. Prior to the assembly, adapters were trimmed and low quality and short reads were removed using Trimmomatic (v0.36) (Bolger *et al.*, 2014). Bases with quality scores (Q) lower than 15 were considered to be low quality reads, while short reads were considered to be less than 25 nucleotides long. The Trinity platform (v2.2.0) (Grabherr *et al.*, 2011) and Trinity-supported program utilities were used for normalization and *de novo* transcriptome assembly of the RNA-Seq dataset, as described in the protocol of Haas *et al.* (2013) (Figure 4.1). Trinity and Transrate (v1.0.3) (Smith-Unna *et al.*, 2016) were used to assemble and assess the *de novo* 'reference' transcriptome of the combined dataset, respectively. For the assembly, the default parameters were used with maximum coverage of 50 reads per each contig in the normalization step.

For transcript identification, the estwisedb program from the Wise2 package (v2.4.1) (Birney & Copley, 2016) and BLAST were employed. The estwisedb program was used to identify mitochondrial transcripts by comparing a protein profile HMM (Hidden Markov Model) to cDNA sequences. In order to build the profile HMM, mitochondrial amino acid sequences from *B. mori, Melitaea cinxia* L. (Lepidoptera: Nymphalidae)*, Papilio machaon* L. (Lepidoptera: Papilionidae) and *P. xylostella* (GenBank accession numbers: NC_002355, CM002851, NC_018047, NC_024742 and NC_025322, respectively) were aligned using MEGA (v7.0.21) (Kumar *et al.*, 2016). Subsequently, the profile HMM was built using HMMbuilder (v2.0). For the identification of the remaining transcripts, BLAST was used. Firstly, two local lepidopteran libraries were

created from nucleotide and amino acid sequences available in the NCBI database using the makeblastdb program from the BLAST toolkit. The two libraries consisted of 2,547,580 nucleotide and 722,405 amino acid sequences, respectively. Both libraries were used to identify transcripts by BLASTn (nucleotide vs. nucleotide comparison), BLASTp (protein vs. protein comparison) and tBLASTx (translated to protein nucleotide vs. translated to protein nucleotide comparison) tools.

4.2.4 Amino acid sequence alignments and mutation detection

Amino acid sequences of Cry receptors (ALP, APN and CDH), ABCC transporters and MAPKs that were identified in the *de novo* transcriptome of *B. fusca* larvae were aligned with MEGA (v7.0.21) (Kumar *et al.*, 2016). ClustalW DNA matrix with default values for gap and extension penalties was used and the longest open-reading frame for each sequence was translated into amino acid sequences according to NCBI translation table no. 1. These sequence alignments were examined for mutations, since mutations in these specific genes have previously been implicated in Cry toxin resistance (Zhang *et al.*, 2009; Gahan *et al.*, 2010; Fabrick *et al.*, 2014). The aligned amino acid sequences span multiple pages and are therefore available on request.

4.2.5 Abundance estimation and differential expression (DE) analysis

To estimate the expression levels of the Trinity-reconstructed transcripts, the RNA-Seq by Expectation-Maximization (RSEM) software package (v1.3.0) was used (Li & Dewey, 2011; 2016). Firstly, the raw RNA-Seq reads were aligned against the normalized Trinity assembly, after which RSEM was used to estimate the number of RNA-Seq fragments mapping to each isocontig. Since the RSEM software (embedded in Trinity) is currently only compatible with gap-free alignments, it was used in conjunction with the Bowtie aligner (v2.2.9) (Langmead & Salzberg, 2012). Subsequently, abundance estimation with RSEM was done for each sample group (S0-Bt, S0-nBt, S1-Bt and S1-nBt) separately, and then the results were combined for counts matrix generation and cross-sample normalization (Figure 4.1). Finally, expression values (measured in RPKM, reads per kilobase of transcript per million reads) of mapped RNA-Seq fragments were normalized for sequencing depth and transcript length and then scaled via TMM normalization under the assumption that most transcripts are not differentially expressed (Robinson & Oshlack, 2010).

All transcripts (329,194) of the assembly were used for DE analysis using the Bioconductor package Empirical analysis of Digital Gene Expression data in R (edgeR) (v3.16.5) (Robinson *et al.*, 2010; McCarthy *et al.*, 2012). Differentially expressed transcripts in each pairwise group comparison were extracted based on the following criteria: at least 16-fold expression at a false-

discovery corrected statistical significance (*p*) of ≤ 0.001 and a negative binomial (NB) dispersion rate of 0.1. Subsequently, identified transcripts were extracted from this list and filtered to retain only those differentially expressed transcripts that were fully characterized as proteins. Given that visualisation of the latter was unmanageable due to the high number of transcripts, the NB dispersion rate was increased to 0.5 to yield only the most highly differentially expressed transcripts. Only those which fit the criteria were extracted and visualized with a heatmap, MA and volcano plots using R software (v3.3.1) packages (RStudio Team, 2015).

4.2.6 Gene Ontology (GO) and metabolic pathway analysis

The Blastx algorithm of Blast2GO software (v4.1) (Conesa *et al.*, 2005) was employed to functionally annotate the assembled transcript sequences of *B. fusca* by searching for similarities against the insect taxonomy of the NCBI non-redundant database with a default 1e-3 cut-off. Based on the Blastx annotations, gene ontology (GO) terms were inferred for each annotated transcript. For metabolic pathway analysis, InterProScan Annotation in Blast2GO was used to firstly retrieve domain and motif information about the differentially expressed transcripts. Secondly, GO results from InterProScan were added and merged with previously obtained GOs, therefore validating all GO terms. Thirdly, Annotation Expander (ANNEX) was run to suggest new annotations for biological processes and cellular components, based on the existing molecular function annotations of the genes. Lastly, enzyme classification (EC) mapping was performed to assign EC codes to the sequences, after which pathway maps were loaded from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (http:///www.genome.jp/ kegg/). The latter application allowed the assignment of EC codes to the GO annotations, whilst displaying their enzymatic functions with reference to the metabolic pathways in which they partake. The complete list of KEGG pathways that were identified are provided in Annexure C, while the top ten pathways are shown in Table 4.3.

4.3 Results

4.3.1 RNA-Sequencing, *de novo* assembly and annotation

More than 226 billion read pairs of 126 bp in length were obtained with RNA-Sequencing of all 15 samples. The sequencing reads had excellent base call quality, where all reads had Phred quality scores of Q36, i.e. 99.97 % base call accuracy. The average GC content of the reads was 45.25 %. As a result of sequence trimming, a total of 167,497,181 (73.94 %) clean read pairs were used to construct a *de novo* reference transcriptome after *in silico* normalization of reads was performed. Only fragments with a minimum overlap of 50 bp or more were

assembled into contigs. A summary of the transcriptome assembly statistics is provided in Table 4.1.

Table 4.1: Summary of statistics of the *de novo* reference transcriptome of *B. fusca*.

Description	Result
Number of transcripts/contigs	329,194
N50 (bp)	1,019
Average transcript length (bp)	627
Number of contigs over 10 kbp	110
Number of contigs with ORFs	42,517
Largest contig (bp)	29,395

The final transcriptome assembly resulted in 329,194 transcripts with an average transcript length of 627 bp and N50 of 1,019 bp. Furthermore, 110 contigs were over 10 kbp long, of which the largest contig was 29,395 bp. In total, 42,517 contigs with open-reading frames (ORFs) could be identified (Table 4.1). By using the estwisedb program from the Wise2 package, 0.01 % mitochondrially expressed transcripts were found within all the obtained transcripts. The remaining transcripts were annotated by comparing them against a locally created Lepidoptera library using BLAST with an E-value cut-off of 1e-5. This low cut-off value was chosen to obtain the longest possible alignments to reference sequences, thus setting a threshold for obtaining the most significant results only. The majority (17.27 %) of these transcripts aligned against *Amyelois transitella* Walker (Lepidoptera: Pyralidae), followed by *B. mori* (16.72 %) and *Heliothis subflexa* Guenée (Lepidoptera: Noctuidae) (7.45 %). In total, 157,099 (47.72 %) transcripts could be identified, including 0.08 % Cry toxin receptors, 1.72 % ABCC transporters and 0.02 % MAPKs (Figure 4.2). Interestingly, 4.06 % of the identified transcripts were virus-related, thus suggesting that the larvae from this study were infected mainly with baculovirus.

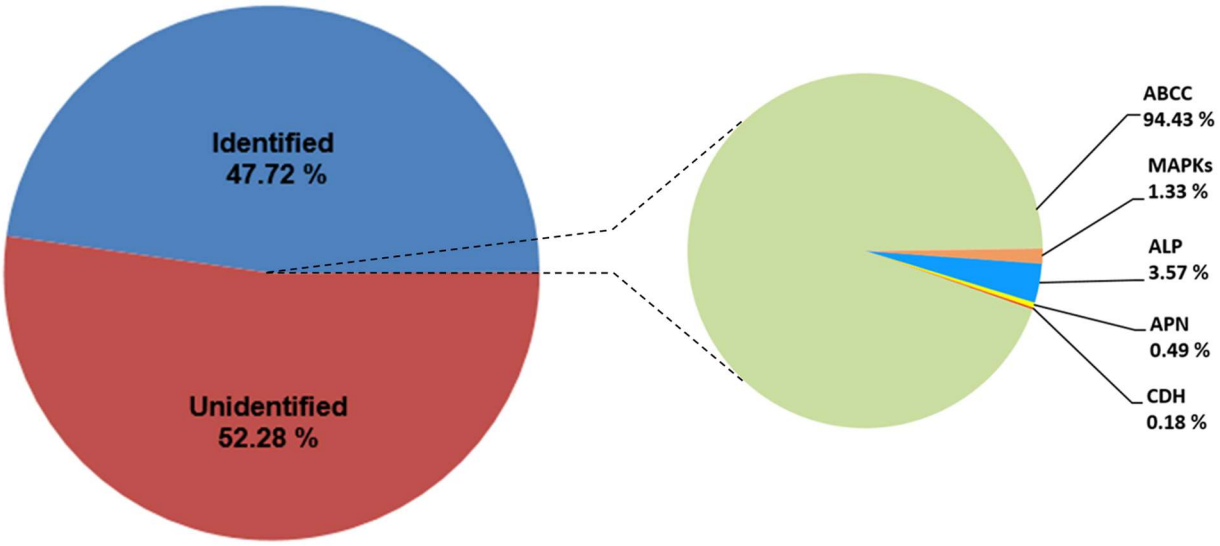


Figure 4.2: Pie chart illustrating the quantity of transcripts identified from the *de novo* transcriptome assembly of *Busseola fusca*. The secondary pie chart focuses on ATP-binding cassette C (ABCC) transporter proteins, mitogen-activated protein kinases (MAPKs) and Cry toxin receptors (alkaline phosphatase (ALP), aminopeptidase N (APN) and cadherin (CDH)) potentially involved in Cry1Ab toxin resistance in *B. fusca*.

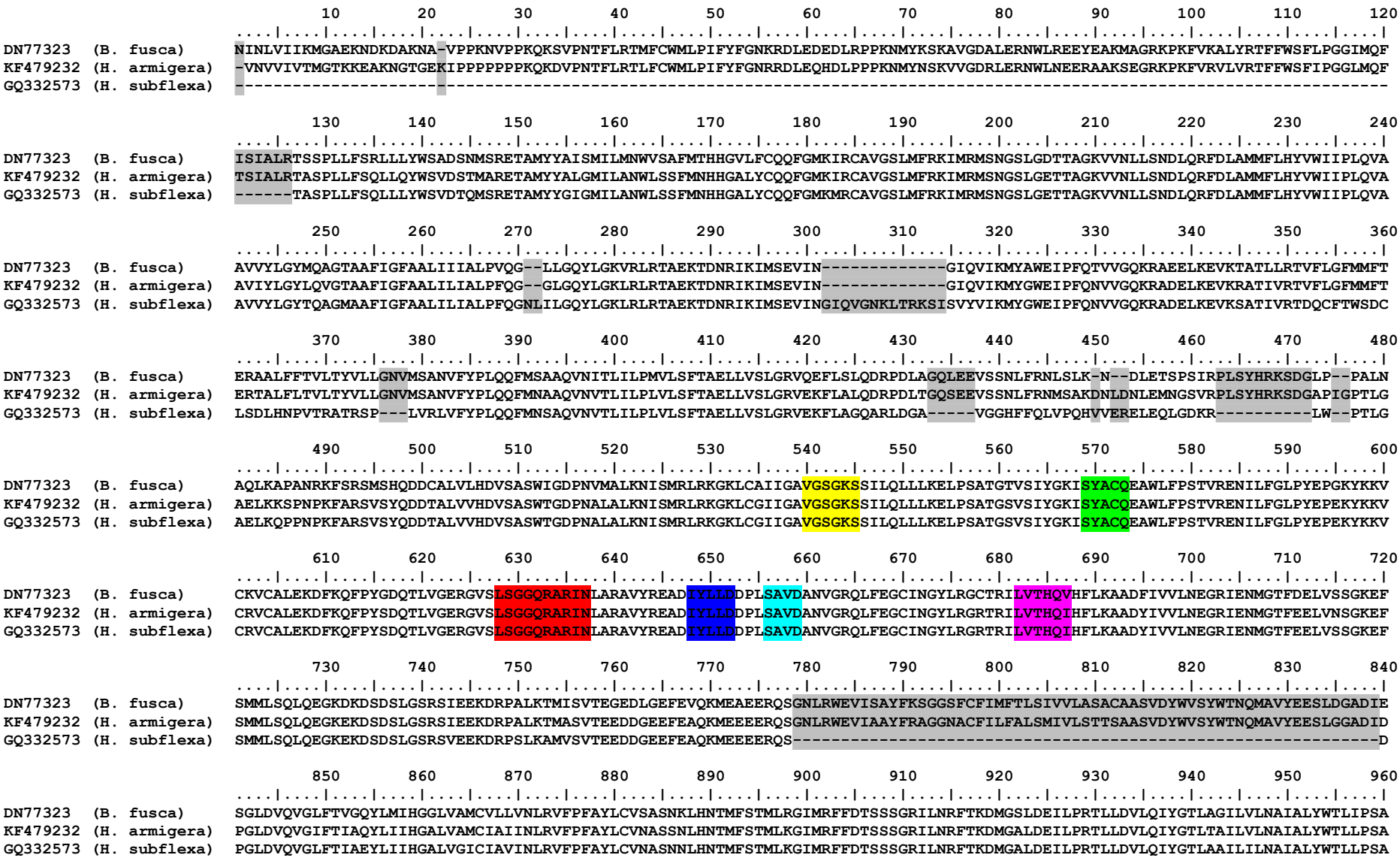
4.3.2 Amino acid sequence alignments and mutation detection

A section of an amino acid sequence alignment for the ABCC transporter protein is shown in Figure 4.3. All amino acid sequence alignments for ALP, APN and CDH Cry receptors, as well as ABC transporters and MAPKs are available on request. The amino acid sequence alignment shown in Figure 4.3 contains the query ABCC sequence of *B. fusca* (DN77323) compared with reference ABCC3 sequences from two heliothine species, *H. armigera* and *H. subflexa*, both in the family Noctuidae. Several mutations (non-synonymous mutations, insertions and deletions) were detected for this ABCC3 gene, as well as all the other above-mentioned genes, where many of the mutations spanned several amino acids. Since the Lepidoptera is a genetically diverse order, differences between these amino acid sequence alignments might occur due to the phylogenetic relationships and speciation between these species.

Nevertheless, a number of studies have previously demonstrated that mutations in Cry toxin-receptor genes (ALP, APN and CDH) and ABC transporters were associated with Cry toxin resistance in some lepidopteran species (Zhang *et al.*, 2009; Gahan *et al.*, 2010; Fabrick *et al.*, 2014). This does not, however, imply that the mutations that were detected in all these genes of *B. fusca* are responsible for the observed Cry toxin resistance. Genetic linkage analysis may shed light on the involvement, or non-involvement, of these mutations in Cry1Ab toxin

resistance of *B. fusca*. Additional studies could determine if these mutations confer, or only additively contribute to, *B. fusca* resistance.

Several conserved motifs, which comprise the nucleotide-binding domains of ABC transporters, were identified in this gene for *B. fusca*, thus validating adequate coverage of the transcriptome assembly. These motifs were Walker A/P-loop, Q-loop, ABC transporter signature, Walker B, D-loop and H-loop/switch region (Figure 4.3).


DN77323 (B. fusca)
KF479232 (H. armigera)
GQ332573 (H. subflexa)

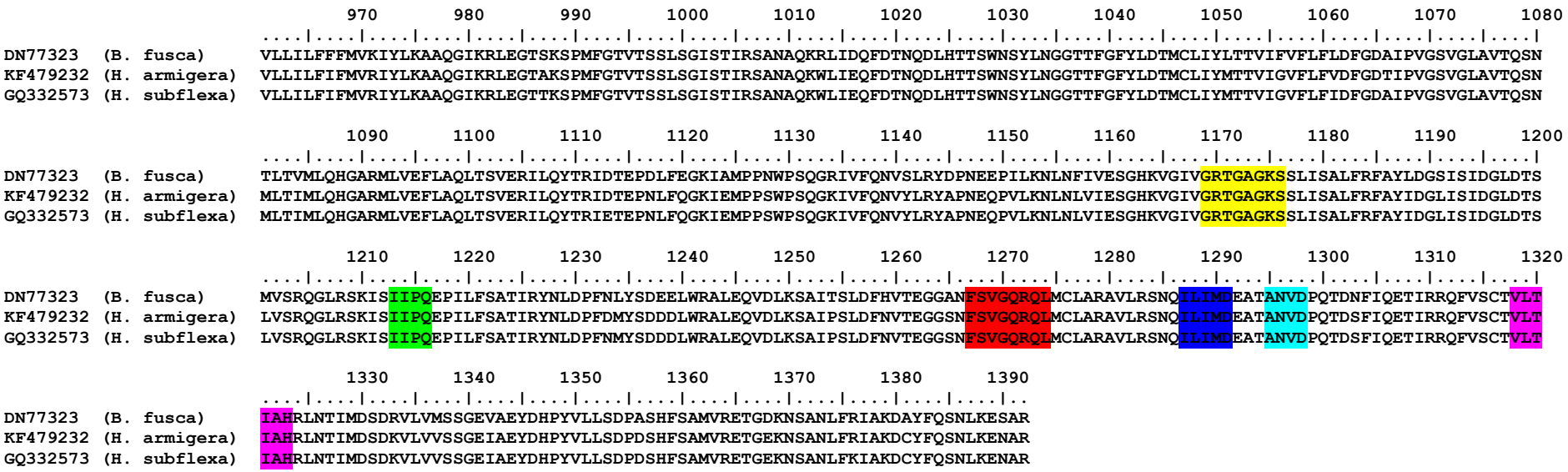


Figure 4.3: The amino acid sequence alignment for the ABCC3 transporter protein, illustrating some of the insertions and deletions (grey) that were detected in the query sequence (DN77323), with respect to the reference sequences (KF479232 and GQ332573). Furthermore, several non-synonymous mutations are present (not shaded). Conserved sequence features include the Walker A motif / P-loop (yellow), Q-loop / lid (green), ABC transporter signature motif (red), Walker B motif (blue), D-loop (turquoise) and H-loop / switch region (pink).

4.3.3 Differential expression (DE) analysis (*Bt*-challenged vs. -unchallenged)

From the 329,194 transcripts, 25,319 (7.69 %) transcripts showed significant expression differences between the toxin-challenged and toxin-unchallenged *B. fusca* larvae, in both the neonates and 4th instars, according to the applied criteria ($p \leq 0.001$, t-test; fold change (FC) ≥ 16 or ≤ -16; NB dispersion rate = 0.1). Of these differentially expressed genes (DEGs), 19,385 (76.56 %) could be identified, of which only 3,718 (19.18 %) could be fully characterized as proteins. Among these DEGs were previously identified Cry toxin receptors (ALP, APN and CDH) and ABCC transporters, which have been implicated in Cry toxin resistance in several lepidopterans (see Chapter 3). Additionally, several transcripts involved in the MAPK signalling pathway were also found to be significantly differentially expressed. This significant phenomenon may serve to support the postulated hypothesis that Cry1Ab toxin resistance of *B. fusca* might be controlled by a MAPK signalling pathway, as was demonstrated for Cry1Ac resistant *P. xylostella* strains (Guo *et al.*, 2015a). However, of all the DEGs (total 25,319), 5,934 (23.44 %) could not be identified.

To identify the most highly DEGs between the *Bt*-challenged and -unchallenged *B. fusca* larvae, a NB dispersion rate of 0.5 was used, resulting in the top 143 DEGs. These were later used to generate a clustered heatmap to show the highest transcript expression levels between the four sample groups (Figure 4.4a). Changes in gene expression (not absolute values) are represented by the colour and intensity of the boxes in Figure 4.4.

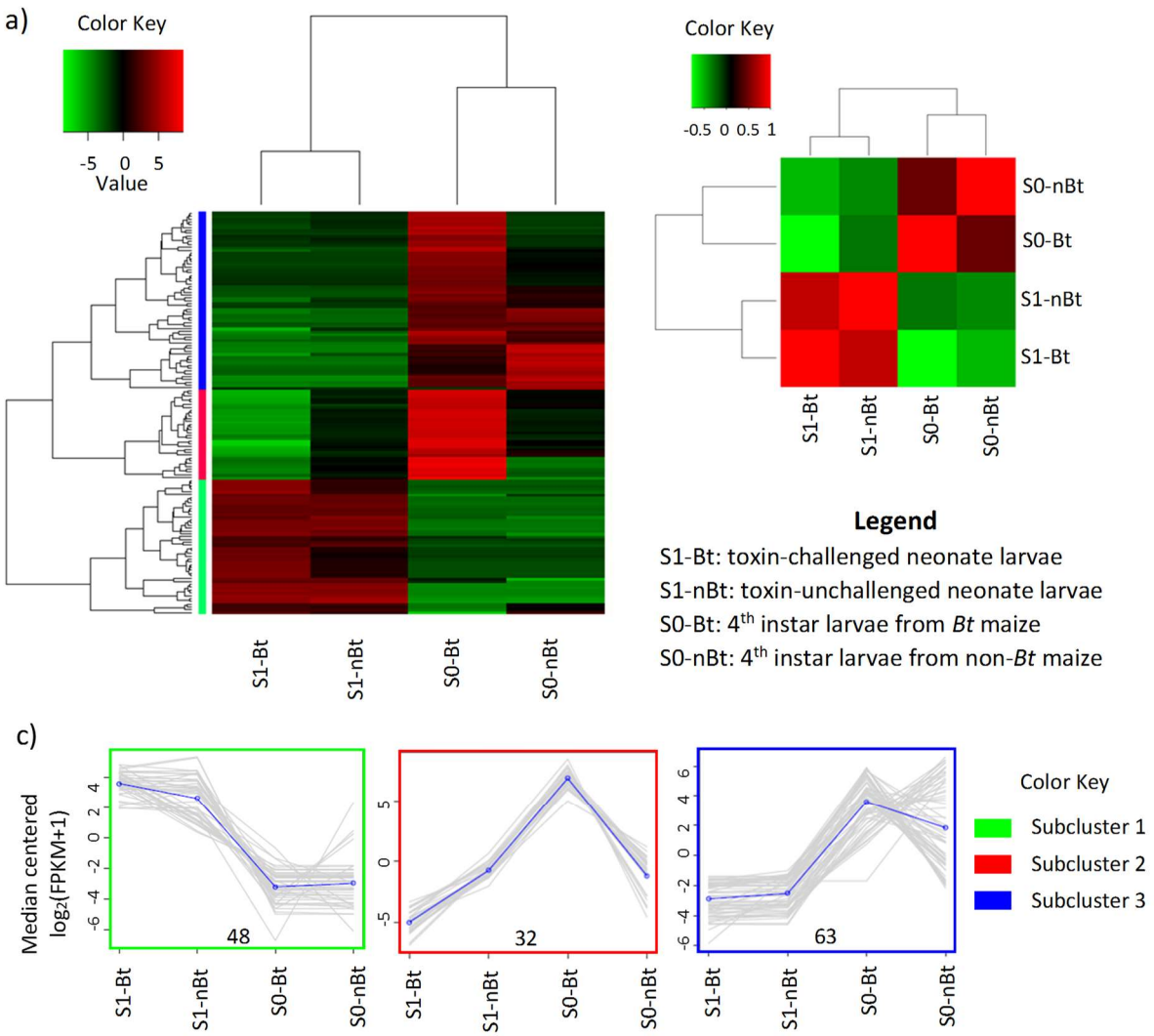


Figure 4.4: (a) Hierarchically clustered heatmap of the top 143 most highly differentially expressed transcripts between four sample groups of *Busseola fusca* larvae. Relative expression levels (median-centered log_2-transformed FPKM values) of each transcript (row) in each sample (column) are illustrated as unchanged (black), under-expressed (green) or over-expressed (red). Transcripts are grouped according to expression patterns and are illustrated in green, red and blue on the left side of the heatmap. (b) Hierarchically clustered Pearson correlation matrix of each sample pair based on compared transcript expression values. Strong correlations are indicated by values close to +1 (red) or -1 (green), and weak correlations are indicated by values close to zero (black). (c) Cluster plots extracted from the hierarchically clustered heatmap. Colours of plots correspond to colours of subclusters on the heatmap. The number of transcripts in each cluster is indicated in the centre at the bottom of each cluster plot. Grey lines: individual transcripts; blue lines: average expression values per cluster; x-axis: samples; y-axis: median-centered log_2(FPKM+1). FPKM: Fragments Per Kilobase of transcript per Million fragments mapped.

According to the dendrogram of samples (on top of the heatmap, Figure 4.4a), samples divided into two groups based on transcript expression correlation. It is evident that there was a distinct difference in profiles of transcript expression between neonate (S1-Bt and S1-nBt) and 4th instar stage (S0-Bt and S0-nBt) *B. fusca* larvae. The sample correlation matrix (Figure 4.4b) demonstrated that there was a moderate-to-strong negative correlation between the expression of genes in neonates (S1-Bt and S1-nBt) and 4th instars (S0 and S0). Conversely, there was a strong positive correlation between gene expression in the toxin-challenged (S1-Bt) and -unchallenged (S1-nBt) neonate larvae. This was due to the fact that only a few DEGs were differently regulated between the two neonate groups. Interestingly, there was a weak positive correlation between the expression of genes in 4th instars feeding on *Bt* (S0-Bt) and non-*Bt* (S0-nBt) maize. This weak correlation is most likely the result of rather different expression patterns in the majority of the DEGs between these two 4th instar larval groups.

Transcript expression profiles of *B. fusca* neonates (S1-Bt and S1-nBt, Figure 4.4a) demonstrated that 96 (67.13 %) of the top 143 DEGs responded to the Cry1Ab toxin in the susceptible *B. fusca* neonate larvae. In comparison to the toxin-unchallenged neonate larvae (S1-nBt), 30 (31.25 %) and 66 (68.75 %) of these 96 significantly DEGs were upregulated and downregulated in the toxin-challenged neonate larvae (S1-Bt), respectively. On the other hand, transcript expression profiles of 4th instars demonstrated that 110 (76.92 %) of the 143 DEGs were differently regulated between the *Bt* feeding (S0-Bt) and non-*Bt* feeding (S0-nBt) groups. In comparison to the 4th instars feeding on non-*Bt* maize (S0-nBt), 82 (74.55 %) and 28 (25.45 %) of these 110 significantly DEGs were upregulated and downregulated in the 4th instars feeding on *Bt* maize (S0-Bt), respectively.

Transcripts were grouped into three subclusters according to the similarity of their expression patterns (Figures 4.4a, c). Regulation patterns of the top 143 most highly DEGs, along with their putative functions, are listed in Table 4.2. Transcripts in subcluster 1 (green, Figure 4.4c) were mostly upregulated in the neonate larvae (S1-Bt and S1-nBt), and, conversely, downregulated in the 4th instars (S0-Bt and S0-nBt). This subcluster comprised 48 transcripts, including enzymes involved in respiration and energy metabolism (NADH dehydrogenase), as well as baculovirus-related proteins (e.g. polyhedron). Conversely, transcripts in subcluster 2 (red, Figure 4.4c) were extremely downregulated in the toxin-challenged neonate larvae (S1-Bt), and extremely upregulated in the 4th instars feeding on *Bt* maize (S0-Bt). Expression of the transcripts in this subcluster were either downregulated or unchanged for both neonates (S1-nBt) and 4th instars (S0-nBt) feeding on non-*Bt* maize. Subcluster 2 comprised only 32 transcripts and included a hemocyanin copper containing domain and arylphorin, which are respectively involved in oxygen transport and epithelial healing. The biggest subcluster of

transcripts, namely subcluster 3 (blue, Figure 4.4c), comprised a total of 63 transcripts. Transcripts in this subcluster were downregulated in the toxin-challenged and non-challenged neonate larvae (S1-Bt and S1-nBt), and upregulated in the 4th instars feeding on *Bt* maize (S0-Bt). For the 4th instars feeding on non-*Bt* maize (S0-nBt), transcripts in subcluster 3 were either extremely upregulated or moderately downregulated. Most of these are genes involved in insect immunity, specifically the prophenoloxidase (proPO)-activating system (Figure 4.5). These genes included immulectins or peptidoglycan recognition receptors (PRRs), as well as antimicrobial peptides (AMPs). Interestingly, these genes were not at all upregulated in the neonate larvae exposed to the Cry1Ab toxin, which might be due to the short period in which the neonates fed on the *Bt* maize. Three days might have been too little to elicit an observable response to the toxin. The proPO-activating system is discussed in detail in Section 4.5.

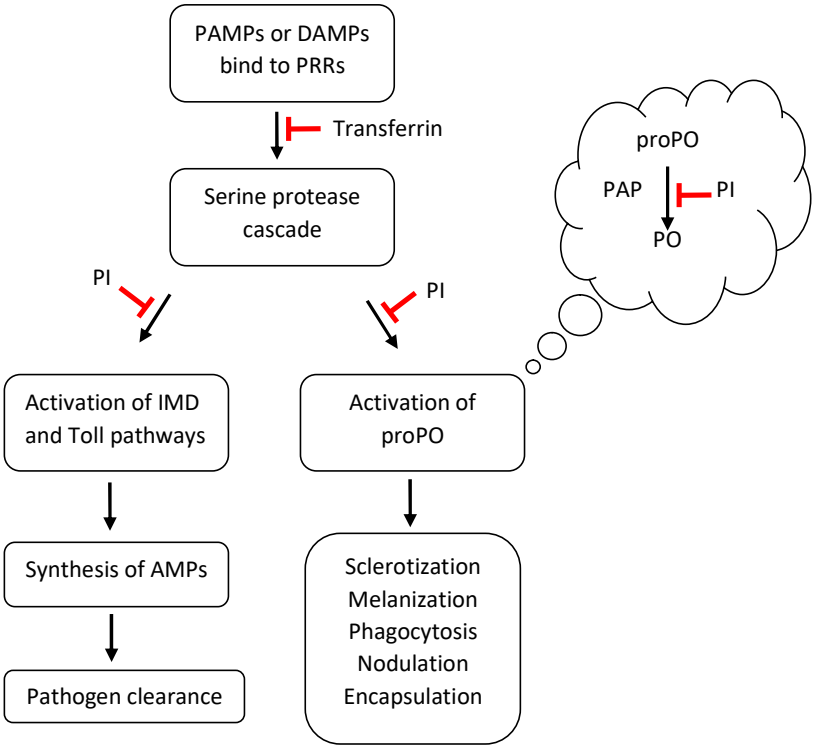


Figure 4.5: Illustration of the prophenoloxidase (proPO)-activating system of the innate immune system in insects (adapted from Cerenius and Söderhäll (2004), Cerenius *et al.* (2008) and Franssens (2006)). PAMPs: pathogen associated molecular patterns (e.g. lipopolysaccharides (LPS), lipoteichoic acids (LTA), peptidoglycans (PGN) and β-1,3-glucans); DAMPs: damage associated molecular patterns (e.g. collagen fragments, glycolipids, glycoproteins, reactive oxygen species (ROS) and calcium); PRR: pattern recognition receptors (e.g. C-type lectins); proPO: prophenoloxidase; PAP: prophenoloxidase-activating protease (e.g. serine proteinase); PO: phenoloxidase (activated); IMD: immune deficiency; AMPs: antimicrobial peptides; PI: proteinase inhibitor (e.g. serine protease inhibitors, also called serpins); T: inhibiting effect.

Table 4.2: Expression patterns and putative functions of the most highly differentially expressed transcripts in the transcriptome of *Busseola fusca*.

Differentially expressed genes (DEGs)	Neonate larvae		4th instar larvae		Function(s) of specific DEGs	References
	S1-Bt	S1-nBt	S0-Bt	S0-nBt		
Fatty acid synthase	↑	↑	↓	↓	Central role in lipid synthesis, stress tolerance and diapause preparation.	Tan *et al.*, 2016
Acyl-CoA desaturase	↑	↑	↓	↓	Pheromone biosynthesis.	Ding *et al.*, 2011
NADH dehydrogenase subunit 3	↑	↑	↓	↓	Respiration and energy metabolism.	Fang *et al.*, 2010
Papain family cysteine protease	↑	↑	↓	↓	Inhibits larval growth by perforating peritrophic matrix.	Harrison & Bonning, 2010
Glycosyl hydrolases	↑	↑	↓	↓	Glycoprotein processing (i.e. glycosylation) essential for rhodopsin biosynthesis in secretory pathway.	Rosenbaum *et al.*, 2014
ssDNA binding protein	↑	↑	↓	↓	Key component in replication and maintenance of mitochondrial DNA, which is essential for development.	Maier *et al.*, 2001
Fibroin	↑	↑	↓	↓	Insoluble protein present in silk used for cocoon construction.	Yonemura *et al.*, 2009
Polyhedrin	↑	–	↓	↓	Protein that form protective crystals that protect virus particles (virions) and resist solubilization under moderate alkaline conditions.	Rohrmann, 1986
Ribonucleotide reductase barrel domain	↑	–	↓	↓	Catalyzes formation of deoxyribonucleotides from ribonucleotides to provide precursors for DNA synthesis and repair.	Kolberg *et al.*, 2004

Differentially expressed genes (DEGs)	Neonate larvae		4th instar larvae		Function(s) of specific DEGs	References
	S1-Bt	S1-nBt	S0-Bt	S0-nBt		
ATP synthase lipid binding protein	↑	↑	–	↓↓	Participates in juvenile hormone binding protein (JHBP) export and transport from fat body cells to target cells.	Zalewska *et al.*, 2009
Collagen type IV	↑	↑	↓	–	Endogenously derived fragments that represent danger/alarm signals that stimulate the immune system.	Altincicek *et al.*, 2009
H transporting ATPase	↑	↑	↓	–	Acidify specific intracellular organelles; alkalinize the gut lumen; vital role in transepithelial transport of cations.	Lu *et al.*, 2014
Ribosomal protein L5	↑	↑	↓	↑	Protein that is a component of the 60S subunit and has high proteosynthetic potential.	Yang & Sehnal, 1998
Arylphorin	↓↓	↓	↑↑	↓	Functions as a storage protein that provides energy and amino acids for non-feeding stages (i.e. metamorphosis).	Burmester, 2015
Arylphorin	↓	↓	↑↑	↑		
Arylphorin	↓	↑	↑↑	↓↓		
Hemocyanin copper containing domain	↓	↓	↑↑	↓	Oxygen transport in the immuno-metabolome and also serve as potent immune effectors under certain physiological conditions.	Coates & Decker, 2017
Basic juvenile hormone suppressible protein	↓	↓	↑	↓	Cell cycle and apoptosis.	Fang *et al.*, 2010
Hexamerine	↓	↓	↑	↓	Functions as a storage protein that provides energy and amino acids for non-feeding stages, and is also involved in cuticle formation, transport and immune response.	Burmester, 2015

Differentially expressed genes (DEGs)	Neonate larvae		4th instar larvae		Function(s) of specific DEGs	References
	S1-Bt	S1-nBt	S0-Bt	S0-nBt		
Diapausin precursor mRNA	↓	↓	↑	↓	Antimicrobial peptides involved in immune response.	Crava *et al.*, 2015
Hemocyanin copper containing domain	↓	↓	↑↑	↓	Oxygen transport in the immuno-metabolome and also serve as potent immune effectors under certain physiological conditions.	Coates & Decker, 2017
Microsatellite sequence	↓	↓	↑↑	↓	Might function to regulate gene expression, and perhaps also interchromosomal recombination.	Vargas Jentzsch *et al.*, 2008
Attacin	↓	↓	↑	↓	Antimicrobial peptides involved in immune response.	Fang *et al.*, 2010
Serine protease inhibitor	↓	↓	↑	↓	Inhibits protease enzymes required for proPO activation, thus inhibiting blood coagulation and melanization.	Fang *et al.*, 2010; Harrison & Bonning, 2010
Serine protease inhibitor	↓	↓	↑	↑		
Collagen alpha	↓	↓	↑	↓	Involved in extracellular matrix assembly.	Barat-Houari *et al.*, 2006
Synaptic vesicle glycoprotein	↓	↓	↑	↓	Associated with organization and scaffolding of the cytocskeleton, transport, channels and signal transduction.	Yanay *et al.*, 2008
Beta tubulin	↓	↓	↑↑	↓	Fundamental cytoskeleton components that mediate processes such as cell division, shape, motility and intracellular trafficking.	Nielsen *et al.*, 2010
Alpha tubulin	↓	↓	↑	↓		
Transferrin	↓	↓	↑	↓	Involved in iron metabolism and physiology of host immunity.	Güz *et al.*, 2013
Glutamate dehydrogenase	↓	↓	↑	↓	Role in energy metabolism in mitochondria.	Papadopoulou & Louis, 2000
Zonadhesin-like mRNA	↓	↓	↑	↓	Implicated in species-specific egg-sperm binding interactions.	Hunt *et al.*, 2005

Differentially expressed genes (DEGs)	Neonate larvae		4th instar larvae		Function(s) of specific DEGs	References
	S1-Bt	S1-nBt	S0-Bt	S0-nBt		
Oxoglutarate (alpha-ketoglutarate) dehydrogenase	↓	↓	↑	↓	Participates in the proline metabolism pathway to support pupation, diapause and flight (i.e. energy metabolism).	Nation, 2001; Denton, 2009
Cingulin-like mRNA	↓	↓	↑	↓	Regulates expression of tight junction proteins in differentiating epithelial cells.	Guillemot *et al.*, 2013
Arylphorin	↓↓	↓	↑↑	–	Functions as a storage protein that provides energy and amino acids for non-feeding stages (i.e. metamorphosis), but may also be involved in cuticle formation, transport and immune response.	Burmester, 2015
Glutamine synthetase catalytic domain	↓	↓	↑	↑	Synthesizes and catalyzes the amino acid glutamine, which then activates the target of rapamycin (TOR) pathway that controls cell growth, is involved in metabolism and may regulate fecundity.	Zhai *et al.*, 2015
C type lectin mRNA	↓	↓	↑	↑	Pathogen recognition receptors and promoters of proPO activation in hemolymph, hemocyte nodule formation and encapsulation.	Fang *et al.*, 2010
Aliphatic nitrilase	↓	↓	↑	↑	Hydrolyzes substrates (e.g. carboxamide sidechain of glutamine) by utilizing conserved cysteine.	Pace & Brenner, 2001
Delta 1 pyrroline 5 carboxylate synthetase	↓	↓	↑	↑	Enzyme involved in proline and arginine biosynthesis. Proline is essential for many physiological and biochemical processes, such as reducing oxidative damage by ROS, involvement in cell apoptosis and survival, serving as a major energy substrate, assimilating and detoxifying ammonium.	Wan *et al.*, 2014

Differentially expressed genes (DEGs)	Neonate larvae		4th instar larvae		Function(s) of specific DEGs	References
	S1-Bt	S1-nBt	S0-Bt	S0-nBt		
GMC oxidoreductase	↓	↓	↑	↑	Common developmental or physiological function related to ecdysteroid metabolism; developmental process, glucose metabolism and immune function.	Iida *et al.*, 2007
Microsatellite sequence	↓	↓	↑	↑	Might function to regulate gene expression, and perhaps also interchromosomal recombination.	Vargas Jentzsch *et al.*, 2008
mRNA cap binding protein	↓	↓	↑	↑	Required for mRNA translation.	Maroto & Sierra, 1989
Glycosyl hydrolases	↓	↓	↑	↑↑	Glycoprotein processing (i.e. glycosylation) essential for rhodopsin biosynthesis in secretory pathway.	Rosenbaum *et al.*, 2014
Ribosomal protein S20	↓↓	↓	↑	↑	Catalyzes protein synthesis.	Fang *et al.*, 2010
Gloverin mRNA	↓	↓	↑↑	↑	Antimicrobial peptides involved in immune response.	Crava *et al.*, 2015
Cecropin C mRNA	↓	↓	↑	↑	Antimicrobial peptides involved in immune response.	Fang *et al.*, 2010
ATP synthase lipid binding protein	↓	↓	↑	↑	Catalyzes ATP synthesis and is associated with transmembrane transporter activity, ATP synthesis coupled proton transport.	Than *et al.*, 2016
Epoxide hydrolase	↓	↓	↑	↑	Irreversibly hydrolyzes juvenile hormones in certain organs and tissues to regulate physiological processes in development (larval molting) and reproductive maturation.	Seino *et al.*, 2010
Neuroglobin-like	↓	↓	↑	↑↑	Postulated involvement in oxygen homeostasis, detoxification of reactive oxygen or nitrogen, signal transduction pathway, or redox processes critical in prevention of apoptosis by reducing cytochrome *c*.	Burmester & Hankeln, 2009
Acyl-CoA binding domain containing protein 5	↓	↓	↑	↑↑	Speculated to be involved in cell differentiation and metabolism.	Fan *et al.*, 2010

Differentially expressed genes (DEGs)	Neonate larvae		4th instar larvae		Function(s) of specific DEGs	References
	S1-Bt	S1-nBt	S0-Bt	S0-nBt		
ABCC transporter family	↓	↓	↑	↑↑	Involvement in the *Bt* toxin mode of action and mediates *Bt* toxin resistance in several lepidopterans.	Park *et al.*, 2014
Trypsin IIb3 precursor	↓	↓	↑	↑↑	Inducible trypsin that activates/degrades Cry protoxins, thus influencing toxicity or resistance.	Díaz-Mendoza *et al.*, 2005
Endogenous virus rhabdovirus N-like EVE	↓	↓	↓	↑↑	Endogenous viral elements (EVEs) integrated into host cell genome potentially exapted for antiviral defenses.	Geisler & Jarvis, 2016

↑↑ Extremely upregulated expression
↑ Upregulated expression
– Unchanged expression
↓ Downregulated expression
↓↓ Extremely downregulated expression

Overall, the top 143 most highly DEGs indicated that the proPO-activating system of *B. fusca*'s innate immunity played a major role in its response to the Cry1Ab toxin. Comparisons of transcript expression profiles between pairs of *Bt*-challenged and unchallenged *B. fusca* larvae were visualised by means of MA and volcano plots (Figure 4.6).

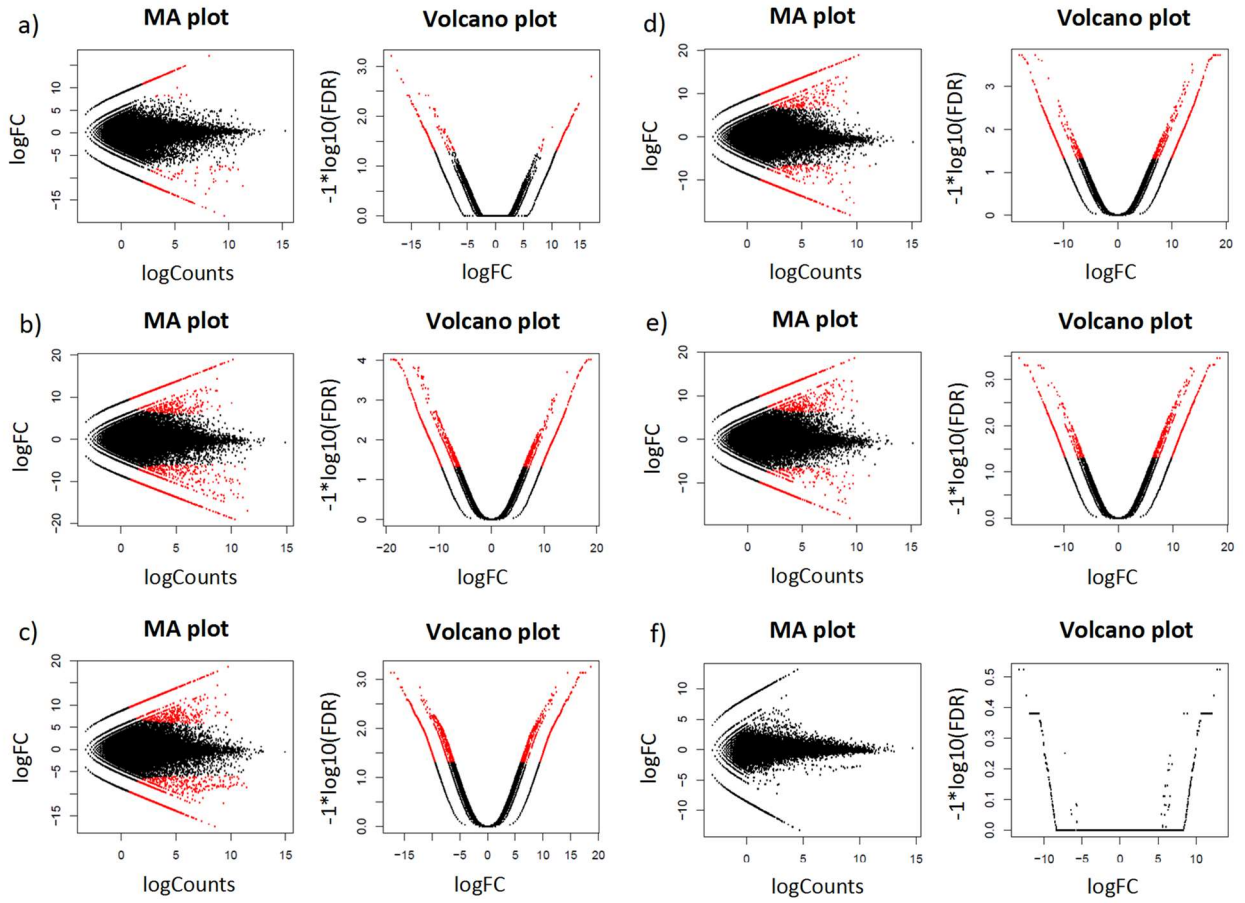


Figure 4.6: Comparisons of transcript expression profiles between pairs of *Busseola fusca* larvae: (a) S0-Bt vs. S0-nBt, (b) S0-Bt vs. S1-Bt, (c) S0-Bt vs. S1-nBt, (d) S0-nBt vs. S1-Bt, (e) S0-nBt vs. S1-nBt and (f) S1-Bt vs. S1-nBt. MA plots demonstrate the log_2(FC: fold change) vs. log_2(Counts: average expression) of each gene between each pair of samples. Volcano plots show the false discovery rate (FDR) as a function of log_2(FC: fold change) between each pair of samples. Both MA and volcano plots are centred at zero. Each dot represents a transcript, and red dots represent transcripts that are significantly differentially expressed with a fold change higher than 16 or less than -16 (i.e. log_2FC = ±4) and an adjusted *p*-value of ≤ 0.001.

Transcript expression profiles were compared between pairs of *B. fusca* larvae (both neonates and 4th instars feeding on *Bt* and non-*Bt* maize) and illustrated with MA and volcano plots (Figure 4.6). Each dot on these plots represents a single transcript. The majority of dots (i.e.

transcripts) occur around the zero on y-axis, which indicates constant expression of these transcripts across each pair of sample groups. Significantly DEGs, according to the applied criteria ($p \leq 0.001$, t-test; FC ≥ 16 or ≤ -16; NB dispersion rate = 0.5), are indicated as red dots. Since the FC between each pair of sample groups is plotted on a log scale, upregulated and downregulated genes appear symmetric on these plots. Transcripts with negative logFC values thus represent downregulated genes, whereas positive logFC values represent upregulated genes.

Significantly DEGs have a FC higher than 16 or less than -16 (i.e. $\log_2$FC = ±4), an adjusted *p*-value of ≤ 0.001 and NB dispersion rate of 0.5. The *p*-values are plotted on a negative log scale on the volcano plots, and therefore smaller *p*-values appear higher up on the plots. Thus, transcripts with both large fold changes and statistical significance appear in the upper corners of the plot. Remarkably, high numbers of these significantly DEGs had fold changes greater than 1000 (i.e. $\log_2$FC = ±10) in the various pairs of *B. fusca* larval groups, which will definitely be of great interest in a follow-up study. Of all the plots, only the pair of neonate *B. fusca* larvae (S1-Bt and S1-nBt, Figure 4.6f) did not contain any significantly DEGs. Although fold changes of the DEGs in these two sample groups were large, according to the applied criteria, differential expression was not statistically significant (i.e. *p*-values were not equal or less than to 0.001).

4.3.4 Gene Ontology (GO) and metabolic pathway analysis

Blast2GO software (Conesa *et al.*, 2005) was employed to perform GO analysis on the 3,718 differentially expressed transcripts, resulting in 1,190 (32.01 %) functionally annotated transcripts with 3,033 total GO annotations. The main GO categories that were revealed for the annotated *B. fusca* transcriptome included biological process, molecular function and cellular component ontologies. Distribution of GO terms at level two for each of these categories is illustrated in Figure 4.7.

For biological process, the most represented GO terms (at level 2) in the DEGs were metabolic process (65.14 %), cellular process (54.03 %) and single-organism process (37.78 %). The metabolic process mainly included the following terms at the third level subcategory: organic substance metabolic process, primary metabolic process, nitrogen compound metabolic process and single-organism metabolic process. On the other hand, the cellular process term mainly included cellular metabolic process, single-organism cellular process and regulation of cellular process at the third level subcategory. Furthermore, 53.28 % and 44.94 % of the DEGs were associated with catalytic activity and binding in the molecular function category, respectively. At third level subcategory, catalytic activity included hydrolase, transferase and oxidoreductase activity, whereas binding included ion, hetero- and organic-cyclic compound

binding and carbohydrate derivative binding. For the cellular component category, membrane (58.51 %), membrane part (49.60 %), cell (40.86 %) and cell part (39.90 %) were the most represented GO terms (at level 2).

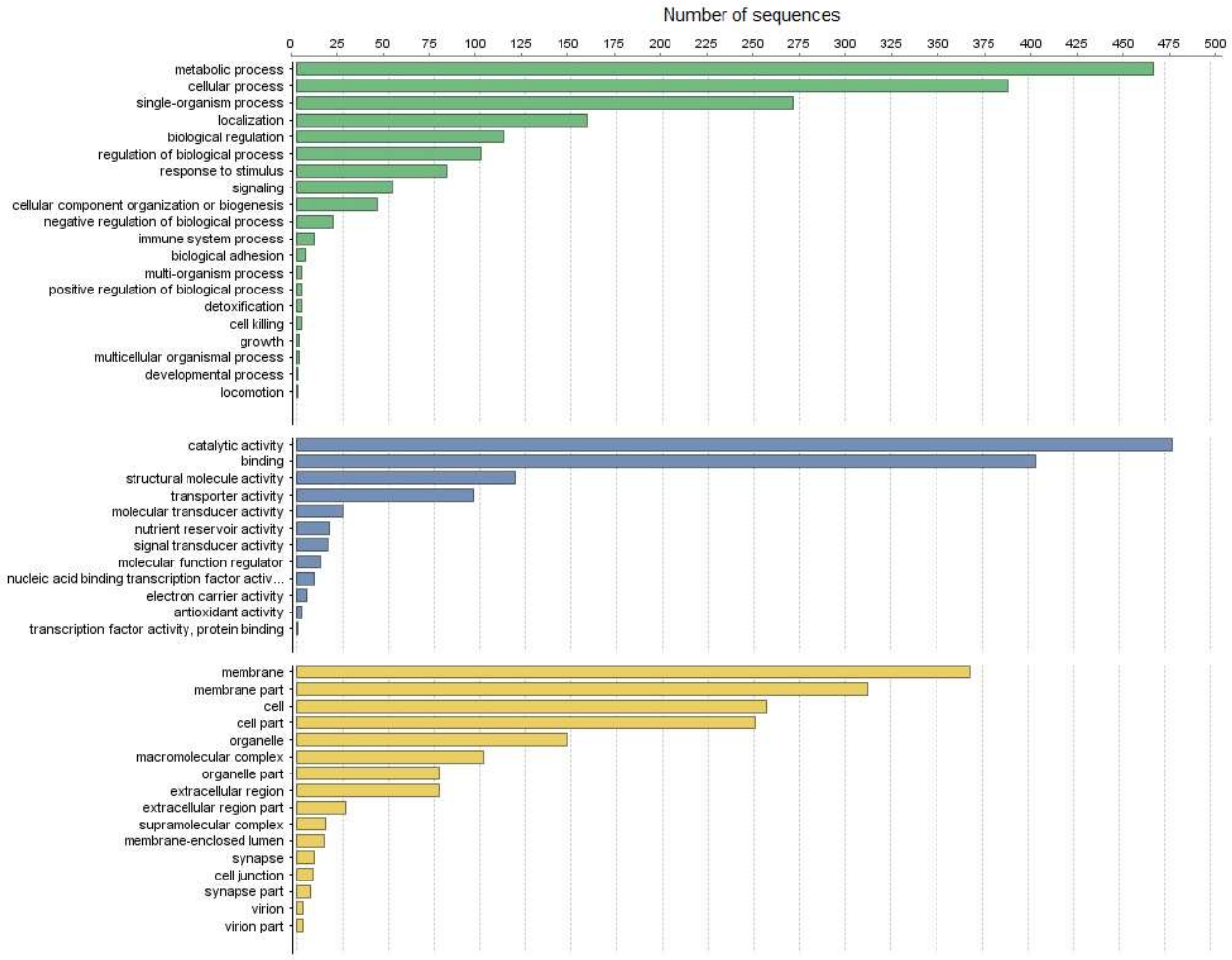


Figure 4.7: Gene Ontology (GO) distribution by level 2, representing the number of DEGs (horizontal axis) for each GO term (vertical axis). The GO terms related to gene functions are grouped according to the three domains of ontology, namely biological process (green), molecular function (blue) and cellular component (yellow).

Biological functions of DEGs were further explored by mapping annotations of all the DEGs to terms in the KEGG database to identify their EC codes and subsequently the main pathways that were significantly enriched in the transcriptome of *B. fusca*. In total, 296 DEGs, corresponding to 186 enzymes, were associated with 80 KEGG pathways (Annexure C). The top ten most frequently mapped KEGG pathways are shown in Table 4.3.

Table 4.3: Top ten most frequently mapped KEGG pathways in the transcriptome of *Busseola fusca*.

Ranking	KEGG pathway	No. of sequences	No. of enzymes
1	Biosynthesis of antibiotics	26	18
2	Purine metabolism	18	9
3	Amino sugar nucleotide sugar metabolism	11	3
4	Drug metabolism – other enzymes	11	2
5	Tyrosine metabolism	8	6
6	Glycolysis / Gluconeogenesis	7	3
7	Alanine, aspartate and glutamate metabolism	7	5
8	Carbon fixation pathways in prokaryotes	7	7
9	Tryptophan metabolism	6	3
10	Oxidative phosphorylation	6	4

KEGG pathway mapping results indicated that transcripts involved in typical insect immune and metabolic functions were present. The most frequently mapped immune pathway identified in this current study was biosynthesis of antibiotics. On the other hand, the major metabolic pathways that were identified included purine, amino sugar nucleotide sugar, drug, tyrosine, alanine, aspartate, glutamate, tryptophan and oxidative metabolisms (Table 4.3). These putative pathways might give some insights on responses and functions that specifically play a role in the molecular processes conferring Cry1Ab toxin resistance in *B. fusca*.

4.4 Discussion

The molecular genetics of this insect is poorly understood and limited genetic data is available to allow for a comprehensive investigation into the molecular basis behind the Cry1Ab toxin resistance of this pest. Thus, in an attempt to address this critical biological question, RNA-Seq analysis was employed to characterize the larval transcriptome of *B. fusca* and compare gene expression profiles between *Bt*-challenged and -unchallenged neonate and 4th instar larvae.

Four sequencing data sets were combined to construct a *de novo* assembly from 167,497,181 high-quality, clean reads using Trinity. Reads were *in silico* normalized prior to the assembly in order to reduce oversampling of abundant transcripts. As a result, 329,194 transcript sequences, representing 238,735 genes, were obtained, of which 157,099 (47.72 %) transcripts could be identified through BLAST. The majority of alignments (17.27 %) was homologous to

sequences of *A. transitella*, followed by *B. mori* (16.72 %) and *H. subflexa* (7.45 %). The high number of hits against *B. mori* might be due to the completeness of its genome database. These results imply that the coverage of the larval transcriptome of *B. fusca* was sufficient.

Of the 157,099 identified transcripts, 2,857 (1.82 %) have been implicated in Cry toxin resistance in lepidopterans. These genes encode Cry receptors (ALP, APN and CDH), ABCC transporters and MAPKs (Figure 4.2) (Zhang *et al.*, 2009; Gahan *et al.*, 2010; Fabrick *et al.*, 2014; Guo *et al.*, 2015a). In the latter studies, resistance in lepidopterans was predominantly due to mutations in and/or differential expression of these genes. Therefore, in the present study the amino acid sequences of these genes' transcriptional counterparts were aligned and examined for mutations. An alignment for an ABCC3 transporter is illustrated in Figure 4.3. Multiple non-synonymous mutations were detected, which could be caused by the phylogenetic divergence between the species used in the comparison. Moreover, several insertions and deletions were identified in the ABCC sequence of *B. fusca*, with respect to ABCC3 sequences of *H. armigera* and *H. subflexa*. However, these results do not necessarily indicate whether these mutations are responsible for the Cry1Ab toxin resistance observed in *B. fusca*. In order to determine the impact of these mutations on Cry resistance of *B. fusca*, genetic linkage studies are recommended.

Nevertheless, several conserved motifs in the nucleotide-binding domains (NBDs) of the ABCC transporter could be identified for *B. fusca*, including Walker A (P-loop), Q-loop, ABC transporter signature, Walker B, D-loop and H-loop. The A-loop, which is the seventh motif that is usually highly conserved in ABC transporters, was absent in *B. fusca*. These results are in accordance with that of *P. xylostella* (Qi *et al.*, 2016). These cytosolic NBDs are ATP hydrolysing enzymes that bind and hydrolyse ATP to facilitate translocation of substrates across the membrane (Rees *et al.*, 2009). According to Dermauw and Van Leeuwen (2014), not much is known about ABC transporters in arthropods. Be that as it may, expression profiling of these ABC transporters may elucidate their roles in physiological and regulatory pathways that may be involved in Cry1Ab toxin resistance in *B. fusca*.

Since differential expression of certain genes has previously been demonstrated to confer Cry toxin resistance in lepidopteran species, DE analysis was performed on four *B. fusca* larval groups. The first two groups consisted of 4th instars collected from *Bt* maize (S0-Bt) and non-*Bt* maize (S0-nBt), respectively. Some of the larvae from the latter group (S0-nBt) were allowed to complete their life cycle, after which neonates from the second generation (S1) were then placed on *Bt* (S1-Bt) and non-*Bt* (S1-nBt) maize plants to feed for three days. Although the pooling strategy is cost-effective, many genes might actually be overlooked due to a dilution effect (Zhao *et al.*, 2013). For instance, genes that are tissue-specific, expressed at low levels,

transiently expressed or bidirectional regulated may be missed (Miller *et al.*, 2012; Zhao *et al.*, 2013). As house-keeping transcripts are expressed in the majority of cells, this dilution effect is exacerbated when whole insects are used for transcriptome sequencing (Miller *et al.*, 2012). Thus some genes, such as those encoding Cry toxin receptors in the midgut, might show insignificant differences in expression levels when whole-body RNA is used (Guo *et al.*, 2012). This dilution effect can, however, be reduced by normalizing cDNA prior to sequencing, plus setting different applied criteria for identification of DEGs (Miller *et al.*, 2012; Zhao *et al.*, 2013). Since whole larvae were used in the current study, normalization and altered criteria were applied in the DE analysis.

In total, 25,319 (7.69 %) DEGs were identified between the toxin-challenged and toxin-unchallenged *B. fusca* larvae (neonates and 4th instars) according to the following applied criteria: FC ≥ 16 or ≤ -16, *p*-value ≤ 0.001 and NB dispersion rate = 0.1. Of these DEGs, 19,385 (76.56 %) could be identified, of which only 3,718 (19.18 %) could be characterized as proteins. Among these DEGs were ALP, APN, CDH, ABCC transporters and MAPKs. It has previously been demonstrated that differential expression of all of these genes conferred Cry toxin resistance in various lepidopteran pests (Jurat-Fuentes *et al.*, 2011; Coates *et al.*, 2013; Guo *et al.*, 2015a; 2015b; Jakka *et al.*, 2015).

Since the heatmap with 3,718 DEGs were indecipherable, an NB dispersion rate of 0.5 was applied to obtain the top 143 DEGs (Figure 4.4). A comparison between the expression profiles of those transcripts demonstrated distinct differences between toxin-challenged and -unchallenged *B. fusca* larvae, with transcript expression being much higher in the former, particularly for the genes involved in insect immunity. These genes included immulectins or PRRs, AMPs and several others involved in the proPO-activating system (Franssens, 2006; Casanova-Torres & Goodrich-Blair, 2013). Although these responses were only acute, it has been suggested that it might be succeeded by adaptive changes and enhanced stress responses to confer Cry toxin resistance in larvae (Candas *et al.*, 2002). The proPO-activating system is illustrated in Figure 4.5.

Damaged tissue resulting from mechanical injuries to insects or pathogens trigger their innate immune system, which comprises both humoral and cellular defence responses (Lavine & Strand, 2002; Cerenius & Söderhäll, 2004). The humoral defence response involves production of AMPs and reactive intermediates, along with cascades that control coagulation and melanisation of hemolymph (Lavine & Strand, 2002). On the other hand, the cellular defence response involves hemocyte-mediated responses that are regulated by signalling factors and effector molecules (Lavine & Strand, 2002). This type of defence response activates the

phenoloxidase (PO) cascade, and also controls cell adhesion and cytotoxicity to promote phagocytosis and encapsulation (Lavine & Strand, 2002; Viljakainen, 2015).

Compounds that elicit activation of the innate system include microbial products (glucans, lipopolysaccharides, lipoteichoic acids and peptidoglycans) and other products (calcium, collagen fragments, glycolipids, glycoproteins and reactive oxygen species) resulting from tissue damage (Lavine & Strand, 2002; Cerenius & Söderhäll, 2004). These products contain either pathogen or damage associated molecular patterns (PAMPs or DAMPs) that are recognized by host humoral and cell surface receptors (Lavine & Strand, 2002; Cerenius *et al.*, 2008). According to Casanova-Torres and Goodrich-Blair (2013), C-type lectins (CTLs) are lepidopteran, calcium-dependent, PRRs with carbohydrate-binding capabilities that are able to induce AMPs in response to infection. On the other hand, downregulation of certain AMPs and lysozymes might indicate that the host was infected by baculoviridae (Crava *et al.*, 2015). Since insect RNA was targeted in this study, detection of virus-related sequences could suggest that these were integrated into the host genome and expressed together with the insect's RNA.

The PAMP- and DAMP-containing compounds cause proPO-activating proteins (PPAs), such as serine proteases, to become proteolytically active (Cerenius & Söderhäll, 2004). In the presence of calcium, inactive proPO precursors are activated through a proteinase cascade that involves limited proteolysis by PPA serine proteases (Cerenius & Söderhäll, 2004; 2008). It has been reported that additional proteinaceous cofactors are required for activation of lepidopteran enzymes (Cerenius & Söderhäll, 2004). Enzymatically active PO then catalyses the initial steps in the melanogenetic pathway (Söderhäll & Cerenius, 1998). Premature or excessive activation is prevented by proteinase inhibitors such as serine proteinase inhibitors (serpins), which is speculated to control proPO activation spatially and temporally in order to protect certain tissues or cells (Cerenius & Söderhäll, 2004; 2008). Other immune reactions are simultaneously produced and are associated with the generation of factors that promote antimicrobial, cytotoxic, opsonic, encapsulation, or wound healing activities (Cerenius & Söderhäll, 2004; Jiravanichpaisala *et al.*, 2006; Cerenius *et al.*, 2008).

According to Gunaratna and Jiang (2013), humoral and cellular immune responses are regulated through receptor-mediated intracellular activation that occurs via several transduction pathways. These include the Toll, IMD, JAK-STAT, JNK and MAPK-JNK-p38 pathways. The IMD and Toll pathways form part of the humoral immune response, while the latter is also involved in developmental processes (Viljakainen, 2015). Nonetheless, effector molecules are produced and melanin is deposited around the intruding object or damaged tissue (Cerenius & Söderhäll, 2004), while AMPs, lysozymes and other substances disrupt microbial membranes to promote pathogen clearance (Casanova-Torres & Goodrich-Blair, 2013; Gunaratna & Jiang,

2013; Viljakainen, 2015). Likewise, several short-lived reaction intermediates such as toxic quinone substances are produced (Cerenius *et al.*, 2008). These reaction intermediates are involved in wound healing by means of sclerotisation (tissue repair) (Cerenius *et al.*, 2008).

It has also been implied that melanisation is accompanied by cellular defence, which is stimulated by factors that support phagocytosis and encapsulation reactions (Cerenius *et al.*, 2008). It is speculated that cellular defence and AMP synthesis are slower processes that succeed the early rapid melanisation response to pathogens (Cerenius *et al.*, 2008). According to Cerenius *et al.* (2008), melanisation is a crucial defence reaction in response to infection, which may determine whether the insect dies or becomes resistant.

Results from DE analysis should advance our understanding of the molecular events elicited upon toxin challenge. For instance, DEGs related to cellular processes such as respiration and energy metabolism were downregulated in the toxin-challenged *B. fusca* larvae. Conversely, immune-related DEGs were upregulated in this same group of larvae. A remarkable amount of the significantly DEGs had fold changes greater than 1000, which is of great interest in this ongoing research into *B. fusca* resistance. Sequences of the DEGs obtained in this study provide a good foundation for further functional studies of these genes. On the other hand, 5,934 (23.44 %) of the DEGs could not be matched with any existing genes. Unmistakably the adaptive process is very complex, and these unidentified genes played a central role in the immune response elicited in the toxin-challenged larvae. According to Vera *et al.* (2008), annotating genes of non-model organisms will remain problematic, no matter which methodological approach is followed or how good the quality of the assembly is. Elucidation of the function of these DEGs will greatly increase our knowledge of invertebrate immunity.

For the moment, the high abundance of highly expressed immune-related transcripts, but more specifically, arylphorin transcripts, in response to toxin challenge indicate that enhanced epithelial healing is one of the resistance mechanisms employed by *B. fusca* larvae against the Cry1Ab toxin. In order to establish whether this mechanism confers total resistance or only additively contributes to resistance, gene silencing studies are recommended. Further studies are needed to elucidate the signal and regulatory pathways that directed the observed immune response in toxin-challenged *B. fusca* larvae. Among other things, post-transcriptional gene expression and post-translational protein modification that are affected by regulation of certain genes should be considered (Nascimento *et al.*, 2015). Most likely, several pathways are affected in such an immune response, of which some may be associated with fitness costs or additional mechanisms contributing to an enhanced immune status.

GO analysis was performed in order to interpret the results from DE analysis in a biological context. This analysis yielded 1,190 (32.01 %) functionally annotated transcripts, revealing three main GO categories, namely biological process, molecular function and cellular component ontologies (Figure 4.7).

For biological process, the most represented GO terms (at level 2) in the DEGs were metabolic process (65.14 %), cellular process (54.03 %) and single-organism process (37.78 %). Likewise, 53.28 % and 44.94 % of the DEGs were associated with catalytic activity and binding in the molecular function category, respectively. For the cellular component category, membrane (58.51 %), membrane part (49.60 %), cell (40.86 %) and cell part (39.90 %) were the most represented GO terms (at level 2). KEGG pathway mapping results demonstrated that typical insect immune and metabolic pathways were significantly enriched in the transcriptome of *B. fusca*. In total, 296 DEGs, corresponding to 186 enzymes, were associated with 80 KEGG pathways (Annexure C). Enriched pathways identified in the current study concur with those that were obtained for *Pectinophora gossypiella* (Tassone *et al.*, 2016), *Heliothis virescens* (Perera et al., 2015) and other lepidopterans (Li *et al.*, 2012; Nguyen *et al.*, 2012; Lei *et al.*, 2014). These results will be very valuable for future research of *B. fusca*, especially for investigating genes with specific functions that are involved in certain pathways or processes in toxin-challenged or resistant strains.

4.5 Conclusion

Transcriptomic data generated in this study allowed identification of potential *Bt* resistance genes as well as screening of other genes that can be targeted when developing resistance management strategies for this pest. This paper is the first to provide a resource base and some insights into one of the potential mechanisms of Cry1Ab toxin resistance in *B. fusca*, which seems to be an enhanced immune response, and possibly greater epithelial healing. Similar to findings in this current study, it was previously demonstrated that resistance in *D. saccharalis* resistant to Cry1Ab was associated with increased metabolic processes (Guo *et al.*, 2012). It was recently proposed that the MAPK signalling pathway might be regulating the expression of various genes encoding Cry toxin receptors, thus acting as a common pathway that facilitates Cry toxin resistance (Guo *et al.*, 2015a). It is therefore hypothesised that Cry1Ab toxin resistance in *B. fusca* could also be controlled by a MAPK signalling pathway, in the same manner that Cry1Ac toxin resistance was conferred to *P. xylostella* strains (Guo *et al.*, 2015a). In order to test this hypothesis, tailored experiments should be designed for *B. fusca*. Among other things, genetic linkage analysis and targeted gene silencing would be extremely valuable for elucidating potential mechanisms of resistance employed by *B. fusca*.

Chapter 5
Final discussions, conclusions and recommendations

5.1 General discussion and conclusions

The aim of this research was to conduct a study of South African *B. fusca* to determine the genetic diversity of this population and potentially elucidate a mechanism of Cry1Ab toxin resistance through differential gene expression and comparative analyses. To achieve the aim of this study, several specific objectives were formulated. Each objective together with concluding remarks are presented below.

Objective 1: Collect *B. fusca* larvae from different geographic regions across the maize production area of SA and sequence the *COI* and *cyt b* mitochondrial genes for statistical and network analyses to determine the genetic diversity within this population (Chapter 2).

Despite the economic importance and *Bt* resistance status of *B. fusca* (Kfir *et al.*, 2002; Gouse *et al.*, 2005; Kruger *et al.*, 2009), limited population genetic and phylogeography data are available for this pest (Sezonlin *et al.*, 2006b). As a result, not much is understood about the molecular genetics of this insect, which is a great hindrance for the development of sustainable management strategies. This research generated COI and cyt b mitochondrial gene sequence data, which is novel for South African *B. fusca* populations and provides the basis for the DNA barcode database of this insect. This data has shown that South African *B. fusca* populations have low genetic diversity. Together with cyt b sequence data from West, Central and East African *B. fusca* populations (Sezonlin *et al.*, 2006a), it was confirmed that three clades (*W, KI* and *KII*) were dominant and were associated with geographic differences and ecological preferences. This clade distribution corresponded to the distribution patterns of various plant and animal species, as well as climatic conditions, and could be linked to the paleogeography of the region.

Results from this study contribute to future studies on the evolution of *B. fusca* within diverse sub-Saharan environments. This low genetic diversity and high dispersal capabilities (Dupas *et al.*, 2014) might suggest that the geographic expansion of a *B. fusca* strain with competitive traits (e.g. resistance to Cry1Ab proteins) may be rapid, especially since it was reported that *Bt* resistance was non-recessively inherited in *B. fusca* (Campagne *et al.*, 2013). Fairly recently, Hauptfleisch *et al.* (2014) assessed the potential of *B. fusca* to establish and become a pest of sorghum and maize in other countries. Results from that study indicated that *B. fusca* can indeed become a global maize pest and could potentially also extend its range to warmer, drier

regions where irrigation is employed. This may have implications for IRM strategies for *Bt* maize in SA and elsewhere.

Objective 2: Review all Cry toxin resistance mechanisms that have been reported for lepidopteran pests to identify the most common mechanism to be investigated for *B. fusca* (Chapter 3).

A vast body of literature is available on resistance mechanisms to various Cry toxins in lepidopteran species (see Chapter 3). This comprehensive overview discusses all the literature that has been generated since the first reports of Cry toxin resistance in 1985 (McGaughey, 1985) and includes data from laboratory, greenhouse and field studies. According to the reviewers' comments received after submission of this chapter for publication, this simplistic and explicit summary of all the resistance mechanisms will be valuable to professionals of the subject.

Elucidation of resistance mechanisms is crucial for developing IRM strategies to ensure sustainable use of GM crops. From the comprehensive overview of mechanisms of Cry toxin resistance that have been reported for lepidopteran pests, the most common resistance mechanism was identified and investigated for *B. fusca* in the succeeding chapter. The most common mechanism of resistance is altered binding sites. The latter is mainly due to mutations (insertions or deletions) in or differential expression of certain Cry toxin-receptor genes (Kumar & Kumari, 2015).

Even though fitness costs were not included in this review, it is crucial to also consider this factor when developing IRM strategies. It has been demonstrated that fitness costs and cross-resistance patterns varied in resistant *P. xylostella* strains from different locations (Zhu *et al.*, 2015), laying emphasis on the importance of geographical differences. Phenotypic plasticity, where multiple phenotypes can result from a single genotype, may occur under different environmental conditions and should thus also be considered in IRM strategies (Deans *et al.*, 2016). Other factors that also need to be taken into account for IRM strategies are the biology/physiology of insects, environmental factors (including diet-Cry interactions), Cry toxin mode of action, dominancy and initial frequency of *Bt* resistance alleles, mode of inheritance and genetic differentiation of populations. The latter is important since susceptible insect populations with low genetic diversity may independently procure the same mechanism of resistance as the existing resistant populations.

Objective 3: Sequence, assemble and annotate the transcriptome of *B. fusca* to detect potential genes encoding Cry toxin receptor proteins (Chapter 4).

Transcriptome sequencing provides molecular information about functional and protein coding RNAs which can be employed in several studies (Wang *et al.*, 2009). This current study generated a novel transcriptomic resource base for *B. fusca*. Several genes encoding for Cry toxin receptors, which have previously been implicated in resistance in lepidopterans, were identified for *B. fusca*. These included genes encoding Cry toxin receptors (ALP, APN and CDH), ABCC transporters and MAPKs. Furthermore, amino acid sequences of all these genes revealed several mutations (non-synonymous mutations, insertions and deletions), which have been associated with Cry toxin resistance in some lepidopteran species (Zhang *et al.*, 2009; Gahan *et al.*, 2010; Fabrick *et al.*, 2014).

Objective 4: Challenge neonate *B. fusca* larvae (collected from non-*Bt* maize) with Cry1Ab toxin and perform Next-Generation Sequencing to determine if these potential Cry toxin-receptor genes are differentially expressed in the *Bt*-challenged group, which may indicate a probable mechanism of *Bt* resistance in *B. fusca* (Chapter 4).

Some studies have previously reported that *Bt* toxin resistance correlated with differences in expression levels of certain genes (Tiewsiri & Wang, 2011; Vellichirammal *et al.*, 2015). Transcriptomic data generated in this study allowed quantification of gene expression levels and analysis of the transcriptional immune response in *B. fusca* larvae challenged with the Cry1Ab toxin. Cry toxin receptors (ALP, APN and CDH), ABCC transporters and MAPKs were among the differentially expressed transcripts that were identified. Differential expression of these genes have previously been associated with Cry toxin resistance in various lepidopteran species (Jurat-Fuentes *et al.*, 2011; Coates *et al.*, 2013; Guo *et al.*, 2015a; 2015b; Jakka *et al.*, 2015). Results indicated distinct differences between toxin-challenged and -unchallenged *B. fusca* larvae, with transcript expression being much higher in the former, particularly for the genes involved in insect immunity. Factors generated from these genes promote antimicrobial, cytotoxic, opsonic, encapsulation, or wound healing activities (Cerenius & Söderhäll, 2004; Jiravanichpaisala *et al.*, 2006; Cerenius *et al.*, 2008), thus suggesting that an enhanced immune response is elicited in *B. fusca* when challenged with Cry1Ab toxin.

Apparently, receptor-mediated intracellular activation regulates humoral and cellular immune responses through transduction pathways such as the Toll, IMD, JAK-STAT, JNK and MAPK-JNK-p38 pathways (Gunaratna & Jiang, 2013). Moreover, a recent study by Guo *et al.* (2015a) proposed that the MAPK signalling pathway might be regulating the expression of various receptor genes, thus acting as a common pathway that facilitates Cry1Ac toxin resistance in *P. xylostella* strains. It is therefore hypothesized that Cry1Ab toxin resistance in *B. fusca* could also be controlled by a MAPK signalling pathway. Comprehensively, results from this research provide key insights into potential resistance mechanisms employed by *B. fusca*. In the interest

of managing *B. fusca* resistance evolution, these results may aid in developing tailored and improved resistance monitoring and management approaches for this insect.

5.2 Recommendations

With regard to the genetic diversity of South African *B. fusca* populations, a follow-up study with a much greater sample size, as well as including other host crops, is necessary. It is also recommended that studies employing mitochondrial genes are supported by nuclear markers to gain better resolution (Jin *et al.*, 2013). Subsequently, the genetic structure of South African *B. fusca* populations could be characterised and its evolutionary history inferred, as was done for Cameroonian *B. fusca* populations by Sezonlin *et al.* (2012). Results could then also be correlated with specific geographic regions and the associated host crops. Moreover, the diversity trend could then be studied through time, which will be invaluable for IRM purposes. The latter approaches must be adapted to delay evolution of resistance to Cry proteins in other *B. fusca* populations, since these populations might be predisposed to develop the same mechanism of resistance.

However, *Bt* resistance development is extremely complex and influenced by interactions among various factors. For example, host protease activity can be influenced by the developmental stage, starvation, dietary changes and the presence of inhibitors or toxic compounds in larvae, thus affecting toxin activation (Rausell *et al.*, 2004). Furthermore, geographical differences demonstrated varied fitness costs and cross-resistance patterns in the same species (Zhu *et al.*, 2015). Other major factors that influence resistance development include the biology and physiology of insects, environmental factors, toxin mode of action, dominancy and initial frequency of resistance alleles, mode of inheritance and genetic differentiation of populations. Consequently, studies on resistance mechanisms should be done on a case-by-case basis and IRM strategies should be adapted accordingly. An understanding of biological, ecological and genetic data is thus crucial to devise long-term sustainable IRM strategies.

Transcriptomic data revealed mutations in, and differential expression of, several Cry toxin receptors (ALP, APN and CDH), ABCC transporters and MAPKs. It is hypothesized that Cry1Ab toxin resistance in *B. fusca* might be controlled by a MAPK signalling pathway. In order to test this hypothesis, tailored experiments should be designed for *B. fusca*. Among other things, genetic linkage analysis and targeted gene silencing would be extremely valuable for elucidating the mechanism of resistance employed by *B. fusca*. Specifically, genetic linkage analysis will demonstrate whether, and to which extent, the observed mutations are associated with Cry1Ab toxin resistance in this pest. Also, validation of differential expression patterns of

specific transcripts between susceptible and resistant larvae by real-time/quantitative polymerase chain reaction (qPCR) is recommended. Moreover, field experiments on groups of larvae feeding on *Bt* and non-*Bt* maize, followed by tissue-specific differential gene expression analysis, could potentially establish the mechanism of Cry1Ab toxin resistance in *B. fusca*. This will also provide insights into pathways that are activated for insect survival. Furthermore, qPCR on tissue samples from larvae in different developmental stages could reveal gene silencing targets that can be exploited for disrupting basic mechanisms underlying resistance using RNAi. Target genes typically include genes that are essential for survival, such as insect development, hormone biosynthesis, hormone signalling pathway, growth and detoxification (Li *et al.*, 2012).

Collectively, results from this research provide key insights into potential resistance mechanisms employed by *B. fusca*. In the interest of managing *B. fusca* resistance evolution, these results could be used to augment current pest management strategies or devise tailored and improved resistance monitoring. In that way, resistant populations could be controlled and resistance evolution in other populations of *B. fusca* prevented.

Reference list

Abdullah, M.A., Moussa, S., Taylor, M.D. & Adang, M.J. 2009. *Manduca sexta* (Lepidoptera: Sphingidae) cadherin fragments function as synergists for Cry1A and Cry1C *Bacillus thuringiensis* toxins against noctuid moths *Helicoverpa zea*, *Agrotis ipsilon* and *Spodoptera exigua*. *Pest Management Science*, 65(10):1097-1103.

Adenle, A.A. 2011. Response to issues on GM agriculture in Africa: are transgenic crops safe? *BMC Research Notes*, 4:388.

Akhurst, R.J., James, W., Bird, L.J. & Beard, C. 2003. Resistance to the Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Economic Entomology*, 96(4):1290-1299.

Alcantara, E., Estrada, A., Alpuerto, V. & Head, G. 2011. Monitoring Cry1Ab susceptibility in Asian corn borer (Lepidoptera: Crambidae) on Bt corn in the Philippines. *Crop Protection*, 30(5):554-559.

Altincicek, B., Berisha, A., Mukherjee, K., Spengler, B., Rompp, A. & Vilcinskas, A. 2009. Identification of collagen IV derived danger/alarm signals in insect immunity by nanoLC-FTICR MS. *Biological Chemistry*, 390(12):1303-1311.

Alves, A.P., Spencer, T.A., Tabashnik, B.E. & Siegfried, B.D. 2006. Inheritance of resistance to the Cry1Ab *Bacillus thuringiensis* toxin in *Ostrinia nubilalis* (Lepidoptera: Crambidae). *Journal of Economic Entomology*, 99(2):494-501.

Alvi, A.H.K., Sayyed, A.H., Naeem, M. & Ali, M. 2012. Field evolved resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis* toxin Cry1Ac in Pakistan. *PLoS One*, 7(10):e47309.

Andow, D.A. & Hilbeck, A. 2004. Science-based risk assessment for non-target effects of transgenic crops. *BioScience*, 54(7):637-649.

Angst, B.D., Marcozzi, C. & Magee, A.I. 2001. The cadherin superfamily: diversity in form and function. *Journal of Cell Science*, 114(4):629-641.

Anilkumar, K.J., Rodrigo-Simón, A., Ferré, J., Pusztai-Carey, M., Sivasupramaniam, S. & Moar, W.J. 2008. Production and characterization of *Bacillus thuringiensis* Cry1Ac-resistant cotton bollworm *Helicoverpa zea* (Boddie). *Applied and Environmental Microbiology*, 74(2):462-469.

Arenas, I., Bravo, A., Soberon, M. & Gomez, I. 2010. Role of alkaline phosphatase from *Manduca sexta* in the mechanism of action of *Bacillus thuringiensis* Cry1Ab toxin. *The Journal of Biological Chemistry*, 285(17):12497-12503.

Assefa, Y., Conlong, D. & Mitchell, A. 2006a. Differences in mitochondrial DNA and fertility of crosses between populations of *Eldana saccharina* (Lepidoptera: Pyralidae) from Kenya and South Africa: possible evidence for cryptic species? *Sugar Cane International*, 24:16-20.

Assefa, Y., Conlong, D.E., Mitchell, A. & Moyal, P.L. 2006b. *Busseola* species complex (Lepidoptera: Noctuidae) in Ethiopian sugarcane: identification based on mitochondrial DNA data. (Unpublished).

Assefa, Y., Conlong, D.E., Van Den Berg, J. & Martin, L.A. 2015. Ecological genetics and host range expansion by *Busseola fusca* (Lepidoptera: Noctuidae). *Environmental Entomology*, 44(4):1265-1274.

Assefa, Y., Mitchell, A. & Conlong, D.E. 2006c. Phylogeography of *Eldana saccharina* Walker (Lepidoptera: Pyralidae). *Annales de la Société Entomologique de France*, 42(3-4):331-337.

Atsumi, S., Miyamoto, K., Yamamoto, K., Narukawa, J., Kawai, S., Sezutsu, H., Kobayashi, I., Uchino, K., Tamura, T., Mita, K., Kadono-Okuda, K., Wada, S., Kanda, K., Goldsmith, M.R. & Noda, H. 2012. Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, *Bombyx mori*. *Proceedings of the National Academy of Sciences*, 109(25):E1591-1598.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A. & Saunders, N.C. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics*, 18:489-522.

Ballester, V., Granero, F., Tabashnik, B.E., Malvar, T. & Ferré, J. 1999. Integrative model for binding of *Bacillus thuringiensis* toxins in susceptible and resistant larvae of the diamondback moth (*Plutella xylostella*). *Applied and Environmental Microbiology*, 65(4):1413-1419.

Banks, D.J., Hua, G. & Adang, M.J. 2003. Cloning of a *Heliothis virescens* 110 kDa aminopeptidase N and expression in *Drosophila* S2 cells. *Insect Biochemistry and Molecular Biology*, 33(5):499-508.

Banks, D.J., Jurat-Fuentes, J.L., Dean, D.H. & Adang, M.J. 2001. *Bacillus thuringiensis* Cry1Ac and Cry1Fa δ-endotoxin binding to a novel 110 kDa aminopeptidase in *Heliothis*

virescens is not N-acetylgalactosamine mediated. *Insect Biochemistry and Molecular Biology*, 31(9):909-918.

Barat-Houari, M., Hilliou, F., Jousset, F.X., Sofer, L., Deleury, E., Rocher, J., Ravallec, M., Galibert, L., Delobel, P., Feyereisen, R., Fournier, P. & Volkoff, A.N. 2006. Gene expression profiling of *Spodoptera frugiperda* hemocytes and fat body using cDNA microarray reveals polydnavirus-associated variations in lepidopteran host genes transcript levels. *BMC Genomics*, 7:160.

Barkhade, U.P. & Thakare, A.S. 2010. Protease mediated resistance mechanism to Cry1C and Vip3A in *Spodoptera litura*. *Egyptian Academic Journal of Biological Sciences*, 3(2):43-50.

Bates, S.L., Zhao, J., Roush, R.T. & Shelton, A.M. 2005. Insect resistance management in GM crops: past, present and future. *Nature Biotechnology*, 23(1):57-62.

Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T. & Roberts, J. 2007. Control of coleopteran insect pests through RNA interference. *Nature Biotechnology*, 25(11):1322-1326.

Baxter, S.W., Badenes-Pérez, F.R., Morrison, A., Vogel, H., Crickmore, N., Kain, W., Wang, P., Heckel, D.G. & Jiggins, C.D. 2011. Parallel evolution of *Bacillus thuringiensis* toxin resistance in Lepidoptera. *Genetics*, 189(2):675-679.

Behere, G.T., Tay, W.T., Russell, D.A., Heckel, D.G., Appleton, B.R., Kranthi, K.R. & Batterham, P. 2007. Mitochondrial DNA analysis of field populations of *Helicoverpa armigera* (Lepidoptera: Noctuidae) and of its relationship to *H. zea*. *BMC Evolutionary Biology*, 7:117.

Bel, Y. & Escriche, B. 2006. Common genomic structure for the Lepidoptera cadherin-like genes. *Gene*, 381:71-80.

Bel, Y., Siqueira, H.A., Siegfried, B.D., Ferre, J. & Escriche, B. 2009. Variability in the cadherin gene in an *Ostrinia nubilalis* strain selected for Cry1Ab resistance. *Insect Biochemistry and Molecular Biology*, 39(3):218-223.

Birney, E. & Copley, R. 2016. Wise2 - Documentation (version 2.1.20 stable). http://dendrome.ucdavis.edu/resources/tooldocs/wise2/doc_wise2.html Date of access2016.

Bolger, A.M., Lohse, M. & Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, btu170.

Boudabous, R., Haouas, N., Bdira, S., Amor, S., Khayech, F., Babba, H. & Azaiez, R. 2011. Identification of mitochondrial cytochrome *b* haplotypes by single strand conformation polymorphism in *Phlebotomus chabaudi* Croset, Abonnenc and Rioux, 1970 (Diptera, Psychodidae). *International Journal of Biodiversity and Conservation*, 3:57-61.

Bradley, D., Harkey, M.A., Kim, M.K., Biever, K.D. & Bauer, L.S. 1995. The insecticidal Cry1B crystal protein of *Bacillus thuringiensis* ssp. *thuringiensis* has dual specificity to coleopteran and lepidopteran larvae. *Journal of Invertebrate Pathology*, 65(2):162-173.

Bravo, A., Gill, S.S. & Soberon, M. 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*, 49(4):423-435.

Bravo, A., Gómez, I., Conde, J., Muñoz-Garay, C., Sánchez, J., Miranda, R., Zhuang, M., Gill, S.S. & Soberón, M. 2004. Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1667(1):38-46.

Bravo, A., Likitvivatanavong, S., Gill, S.S. & Soberón, M. 2011. *Bacillus thuringiensis*: a story of a successful bioinsecticide. *Insect Biochemistry and Molecular Biology*, 41(7):423-431.

Bravo, A. & Soberón, M. 2008. How to cope with insect resistance to Bt toxins? *Trends in Biotechnology*, 26(10):573-579.

Broderick, N.A., Raffa, K.F. & Handelsman, J. 2006. Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. *Proceedings of the National Academy of Sciences*, 103(41):15196-15199.

Broderick, N.A., Robinson, C.J., McMahon, M.D., Holt, J., Handelsman, J. & Raffa, K.F. 2009. Contributions of gut bacteria to *Bacillus thuringiensis*-induced mortality vary across a range of Lepidoptera. *BMC Biology*, 7:11.

Burmester, T. 2015. Expression and evolution of hexamerins from the tobacco hornworm, *Manduca sexta*, and other Lepidoptera. *Insect Biochemistry and Molecular Biology*, 62:226-234.

Burmester, T. & Hankeln, T. 2009. What is the function of neuroglobin? *The Journal of Experimental Biology*, 212:1423-1428.

Caccia, S., Di Lelio, I., La Storia, A., Marinelli, A., Varricchio, P., Franzetti, E., Banyuls, N., Tettamanti, G., Casartelli, M., Giordana, B., Ferré, J., Gigliotti, S., Ercolini, D. & Pennacchio, F.

2016. Midgut microbiota and host immunocompetence underlie *Bacillus thuringiensis* killing mechanism. *Proceedings of the National Academy of Sciences of the United States of America*, 113(34):9486.

Caccia, S., Hernández-Rodríguez, C.S., Mahon, R.J., Downes, S., James, W., Bautsoens, N., Van Rie, J. & Ferré, J. 2010. Binding site alteration is responsible for field-isolated resistance to *Bacillus thuringiensis* Cry2A insecticidal proteins in two *Helicoverpa species*. *PLoS One*, 5(4):e9975.

Caccia, S., Moar, W.J., Chandrashekhar, J., Oppert, C., Anilkumar, K.J., Jurat-Fuentes, J.L. & Ferré, J. 2012. Association of Cry1Ac toxin resistance in *Helicoverpa zea* (Boddie) with increased alkaline phosphatase levels in the midgut lumen. *Applied and Environmental Microbiology*, 78(16):5690-5698.

Calatayud, P.-A., Le Rü, B.P., Van den Berg, J. & Schulthess, F. 2014. Ecology of the African maize stalk borer, *Busseola fusca* (Lepidoptera: Noctuidae) with special reference to insect-plant interactions. *Insects*, 5(3):539-563.

Camargo, R., Herai, R.H., Santos, L.N., Bento, F.M.M., Lima, J.E., Marques-Souza, H. & Figueira, A. 2015. *De novo* transcriptome assembly and analysis to identify potential gene targets for RNAi-mediated control of the tomato leafminer (*Tuta absoluta*). *BMC Genomics*, 16:635-652.

Campagne, P., Kruger, M., Pasquet, R., Le Rü, B. & Van den Berg, J. 2013. Dominant inheritance of field-evolved resistance to *Bt* corn in *Busseola fusca*. *PLOS One*, 8(7):e69675.

Cancino-Rodezno, A., Alexander, C., Villasenor, R., Pacheco, S., Porta, H., Pauchet, Y., Soberón, M., Gill, S.S. & Bravo, A. 2010. The mitogen-activated protein kinase p38 is involved in insect defense against Cry toxins from *Bacillus thuringiensis*. *Insect Biochemistry and Molecular Biology*, 40(1):58-63.

Candas, M., Loseva, O., Oppert, B., Kosaraju, P. & Bulla, L.A., Jr. 2002. Insect resistance to *Bacillus thuringiensis*: alterations in the indianmeal moth larval gut proteome. *Molecular & Cellular Proteomics*, 2(1):19-28.

Cao, G., Zhang, L., Liang, G., Li, X. & Wu, K. 2013. Involvement of nonbinding site proteinases in the development of resistance of *Helicoverpa armigera* (Lepidoptera: Noctuidae) to Cry1Ac. *Journal of Economic Entomology*, 106(6):2514-2521.

Carrière, Y., Ellers-Kirk, C., Biggs, R., Higginson, D.M., Dennehy, T.J. & Tabashnik, B.E. 2004. Effects of gossypol on fitness costs associated with resistance to Bt Cotton in pink bollworm. *Journal of Economic Entomology*, 97(5):1710-1718.

Carriére, Y. & Tabashnik, B.E. 2001. Reversing insect adaptation to transgenic insecticidal plants. *Proceedings of the Royal Society of London B*, 268(1475):1475-1480.

Casanova-Torres, Á.M. & Goodrich-Blair, H. 2013. Immune signaling and antimicrobial peptide expression in Lepidoptera. *Insects*, 4:320-338.

Castagnola, A. & Jurat-Fuentes, J.L. 2009. Resistance to Cry toxins and epithelial healing. *IOBC/WPRS Bulletin Proceedings from Annual Meeting*, 45:27-32.

Cerenius, L., Lee, B.K. & Söderhäll, K. 2008. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in Immunology*, 29(6):263-271.

Cerenius, L. & Söderhäll, K. 2004. The prophenoloxidase-activating system in invertebrates. *Immunological Reviews*, 198:116-126.

Chang, W.S., Zachow, K.R. & Bentley, D. 1993. Expression of epithelial alkaline phosphatase in segmentally iterated bands during grasshopper limb morphogenesis. *Development*, 118:651-663.

Chen, J., Brown, M.R., Hua, G. & Adang, M.J. 2005. Comparison of the localization of *Bacillus thuringiensis* Cry1A δ-endotoxins and their binding proteins in larval midgut of tobacco hornworm, *Manduca sexta*. *Cell and Tissue Research*, 321(1):123-129.

Chen, L.Z., Liang, G.M., Zhang, J., Wu, K.M., Guo, Y.Y. & Rector, B.G. 2010. Proteomic analysis of novel Cry1Ac binding proteins in *Helicoverpa armigera* (Hübner). *Archives of Insect Biochemistry and Physiology*, 73(2):61-73.

Chen, W., Liu, C., Xiao, Y., Zhang, D., Zhang, Y., Li, X., Tabashnik, B.E. & Wu, K. 2015. A toxin-binding alkaline phosphatase fragment synergizes Bt toxin Cry1Ac against susceptible and resistant *Helicoverpa armigera*. *PLoS One*, 10(4):e0126288.

Chilcutt, C.F. & Tabashnik, B.E. 2004. Contamination of refuges by *Bacillus thuringiensis* toxin genes from transgenic maize. *Proceedings of the National Academy of Sciences*, 101(20):7526-7529.

Coates, B.S., Sumerford, D.V., Siegfried, B.D., Hellmich, R.L. & Abel, C.A. 2013. Unlinked genetic loci control the reduced transcription of aminopeptidase N 1 and 3 in the European corn

borer and determine tolerance to *Bacillus thuringiensis* Cry1Ab toxin. *Insect Biochemistry and Molecular Biology*, 43(12):1152-1160.

Coates, C.J. & Decker, H. 2017. Immunological properties of oxygen-transport proteins: hemoglobin, hemocyanin and hemerythrin. *Cellular and Molecular Life Sciences*, 74(2):293-317.

Conesa, A., Götz, S., Garcia-Gomez, J.M., Terol, J., Talon, M. & Robles, M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*, 21:3674-3676.

Cox, A.J. & Hebert, P.D. 2001. Colonization, extinction, and phylogeographic patterning in a freshwater crustacean. *Molecular Ecology*, 10:371-386.

Crava, C.M., Bel, Y., Lee, S.F., Manachini, B., Heckel, D.G. & Escriche, B. 2010. Study of the aminopeptidase N gene family in the lepidopterans *Ostrinia nubilalis* (Hübner) and *Bombyx mori* (L.): sequences, mapping and expression. *Insect Biochemistry and Molecular Biology*, 40(7):506-515.

Crava, C.M., Jakubowska, A.K., Escriche, B., Herrero, S. & Bel, Y. 2015. Dissimilar regulation of antimicrobial proteins in the midgut of *Spodoptera exigua* larvae challenged with *Bacillus thuringiensis* toxins or baculovirus. *PLoS One*, 10(5):e0125991.

Crespo, A.L., Rodrigo-Simón, A., Siqueira, H.A.A., Pereira, E.J., Ferré, J. & Siegfried, B.D. 2011. Cross-resistance and mechanism of resistance to Cry1Ab toxin from *Bacillus thuringiensis* in a field-derived strain of European corn borer, *Ostrinia nubilalis*. *Journal of Invertebrate Pathology*, 107(3):185-192.

Crespo, A.L.B., Spencer, T.A., Alves, A.P., Hellmich, R.L., Blankenship, E.E., Magalhães, L.C. & Siegfried, B.D. 2009. On-plant survival and inheritance of resistance to Cry1Ab toxin from *Bacillus thuringiensis* in a field-derived strain of European corn borer, *Ostrinia nubilalis*. *Pest Management Science*, 65(10):1071-1081.

Crickmore, N., Baum, J.A., Bravo, A., Lereclus, D., Narva, K.E., Sampson, K., Schnepf, H.E., Sun, M. & Zeigler, D.R. 2014. *Bacillus thuringiensis* toxin nomenclature. http://www.btnomenclature.info/ Date of access: 11 June 2015.

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. & Dean, D.H. 1998. Revision of the nomenclature of the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews*, 62(3):807-813.

Cunningham, D.F. & O'Connor, B. 1997. Proline specific peptidases. *Microbiology and Molecular Biology Reviews*, 62(3):807-813.

De Maagd, R.A., Kwa, M.S.G., Van der Klei, H., Yamamoto, T., Schipper, B., Vlak, J.M., Stiekema, W.J. & Bosch, D. 1996. Domain III substitutions in *Bacillus thuringiensis* delta-endotoxin Cry1A(b) results in superior toxicity for *Spodoptera exigua* and altered membrane protein recognition. *Applied and Environmental Microbiology*, 62:1537-1543.

Deans, C.A., Sword, G.A. & Behmer, S.T. 2016. Nutrition as a neglected factor in insect herbivore susceptibility to *Bt* toxins. *Current Opinion in Insect Science*, 15:97-103.

Deist, B.R., Rausch, M.A., Fernandez-Luna, M.T., Adang, M.J. & Bonning, B.C. 2014. Bt toxin modification for enhanced efficacy. *Toxins (Basel)*, 6(10):3005-3027.

Denton, R.M. 2009. Regulation of mitochondrial dehydrogenases by calcium ions. *Biochimica et Biophysica Acta*, 1787:1309-1316.

Dermauw, W. & Van Leeuwen, T. 2014. The ABC gene family in arthropods: Comparative genomics and role in insecticide transport and resistance. *Insect Biochemistry and Molecular Biology*, 45:89-110.

Dhurua, S. & Gujar, G.T. 2011. Field-evolved resistance to *Bt* toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), from India. *Pest Management Science*, 67(8):898-903.

Díaz-Mendoza, M., Ortego, F., García de Lacoba, M., Magaña, C., De la Poza, M., Farinós, G.P., Castañera, P. & Hernández-Crespo, P. 2005. Diversity of trypsins in the Mediterranean corn borer *Sesamia nonagrioides* (Lepidoptera: Noctuidae), revealed by nucleic acid sequences and enzyme purification. *Insect Biochemistry and Molecular Biology*, 35:1005-1020.

Ding, B.-J., Liénard, M.A., Wang, H.-L., Zhao, C.-H. & Löfstedt, C. 2011. Terminal fatty-acyl-CoA desaturase involved in sex pheromone biosynthesis in the winter moth (*Operophtera brumata*). *Insect Biochemistry and Molecular Biology*, 41:715-722.

Dong, H.Z. & Li, W.J. 2007. Variability of endotoxin expression in Bt transgenic cotton. *Journal of Agronomy and Crop Science*, 193(1):21-29.

Downes, S. & Mahon, R. 2012. Evolution, ecology and management of resistance in *Helicoverpa* spp. to Bt cotton in Australia. *Journal of Invertebrate Pathology*, 110(3):281-286.

Downes, S., Mahon, R. & Olsen, K. 2007. Monitoring and adaptive resistance management in Australia for Bt-cotton: current status and future challenges. *Journal of Invertebrate Pathology*, 95(3):208-213.

Downes, S., Mahon, R.J., Rossiter, L., Kauter, G., Leven, T., Fitt, G. & Baker, G. 2010a. Adaptive management of pest resistance by *Helicoverpa* species (Noctuidae) in Australia to the Cry2Ab Bt toxin in Bollgard II® cotton. *Evolutionary Applications*, 3(5-6):574-584.

Downes, S., Parker, T. & Mahon, R. 2010b. Incipient resistance of *Helicoverpa punctigera* to the Cry2Ab Bt toxin in Bollgard II cotton. *PLoS One*, 5(9):e12567.

Downes, S., Parker, T.L. & Mahon, R.J. 2010c. Characteristics of resistance to *Bacillus thuringiensis* toxin Cry2Ab in a strain of *Helicoverpa punctigera* (Lepidoptera: Noctuidae) isolated from a field population. *Journal of Economic Entomology*, 103(6):2147-2154.

Dupas, S., Le Rü, B.P., Branca, A., Faure, N., Gigot, G., Campagne, P., Sezonlin, M., Ndemah, R., Ong'amo, G., Calatayud, P.A. & Silvain, J.F. 2014. Phylogeography in continuous space: coupling species distribution models and circuit theory to assess the effect of contiguous migration at different climatic periods on genetic differentiation in *Busseola fusca* (Lepidoptera: Noctuidae). *Molecular Ecology*, 23(9):2313-2325.

Eguchi, M. 1995. Alkaline phosphatase isozymes in insects and comparison with mammalian enzyme. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 111B(2):151-162.

Eguchi, M., Sawaki, M. & Suzuki, Y. 1972. Multiple forms of midgut alkaline phosphatase in the silkworm: new band formation and the relationship between the midgut and digestive fluid enzymes. *Insect Biochemistry*, 2(7):297-304.

Eyster, K.M. 2007. The membrane and lipids as integral participants in signal transduction: lipid signal transduction for the non-lipid biochemist. *Advances in Physiology Education*, 31(1):5-16.

Fabrick, J.A., Mathew, L.G., Tabashnik, B.E. & Li, X. 2011. Insertion of an intact CR1 retrotransposon in a cadherin gene linked with Bt resistance in the pink bollworm, *Pectinophora gossypiella*. *Insect Molecular Biology*, 20(5):651-665.

Fabrick, J.A., Ponnuraj, J., Singh, A., Tanwar, R.K., Unnithan, G.C., Yelich, A.J., Li, X., Carriére, Y. & Tabashnik, B.E. 2014. Alternative splicing and highly variable cadherin transcripts

associated with field-evolved resistance of pink bollworm to Bt cotton in India. *PLoS One*, 9(5):e97900.

Fabrick, J.A. & Tabashnik, B.E. 2007. Binding of *Bacillus thuringiensis* toxin Cry1Ac to multiple sites of cadherin in pink bollworm. *Insect Biochemistry and Molecular Biology*, 37(2):97-106.

Fabrick, J.A. & Tabashnik, B.E. 2012. Similar genetic basis of resistance to Bt toxin Cry1Ac in Boll-selected and diet-selected strains of pink bollworm. *PLoS One*, 7(4):e35658.

Fabrick, J.A. & Wu, Y. 2015. Roles of insect midgut cadherin in Bt intoxication and resistance. *(In* Soberón, M., Gao Y. & Bravo A., *eds*. Bt resistance: characterization and strategies for GM crops producing *Bacillus thuringiensis* toxins. UK: CAB International. p. 69-83).

Fan, J., Liu, J., Culty, M. & Papadopoulos, V. 2010. Acyl-coenzyme A binding domain containing 3 (ACBD3; PAP7; GCP60): an emerging signaling molecule. *Progress in Lipid Research*, 49(3):218-234.

Fang, Q., Wang, L., Zhu, J., Li, Y., Song, Q., Stanley, D.W., Akhtar, Z. & Ye, G. 2010. Expression of immune-response genes in lepidopteran host is suppressed by venom from an endoparasitoid, *Pteromalus puparum*. *BMC Genomics*, 11:484-500.

Farias, I.P., Ortí, G., Sampaio, I., Schneider, H. & Meyer, A. 2001. The cytochrome *b* gene as a phylogenetic marker: the limits of resolution for analyzing relationships among cichlid fishes. *Journal of Molecular Evolution*, 53(2):89-103.

Farias, J.R., Andow, D.A., Horikoshi, R.J., Sorgatto, R.J., Fresia, P., Dos Santos, A.C. & Omoto, C. 2014. Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. *Crop Protection*, 64:150-158.

Faure, N. 2006. Microsatellite families based on similarities among flanking regions in insects. (Unpublished).

Faure, N. & Silvain, J.-F. 2005. Characterization of eight microsatellite loci in the maize stalk borer *Busseola fusca* Fuller, 1901 (Lepidoptera: Noctuidae). *Molecular Ecology Notes*, 5(4):846-848.

Ferré, J., Real, M.D., Van Rie, J., Jansens, S. & Peferoen, M. 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. *Proceedings of the National Academy of Sciences*, 88(12):5119-5123.

Ferré, J. & Van Rie, J. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annual Review of Entomology*, 47:501-533.

Ffrench-Constant, R.H., Steichen, J., Rocheleau, T.A., Aronstein, K. & Roush, R.T. 1993. A single-amino acid substitution in a gamma-aminobutyric acid subtype A receptor locus is associated with cyclodiene insecticide resistance in *Drosophila* populations. *Proceedings of the National Academy of Sciences of the United States of America*, 90:1957-1961.

Flannagan, R.D., Yu, C.G., Mathis, J.P., Meyer, T.E., Shi, X., Siqueira, H.A. & Siegfried, B.D. 2005. Identification, cloning and expression of a Cry1Ab cadherin receptor from European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). *Insect Biochemistry and Molecular Biology*, 35(1):33-40.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3:294-299.

Forcada, C., Alcácer, E., Garcerá, M.D. & Martínez, R. 1996. Differences in the midgut proteolytic activity of two *Heliothis virescens* strains, one susceptible and one resistant to *Bacillus thuringiensis* toxins. *Archives of Insect Biochemistry and Physiology*, 31:257-272.

Forcada, C., Alcácer, E., Garcerá, M.D., Tato, A. & Martínez, R. 1999. Resistance to *Bacillus thuringiensis* Cry1Ac toxin in three strains of *Heliothis virescens*: proteolytic and SEM study of the larval midgut. *Archives of Insect Biochemistry and Physiology*, 42(1):51-63.

Franklin, M.T., Nieman, C.L., Janmaat, A.F., Soberón, M., Bravo, A., Tabashnik, B.E. & Myers, J.H. 2009. Modified *Bacillus thuringiensis* toxins and a hybrid *B. thuringiensis* strain counter greenhouse-selected resistance in *Trichoplusia ni*. *Applied and Environmental Microbiology*, 75(17):5739-5741.

Franssens, V. 2006. Study of two types of immune responses in insects: nodulation in the flesh fly, *Neobellieria bullata*, and prophenoloxidase activation in the desert locust, *Schistocerca gregaria.* Nederland: Katholieke Universiteit Leuven. (Thesis - PhD).

Gahan, L.J., Gould, F. & Heckel, D.G. 2001. Identification of a gene associated with Bt resistance in *Heliothis virescens*. *Science*, 293(5531):857-860.

Gahan, L.J., Gould, F., López, J.D., Jr., Micinski, S. & Heckel, D.G. 2007. A polymerase chain reaction screen of field populations of *Heliothis virescens* for a retrotransposon insertion

conferring resistance to *Bacillus thuringiensis* toxin. *Journal of Economic Entomology*, 100(1):187-194.

Gahan, L.J., Pauchet, Y., Vogel, H. & Heckel, D.G. 2010. An ABC transporter mutation is correlated with insect resistance to *Bacillus thuringiensis* Cry1Ac toxin. *PLoS Genetics*, 6(12):e1001248.

Geisler, C. & Jarvis, D.L. 2016. Rhabdovirus-like endogenous viral elements in the genome of *Spodoptera frugiperda* insect cells are actively transcribed: Implications for adventitious virus detection. *Biologicals*, 44:219-225.

George, D.M., Rind, F.C., Bendall, M.W., Taylor, M.A. & Gatehouse, A.M.R. 2012. Developmental studies of transgenic maize expressing Cry1Ab on the African stem borer, *Busseola fusca*; effects on midgut cellular structure. *Pest Management Science*, 68(3):330-339.

Gill, M. & Ellar, D.J. 2002. Transgenic *Drosophila* reveals a functional in vivo receptor for the *Bacillus thuringiensis* toxin Cry1Ac1. *Peptides*, 28(1):169-173.

Gómez, I., Pardo-López, L., Muñoz-Garay, C., Fernandez, L.E., Pérez, C., Sánchez, J., Soberón, M. & Bravo, A. 2007. Role of receptor interaction in the mode of action of insecticidal Cry and Cyt toxins produced by *Bacillus thuringiensis*. *Peptides*, 28(1):169-173.

Gómez, I., Sánchez, J., Miranda, R., Bravo, A. & Soberón, M. 2002. Cadherin-like receptor binding facilitates proteolytic cleavage of helix α-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin. *FEBS Letters*, 513(2-3):242-246.

Gong, Y., Wang, C., Yang, Y., Wu, S. & Wu, Y. 2010. Characterization of resistance to *Bacillus thuringiensis* toxin Cry1Ac in *Plutella xylostella* from China. *Journal of Invertebrate Pathology*, 104:90-96.

González-Cabrera, J., Escriche, B., Tabashnik, B.E. & Ferré, J. 2003. Binding of *Bacillus thuringiensis* toxins in resistant and susceptible strains of pink bollworm (*Pectinophora gossypiella*). *Insect Biochemistry and Molecular Biology*, 33(9):929-935.

Gould, F. 1988. Evolutionary biology and genetically engineered crops. *BioScience*, 38:26-33.

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. *Annual Review of Entomology*, 43:701-726.

Gould, F. 2000. Testing *Bt* refuge strategies in the field. *Nature Biotechnology*, 18:266-267.

Gould, F., Anderson, A., Reynolds, A., Bumgarner, L. & Moar, W. 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. *Journal of Economic Entomology*, 88(6):1545-1559.

Gould, F., Martínez-Ramírez, A., Anderson, A., Ferré, J., Silva, F.J. & Moar, W.J. 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. *Proceedings of the National Academy of Sciences*, 89:7986-7990.

Gouse, M., Pray, C.E., Kirsten, J. & Schimmelpfennig, D. 2005. A GM subsistence crop in Africa: the case of Bt white maize in South Africa. *International Journal of Biotechnology*, 7(1/2/3):84-94.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., Di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N. & Regev, A. 2011. Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. *Nature Biotechnology*, 29(7):644-652.

Graf, J. 2011. Shifting paradigm on *Bacillus thuringiensis* toxin and a natural model for *Enterococcus faecalis* septicemia. *mBio*, 2(4):e00161-00111.

Grant, W.S. & Bowen, B.W. 1998. Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. *Journal of Heredity*, 89:415-426.

Griffitts, J.S. & Aroian, R.V. 2005. Many roads to resistance: how invertebrates adapt to Bt toxins. *BioEssays*, 27(6):614-624.

Griffitts, J.S., Haslam, S.M., Yang, T., Garczynski, S.F., Mulloy, B., Morris, H., Cremer, P.S., Dell, A., Adang, M.J. & Aroian, R.V. 2005. Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. *Science Reports*, 307(5711):922-925.

Guillemot, L., Spadaro, D. & Citi, S. 2013. The junctional proteins cingulin and paracingulin modulate the expression of tight junction protein genes through GATA-4. *PLoS One*, 8(2):e55873.

Gunaratna, R.T. & Jiang, H. 2013. A comprehensive analysis of the *Manduca sexta* immunotranscriptome. *Developmental and Comparative Immunology*, 39(4):388-398.

Gunning, R.V., Dang, H.T., Kemp, F.C., Nicholson, I.C. & Moores, G.D. 2005. New resistance mechanism in *Helicoverpa armigera* threatens transgenic crops expressing *Bacillus thuringiensis* Cry1Ac toxin. *Applied and Environmental Microbiology*, 71(5):2558-2563.

Guo, Z., Kang, S., Chen, D., Wu, Q., Wang, S., Xie, W., Zhu, X., Baxter, S.W., Zhou, X., Jurat-Fuentes, J.L. & Zhang, Y. 2015a. MAPK signaling pathway alters expression of midgut ALP and ABCC genes and causes resistance to *Bacillus thuringiensis* Cry1Ac toxin in diamondback moth. *PLoS Genetics*, 11(4):e1005124.

Guo, Z., Kang, S., Zhu, X., Xia, J., Wu, Q., Wang, S., Xie, W. & Zhang, Y. 2015b. Down-regulation of a novel ABC transporter gene (*Pxwhite*) is associated with Cry1Ac resistance in the diamondback moth, *Plutella xylostella* (L.). *Insect Biochemistry and Molecular Biology*, 59:30-40.

Guo, Z., Zhu, Y.C., Huang, F., Luttrell, R. & Leonard, R. 2012. Microarray analysis of global gene regulation in the Cry1Ab-resistant and Cry1Ab-susceptible strains of *Diatraea saccharalis*. *Pest Management Science*, 68(5):718-730.

Güz, N., Dağeri, A., Erdoğan, T., Mousavi, M., Bayram, Ş. & Gürkan, M.O. 2013. Transcriptional profiling of *transferrin* gene from Egyptian cotton leaf worm, *Spodoptera littoralis*. *Turkish Journal of Biology*, 37:582-590.

Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B., Eccles, D., Li, B., Lieber, M., Macmanes, M.D., Ott, M., Orvis, J., Pochet, N., Strozzi, F., Weeks, N., Westerman, R., William, T., Dewey, C.N., Henschel, R., Leduc, R.D., Friedman, N. & Regev, A. 2013. *De novo* transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols*, 8(8):1494-1512.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41:95-98.

Han, G., Li, X., Zhang, T., Zhu, X. & Li, J. 2015. Cloning and tisseue-specific expression of a chitin deacetylase gene from *Helicoverpa armigera* (Lepidoptera: Noctuidae) and its response to *Bacillus thuringiensis*. *Journal of Insect Science*, 15(1):95.

Han, L., Han, C., Liu, Z., Chen, F., Jurat-Fuentes, J.L., Hou, M. & Peng, Y. 2014. Binding site concentration explains the differential susceptibility of *Chilo suppressalis* and *Sesamia inferens* to Cry1A-producing rice. *Applied and Environmental Microbiology*, 80(16):5134-5140.

Hara, H., Atsumi, S., Yaoi, K., Nakanishi, K., Higurashi, S., Miura, N., Tabunoki, H. & Sato, R. 2003. A cadherin-like protein functions as a receptor for *Bacillus thuringiensis* Cry1Aa and Cry1Ac toxins on midgut epithelial cells of *Bombyx mori* larvae. *FEBS Letters*, 538(1-3):29-34.

Harrison, R.L. & Bonning, B.C. 2010. Proteases as insecticidal agents. *Toxins*, 2:935-953.

Hauptfleisch, K., Yonow, T., Kriticos, D.J. & Ota, N. 2014. *Busseola fusca. HarvestChoice Pest Geography*, St. Paul, MN:InSTePP-HarvestChoice.

Havird, J.C. & Santos, S.R. 2014. Performance of single and concatenated sets of mitochondrial genes at inferring metazoan relationships relative to full mitogenome data. *PLoS One*, 9(1):e84080.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & De Waard, J.R. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B*, 270:313-321.

Heckel, D.G. 2012. Learning the ABCs of Bt: ABC transporters and insect resistance to *Bacillus thuringiensis* provide clues to a crucial step in toxin mode of action. *Pesticide Biochemistry and Physiology*, 104(2):103-110.

Heckel, D.G. 2015. Roles of ABC proteins in the mechanism and management of Bt resistance. *(In* Soberón, M., Gao Y. & Bravo A., *eds*. Bt resistance: characterization and strategies for GM crops producing *Bacillus thuringiensis* toxins. UK: CAB International. p. 98-106).

Heckel, D.G., Gahan, L.J., Baxter, S.W., Zhao, J.-Z., Shelton, A.M., Gould, F. & Tabashnik, B.E. 2007. The diversity of Bt resistance genes in species of Lepidoptera. *Journal of Invertebrate Pathology*, 95(3):192-197.

Hernández-Martínez, P., Navarro-Cerrillo, G., Caccia, S., De Maagd, R.A., Moar, W.J., Ferré, J., Escriche, B. & Herrero, S. 2010. Constitutive activation of the midgut response to *Bacillus thuringiensis* in Bt-resistant *Spodoptera exigua*. *PLoS One*, 5(9):e12795.

Herrero, S., Gechev, T., Bakker, P.L., Moar, W.J. & De Maagd, R.A. 2005. *Bacillus thuringiensis* Cry1Ca-resistant *Spodoptera exigua* lacks expression of one of four Aminopeptidase N genes. *BMC Genomics*, 6(1):96.

Herrero, S., Oppert, B. & Ferré, J. 2001. Different mechanisms of resistance to *Bacillus thuringiensis* toxins in the indianmeal moth. *Applied and Environmental Microbiology*, 67(3):1085-1089.

Hiller, D., Jiang, H., Xu, W. & Wong, W.H. 2009. Identifiability of isoform deconvolution from junction arrays and RNA-Seq. *Bioinformatics*, 25(23):3056-3059.

Hooper, N.M. 1994. Families of zinc metalloproteases. *FEBS Letters*, 354(1):1-6.

Hossain, D.M., Shitomi, Y., Moriyama, K., Higuchi, M., Hayakawa, T., Mitsui, T., Sato, R. & Hori, H. 2004. Characterization of a novel plasma membrane protein, expressed in the midgut epithelia of *Bombyx mori*, that binds to Cry1A toxins. *Applied and Environmental Microbiology*, 70(8):4604-4612.

Hua, G., Jurat-Fuentes, J.L. & Adang, M.J. 2004. Fluorescent-based assays establish *Manduca sexta* Bt-R_{1a} cadherin as a receptor for multiple *Bacillus thuringiensis* Cry1A toxins in *Drosophila* S2 cells. *Insect Biochemistry and Molecular Biology*, 34(3):193-202.

Huang, F., Buschman, L.L., Higgins, R.A. & McGaughey, W.H. 1999a. Inheritance of resistance to *Bacillus thuringiensis* toxin (Dipel ES) in the European corn borer. *Science*, 284(5416):965-967.

Huang, F., Ghimire, M.N., Leonard, B.R., Daves, C., Levy, R. & Baldwin, J. 2012. Extended monitoring of resistance to *Bacillus thuringiensis* Cry1Ab maize in *Diatraea saccharalis* (Lepidoptera: Crambidae). *GM Crops and Food*, 3(3):245-254.

Huang, F., Zhu, Y., Buschman, L.L., Higgins, R.A. & Oppert, B. 1999b. Comparison of midgut proteinases in *Bacillus thuringiensis*-susceptible and -resistant European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Pesticide Biochemistry and Physiology*, 65(2):132-139.

Hunt, P.N., Wilson, M.D., von Schalburg, K.R., Davidson, W.S. & Koop, B.F. 2005. Expression and genomic organization of zonadhesin-like genes in three species of fish give insight into the evolutionary history of a mosaic protein. *BMC Genomics*, 6:165.

Huyser, E. 2015. Base-line susceptibility of *Busseola fusca* for Bt maize in South Africa. (Dissertation).

Iida, K., Cox-Foster, D.L., Yang, X., Ko, W.Y. & Cavener, D.R. 2007. Expansion and evolution of insect GMC oxidoreductases. *BMC Evolutionary Biology*, 7:75.

Jacquet, F., Nicolas, V., Bonillo, C., Cruaud, C. & Denys, C. 2012. Barcoding, molecular taxonomy, and exploration of the diversity of shrews (Soricomorpha: Soricidae) on Mount Nimba (Guinea). *Zoological Journal of the Linnean Society*, 166(3):672-687.

Jakka, S.R.K., Gong, L., Hasler, J., Banerjee, R., Sheets, J.J., Narva, K., Blanco, C.A. & Jurat-Fuentes, J.L. 2015. Field-evolved mode 1 resistance of the fall armyworm to transgenic Cry1Fa-expressing corn associated with reduced Cry1Fa toxin binding and midgut alkaline phosphatase expression. *Applied and Environmental Microbiology*, 82(4):1023-1034.

James, C. 2010. Executive summary. Global status of commercialized/biotech crops: 2010. *ISAAA Brief*, No. 42.

James, C. 2014. Executive Summary. Global status of commercialized biotech/GM crops: 2014. *ISAAA Brief*, No. 49.

James, C. 2015a. 20th anniversary (1996-2015) of the global commercialization of biotech crops and biotech crop highlights in 2015. *ISAAA Brief*, No. 51.

James, C. 2015b. Executive Summary. Global status of commercialized biotech/GM crops: 2015. *ISAAA Brief*, No. 51.

Jin, Q., Han, H., Hu, X., Li, X., Zhu, C., Ho, S.Y., Ward, R.D. & Zhang, A.B. 2013. Quantifying species diversity with a DNA barcoding-based method: Tibetan moth species (Noctuidae) on the Qinghai-Tibetan Plateau. *PLoS One*, 8(5):e64428.

Jin, T., Chang, X., Gatehouse, A.M.R., Wang, Z., Edwards, M.G. & He, K. 2014. Downregulation and mutation of a cadherin gene associated with Cry1Ac resistance in the Asian corn borer, *Ostrinia furnacalis* (Guenee). *Toxins*, 6(9):2676-2693.

Jiravanichpaisala, P., Leeb, B.L. & Söderhäll, K. 2006. Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization. *Immunobiology*, 211:213-236.

Johnston, P.R. & Crickmore, N. 2009. Gut bacteria are not required for the insecticidal activity of *Bacillus thuringiensis* toward the tobacco hornworm, *Manduca sexta*. *Applied and Environmental Microbiology*, 75(15):5094-5099.

Jurat-Fuentes, J.L. 2010. Characterization of Cry toxin mode of action. http://web.utk.edu/~jurat/Btresearchtable.html Date of access: 16 Nov. 2010.

Jurat-Fuentes, J.L. & Adang, M.J. 2001. Importance of Cry1 δ-endotoxin domain II loops for binding specificity in *Heliothis virescens* (L.). *Applied and environmental microbiology*, 67(1):323-329.

Jurat-Fuentes, J.L. & Adang, M.J. 2004. Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. *European Journal of Biochemistry*, 271(15):3127-3135.

Jurat-Fuentes, J.L. & Adang, M.J. 2006. Cry toxin mode of action in susceptible and resistant *Heliothis virescens* larvae. *Journal of Invertebrate Pathology*, 92(3):166-171.

Jurat-Fuentes, J.L. & Adang, M.J. 2007. A proteomic approach to study Cry1Ac binding proteins and their alterations in resistant *Heliothis virescens* larvae. *Journal of Invertebrate Pathology*, 95(3):187-191.

Jurat-Fuentes, J.L., Gahan, L.J., Gould, F.L., Heckel, D.G. & Adang, M.J. 2004. The HevCaLP protein mediates binding specificity of the Cry1A class of *Bacillus thuringiensis* toxins in *Heliothis virescens*. *Biochemistry*, 43(44):14299-14305.

Jurat-Fuentes, J.L., Gould, F.L. & Adang, M.J. 2002. Altered glycosylation of 63- and 68-kilodalton microvillar proteins in *Heliothis virescens* correlates with reduced Cry1 toxin binding, decreased pore formation, and increased resistance to *Bacillus thuringiensis* Cry1 toxins. *Applied and Environmental Microbiology*, 68(11):5711-5717.

Jurat-Fuentes, J.L., Gould, F.L. & Adang, M.J. 2003. Dual resistance to *Bacillus thuringiensis* Cry1Ac and Cry2Aa toxins in *Heliothis virescens* suggests multiple mechanisms of resistance. *Applied and Environmental Microbiology*, 69(10):5898-5906.

Jurat-Fuentes, J.L., Karumbaiah, L., Jakka, S.R.K., Ning, C., Liu, C., Wu, K., Jackson, J., Gould, F., Blanco, C., Portilla, M., Perera, O. & Adang, M. 2011. Reduced levels of membrane-bound alkaline phosphatase are common to lepidopteran strains resistant to Cry toxins from *Bacillus thuringiensis*. *PLoS One*, 6(3):e17606.

Karim, S. & Dean, D.H. 2000. Pesticidal and receptor binding properties of *Bacillus thuringiensis* Cry1Ab and Cry1Ac δ-endotoxin mutants to *Pectinophora gossypiella* and *Helicoverpa zea*. *Current Microbiology*, 41(6):430-440.

Karumbaiah, L., Oppert, B., Jurat-Fuentes, J.L. & Adang, M.J. 2007. Analysis of midgut proteinases from *Bacillus thuringiensis*-susceptible and -resistant *Heliothis virescens* (Lepidoptera: Noctuidae). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 146(1):139-146.

Keller, M., Sneh, B., Strizhov, N., Prudovsky, E., Regev, A., Koncz, C., Schell, J. & Zilberstein, A. 1996. Digestion of δ-endotoxin by gut proteases may explain reduced sensitivity of

advanced instar larvae of *Spodoptera littoralis*. *Insect Biochemistry and Molecular Biology*, 26(4):365-373.

Kfir, R., Overholt, W.A., Khan, Z.R. & Polaszek, A. 2002. Biology and management of economically important lepidopteran cereal stem borers in Africa. *Annual Review of Entomology*, 47:701-731.

Khajuria, C., Buschman, L.L., Chen, M.S., Siegfried, B.D. & Zhu, K.Y. 2011. Identification of a novel aminopeptidase P-like gene (*OnAPP*) possibly involved in Bt toxicity and resistance in a major corn pest (*Ostrinia nubilalis*). *PLoS One*, 6(8):e23983.

Knight, P.J.K., Knowles, B.H. & Ellar, D.J. 1995. Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* Cry1A(c) toxin. *The Journal of Biological Chemistry*, 270(30):17765-17770.

Knowles, B.H. & Farndale, R.W. 1988. Activation of insect cell adenylate cyclase by *Bacillus thuringiensis* δ-endotoxins and melittin. *Biochemical Journal*, 253(1):235-241.

Kolberg, M., Strand, K.R., Graff, P. & Andersson, K.K. 2004. Structure, function, and mechanism of ribonucleotide reductases. *Biochimica et Biophysica Acta*, 1699(1-2):1-34.

Kotey, D.A., Obi, A., Assefa, Y., Erasmus, A. & Van den Berg, J. 2017. Monitoring resistance to Bt maize in field populations of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) from smallholder farms in the Eastern Cape Province of South Africa. *African Entomology*, 25(1):200-209.

Krishnamoorthy, M., Jurat-Fuentes, J.L., McNall, R.J., Andacht, T. & Adang, M.J. 2007. Identification of novel Cry1Ac binding proteins in midgut membranes from *Heliothis virescens* using proteomic analyses. *Insect Biochemistry and Molecular Biology*, 37(3):189-201.

Kruger, M., Van Rensburg, J.B.J. & Van den Berg, J. 2009. Perspective on the development of stem borer resistance to Bt maize and refuge compliance at the Vaalharts irrigation scheme in South Africa. *Crop Protection*, 28(8):684-689.

Kruger, M., Van Rensburg, J.B.J. & Van den Berg, J. 2011. Resistance to Bt maize in *Busseola fusca* (Lepidoptera: Noctuidae) from Vaalharts, South Africa. *Environmental Entomology*, 40(2):477-483.

Kruger, M., Van Rensburg, J.B.J. & Van den Berg, J. 2012. Transgenic Bt maize: farmers' perceptions, refuge compliance and reports of stem borer resistance in South Africa. *Journal of Applied Entomology*, 136(1-2):38-50.

Kruger, M., Van Rensburg, J.B.J. & Van den Berg, J. 2014. No fitness costs associated with resistance of *Busseola fusca* (Lepidoptera: Noctuidae) to genetically modified Bt maize. *Crop Protection*, 55:1-6.

Kulkarni, G.V. & Deobagkar, D.D. 2002. A cytosolic form of aminopeptidase P from *Drosophila melanogaster*: molecular cloning and characterization. *Journal of Biochemistry*, 131:445-452.

Kumar, S. & Kumari, R. 2015. Occurrence of molecularly diverse *Bt* Cry toxin-resistant mutations in insect pests of Bt^{+} corn and cotton crops and remedial approaches. *Current Science*, 108(8):1483-1490.

Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33:1870-1874.

Kumaraswami, N.S., Maruyama, T., Kurabe, S., Kishimoto, T., Mitsui, T. & Hori, H. 2001. Lipids of brush border membrane vesicles (BBMV) from *Plutella xylostella* resistant and susceptible to Cry1Ac delta-endotoxin of *Bacillus thuringiensis*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 129(1):173-183.

Langmead, B. & Salzberg, S. 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods*, 9:357-359.

Lavine, M.D. & Strand, M.R. 2002. Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology*, 32(10):1295-1309.

Le Rü, B., Capdevielle-Dulac, C., Conlong, D., Pallangyo, B., Van den Berg, J., Ong'amo, G. & Kergoat, G.J. 2015. A revision of the genus *Conicofrontia* Hampson (Lepidoptera, Noctuidae, Apameini, Sesamiina), with description of a new species: new insights from morphological, ecological and molecular data. *Zootaxa*, 3925(1):56-74.

Lee, M.K., Rajamohan, F., Gould, F. & Dean, D.H. 1995. Resistance to *Bacillus thuringiensis* Cry1A δ-endotoxins in a laboratory-selected *Heliothis virescens* strain is related to receptor alteration. *Applied and Environmental Microbiology*, 61(11):3836-3842.

Lee, M.K., You, T.H., Young, B.A., Cotrill, J.A., Valaitis, A.P. & Dean, D.H. 1996. Aminopeptidase N purified form gypsy moth brush border membrane vesicles is a specific

receptor for *Bacillus thuringiensis* Cry1Ac toxin. *Applied and Environmental Microbiology*, 62(8):2845-2849.

Lei, Y., Zhu, X., Xie, W., Wu, Q., Wang, S., Guo, Z., Xu, B., Li, X., Zhou, X. & Zhang, Y. 2014. Midgut transcriptome response to a Cry toxin in the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae). *Gene*, 533(1):180-187.

Li, A., Zhao, Q., Tang, S., Zhang, Z., Pan, S. & Shen, G. 2005a. Molecular phylogeny of the domesticated silkworm, *Bombyx mori*, based on the sequences of mitochondrial *cytochrome b* genes. *Journal of Genetics*, 84(2):137-142.

Li, B. & Dewey, C. 2016. RSEM (RNA-Seq by Expectation-Maximization). http://deweylab.github.io/RSEM/ Date of access: 31 Dec. 2016.

Li, B. & Dewey, C.N. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*, 12:323.

Li, H., Gonzáles-Cabrera, J., Oppert, B., Ferré, J., Higgins, R.A., Buschman, L.L., Radke, G.A., Zhu, K.Y. & Huang, F. 2004a. Binding analyses of Cry1Ab and Cry1Ac with membrane vesicles from *Bacillus thuringiensis*-resistant and -susceptible *Ostrinia nubilalis*. *Biochemical and Biophysical Research Communications*, 323(1):52-57.

Li, H., Oppert, B., Higgins, R.A., Huang, F., Buschman, L.L., Gao, J.R. & Zhu, K.Y. 2005b. Characterization of cDNAs encoding three trypsin-like proteinases and mRNA quantitative analysis in Bt-resistant and -susceptible strains of *Ostrinia nubilalis*. *Insect Biochemistry and Molecular Biology*, 35(8):847-860.

Li, H., Oppert, B., Higgins, R.A., Huang, F., Zhu, K.Y. & Buschman, L.L. 2004b. Comparative analysis of proteinase activities of *Bacillus thuringiensis*-resistant and -susceptible *Ostrinia nubilalis* (Lepidoptera: Crambidae). *Insect Biochemistry and Molecular Biology*, 34(8):753-762.

Li, H., Zhang, H., Guan, R. & Miao, X. 2013a. Identification of differential expression genes associated with host selection and adaptation between two sibling insect species by transcriptional profile analysis. *BMC Genomics*, 14:582.

Li, J., Li, X., Chen, Y., Yang, Z. & Guo, S. 2012. Solexa sequencing based transcriptome analysis of *Helicoverpa armigera* larvae. *Molecular Biology Reports*, 39(12):11051-11059.

Li, L.T., Zhu, Y.B., Ma, J.F., Li, Z.Y. & Dong, Z.P. 2013b. An analysis of the *Athetis lepigone* transcriptome from four developmental stages. *PLoS One*, 8(9):e73911.

Li, Q.Q., Li, D.Y., Ye, H., Liu, X.F., Shi, W., Cao, N. & Duan, Y.Q. 2011. Using *COI* gene sequence to barcode two morphologically alike species: the cotton bollworm and the oriental tobacco budworm (Lepidoptera: Noctuidae). *Molecular Biology Reports*, 38(8):5107-5113.

Liang, G.M., Wu, K.M., Yu, H.K., Li, K.K., Feng, X. & Guo, Y.Y. 2008. Changes of inheritance mode and fitness in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) along with its resistance evolution to Cry1Ac toxin. *Journal of Invertebrate Pathology*, 97(2):142-149.

Librado, P. & Rozas, J. 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25:1451-1452.

Lightwood, D.J., Ellar, D.J. & Jarret, P. 2000. Role of proteolysis in determining potency of *Bacillus thuringiensis* Cry1Ac δ-endotoxin. *Applied and Environmental Microbiology*, 66(12):5174-5181.

Liu, C., Xiao, Y., Li, X., Oppert, B., Tabashnik, B.E. & Wu, K. 2014. *Cis*-mediated down-regulation of a trypsin gene associated with Bt resistance in cotton bollworm. *Scientific Reports*, 4:7219.

Liu, F., Xu, Z., Zhu, Y.C., Huang, F., Wang, Y., Li, H., Li, H., Gao, C., Zhou, W. & Shen, J. 2010. Evidence of field-evolved resistance to Cry1Ac-expressing *Bt* cotton in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in northern China. *Pest Management Science*, 66(2):155-161.

Liu, Y.-B. & Tabashnik, B.E. 1997. Inheritance of resistance to the *Bacillus thuringiensis* toxin Cry1C in the diamondback moth. *Applied and Environmental Microbiology*, 63:2218-2223.

Liu, Y., Qi, M., Chi, Y. & Wuriyanghan, H. 2016. *De novo* assembly of the transcriptome for oriental armyworm *Mythimna separata* (Lepidoptera: Noctuidae) and analysis on insecticide resistance-related genes. *Journal of Insect Science*, 16(1):92-99.

Liu, Y.B., Tabashnik, B.E., Masson, L., Escriche, B. & Ferré, J. 2000. Binding and toxicity of *Bacillus thuringiensis* protein Cry1C to susceptible and resistant diamondback moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology*, 93(1):1-6.

Loeb, M.J., Martin, P.A.W., Hakim, R.S., Goto, S. & Takeda, M. 2001. Regeneration of cultured midgut cells after exposure to sublethal doses of toxin from two strains of *Bacillus thuringiensis*. *Journal of Insect Physiology*, 47(6):599-606.

Lu, L., Qi, Z. & Wu, W. 2014. Cloning, expression and purification of subunit H of vacuolar H^+-ATPase from *Mythimna separata* Walker (Lepidoptera: Noctuidae). *International Journal of Molecular Sciences*, 15(9):15443-15455.

Lu, Y.J. & Adang, M.J. 1996. Conversion of *Bacillus thuringiensis* Cry1Ac-binding aminopeptidase to a soluble form by endogenous phosphatidylinositol phospholipase C. *Insect Biochemistry and Molecular Biology*, 26(1):33-40.

Luo, K., Sangadala, S., Masson, L., Mazza, A., Brousseau, R. & Adang, M.J. 1997a. The *Heliothis virescens* 170 kDa aminopeptidase functions as "receptor A" by mediating specific *Bacillus thuringiensis* Cry1A δ-endotoxin binding and pore formation. *Insect Biochemistry and Molecular Biology*, 27(8-9):735-743.

Luo, K., Tabashnik, B.E. & Adang, M.J. 1997b. Binding of *Bacillus thuringiensis* Cry1Ac toxin to aminopeptidase in susceptible and resistant diamondback moths (*Plutella xylostella*). *Applied and Environmental Microbiology*, 63(3):1024-1027.

Luo, S., Wang, G., Liang, G., Wu, K.M., Bai, L., Ren, X. & Guo, Y. 2006. Binding of three Cry1A toxins in resistant and susceptible strains of cotton bollworm (*Helicoverpa armigera*). *Pesticide Biochemistry and Physiology*, 85(2):104-109.

Luttrell, R.G., Ali, I., Allen, K.C., Young Iii, S.Y., Szalanski, A., Williams, K., Lorenz, G., Parker Jr., C.D. & Blanco, C. 2004. Resistance to Bt in Arkansas populations of cotton bollworm. Paper presented at the Beltwide Cotton Conferences, San Antonio, TX, 5-9 Jan. http://naldc.nal.usda.gov/download/12012/PDF Date of access: 16 Aug. 2012.

Ma, G., Roberts, H., Sarjan, M., Featherstone, N., Lahnstein, J., Akhurst, R. & Schmidt, O. 2005. Is the mature endotoxin Cry1Ac from *Bacillus thuringiensis* inactivated by a coagulation reaction in the gut lumen of resistant *Helicoverpa armigera* larvae? *Insect Biochemistry and Molecular Biology*, 35(7):729-739.

MacIntosh, S.C., Kishore, G.M., Perlak, F.J., Marrone, P.G., Stone, T.B., Sims, S.R. & Fuchs, R.L. 1990. Potentiation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitors. *Journal of Agricultural and Food Chemistry*, 38(4):1145-1152.

MacIntosh, S.C., Stone, T.B., Jokerst, R.S. & Fuchs, R.L. 1991. Binding of *Bacillus thuringiensis* proteins to a laboratory-selected line of *Heliothis virescens*. *Proceedings of the National Academy of Sciences*, 88(20):8930-8933.

Mahon, R.J., Olsen, K.M., Garsia, K.A. & Young, S.R. 2007. Resistance to *Bacillus thuringiensis* toxin Cry2Ab in a strain of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Australia. *Journal of Economic Entomology*, 100(3):894-902.

Maier, D., Farr, C.L., Poeck, B., Alahari, A., Vogel, M., Fischer, S., Kaguni, L.S. & Schneuwly, S. 2001. Mitochondrial single-stranded DNA-binding protein is required for mitochondrial DNA replication and development in *Drosophila melanogaster*. *Molecular Biology of the Cell*, 12:821-830.

Mallet, J. & Porter, P. 1992. Preventing insect adaptation to insect-resistant crops: are seed mixtures or refugia the best strategy? *Proceedings of the Royal Society of London Series B*, 250:165-169.

Mao, Y.B., Cai, W.J., Wang, J.W., Hong, G.J., Tao, X.Y., Wang, L.J., Huang, Y.P. & Chen, X.Y. 2007. Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nature Biotechnology*, 25(11):1307-1313.

Marioni, J.C., Mason, C.E., Mane, S.M., Stephens, M. & Gilad, Y. 2008. RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. *Genome Research*, 18(9):1509-1517.

Maroto, F.G. & Sierra, J.M. 1989. Purification and characterization of mRNA cap-binding protein from *Drosophila melanogaster* embryos. *Molecular and Cellular Biology*, 9(5):2181-2190.

Martínez-Ramírez, A.C., Gould, F. & Ferré, J. 1999. Histopathological effects and growth reduction in a susceptible and a resistant strain of *Heliothis virescens* (Lepidoptera: Noctuidae) caused by sublethal doses of pure Cry1A crystal proteins from *Bacillus thuringiensis*. *Biocontrol Science and Technology*, 9(2):239-246.

Masson, L., Tabashnik, B.E., Mazza, A., Préfontaine, G., Potvin, L., Brousseau, R. & Schwartz, J.L. 2002. Mutagenic analysis of a conserved region of domain III in the Cry1Ac toxin of *Bacillus thuringiensis*. *Applied and Environmental Microbiology*, 68(1):194-200.

McCarthy, D.J., Chen, Y. & Smyth, G.K. 2012. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Research*, 40(10):4288–4297.

McGaughey, W.H. 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. *Science*, 229:193-195.

McKenzie, J.A. & Batterham, P. 1994. The genetic, molecular and phenotypic consequences of selection for insecticide resistance. *Trends in Ecology & Evolution*, 9:166-169.

McNall, R.J. & Adang, M.J. 2003. Identification of novel *Bacillus thuringiensis* Cry1Ac binding proteins in *Manduca sexta* midgut through proteomic analysis. *Insect Biochemistry and Molecular Biology*, 33(10):999-1010.

Mellon, M. & Rissler, J. 2004. Gone to seed: transgenic contaminants in the traditional seed supply. Cambridge, MA: UCS Publications.

Midboe, E., Candas, M. & Bulla, L.A., Jr. 2003. Expression of a midgut-specific cadherin BT-R_1 during the development of *Manduca sexta* larva. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 135(1):125-137.

Miller, N.J., Sun, J. & Sappington, T.W. 2012. High-throughput transcriptome sequencing for SNP and gene discovery in a moth. *Environmental Entomology*, 41(4):997-1007.

Milne, R., Wright, T., Kaplan, H. & Dean, D. 1998. Spruce budworm elastase precipitates *Bacillus thuringiensis* delta-endotoxin by specifically recognizing the C-terminal region. *Insect Biochemistry and Molecular Biology*, 28:1013-1023.

Min, X.J. & Hickey, D.A. 2007. DNA barcodes provide a quick preview of mitochondrial genome composition. *PLoS One*, 2(3):e325.

Moar, W.J., Pusztai-Carey, M., Van Faassen, H., Bosch, D., Frutos, R., Rang, C., Luo, K. & Adang, M.J. 1995. Development of *Bacillus thuringiensis* Cry1C resistance by *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). *Applied and Environmental Microbiology*, 61(6):2086-2092.

Monette, R., Potvin, L., Baines, D., Laprade, R. & Schwartz, J.L. 1997. Interaction between calcium ions and *Bacillus thuringiensis* toxin activity against Sf9 cells (*Spodoptera frugiperda*, Lepidoptera). *Applied and Environmental Microbiology*, 63:440-447.

Monnerat, R., Martins, E., Queiroz, P., Praça, L. & Soares, M. 2015. Insect resistance to Bt toxins in Brazil and Latin America. *(In* Soberón, M., Gao Y. & Bravo A., *eds*. Bt resistance: characterization and strategies for GM crops producing *Bacillus thuringiensis* toxins. UK: CAB International. p. 26-35).

Monsanto. 2009. MON89034 Safety summary, January 2009. http://www.monsanto.com/products/documents/safety-summaries/mon89034_pss.pdf Date of access: 13 April 2017.

Morin, S., Biggs, R.W., Sisterson, M.S., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, L.J., Heckel, D.G., Carriére, Y., Dennehy, T.J., Brown, J.K. & Tabashnik, B.E. 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. *Proceedings of the National Academy of Sciences*, 100(9):5004-5009.

Morin, S., Henderson, S., Fabrick, J.A., Carriére, Y., Dennehy, T.J., Brown, J.K. & Tabashnik, B.E. 2004. DNA-based detection of Bt resistance alleles in pink bollworm. *Insect Biochemistry and Molecular Biology*, 34(11):1225-1233.

Muñoz-Garay, C., Sánchez, J., Darszon, A., De Maagd, R.A., Bakker, P., Soberón, M. & Bravo, A. 2006. Permeability changes of *Manduca sexta* midgut brush border membranes induced by oligomeric structures of different cry toxins. *Journal of Membrane Biology*, 212(1):61-68.

Nair, R., Kalia, V., Aggarwal, K.K. & Gujar, G.T. 2010. Inheritance of Cry1Ac resistance and associated biological traits in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Invertebrate Pathology*, 104(1):31-38.

Nair, R., Kalia, V., Aggarwal, K.K. & Gujar, G.T. 2013. Variation in the cadherin gene sequence of Cry1Ac susceptible and resistant *Helicoverpa armigera* (Lepidoptera: Noctuidae) and the identification of mutant alleles in resistant strains. *Current Science*, 104(2):215-223.

Nakanishi, K., Yaoi, K., Nagino, Y., Hara, H., Kitami, M., Atsumi, S., Miura, N. & Sato, R. 2002. Aminopeptidase N isoforms from the midgut of *Bombyx mori* and *Plutella xylostella* – their classification and the factors that determine their binding specificity to *Bacillus thuringiensis* Cry1A toxin. *FEBS Letters*, 519:215-220.

Nascimento, A.R.B., Fresia, P., Cônsoli, F.L. & Omoto, C. 2015. Comparative transcriptome analysis of lufenuron-resistant and susceptible strains of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *BMC Genomics*, 16:985-997.

Nation, J.L. 2001. Insect physiology and biochemistry. Boca Raton: CRC Press. Date of access: 28 Oct. 2016.

Nguyen, Q., Palfreyman, R.W., Chan, L.C., Reid, S. & Nielsen, L.K. 2012. Transcriptome sequencing of and microarray development for a *Helicoverpa zea* cell line to investigate in vitro insect cell-baculovirus interactions. *PLoS One*, 7(5):e36324.

Nielsen, M.G., Gadagkar, S.R. & Gutzwiller, L. 2010. Tubulin evolution in insects: gene duplication and subfunctionalization provide specialized isoforms in a functionally constrained gene family. *BMC Evolutionary Biology*, 10:113-133.

Ningshen, T.J., Aparoy, P., Ventaku, V.R. & Dutta-Gupta, A. 2013. Functional interpretation of a non-gut hemocoelic tissue aminopeptidase N (APN) in a lepidopteran insect pest *Achaea janata*. *PLoS One*, 8(11):e79468.

Ong'amo, G.O., Le Rü, B.P., Moyal, P., Calatayud, P.A., Le Gall, P., Ogol, C.K.P.O., Kokwaro, E.D., Capdevielle-Dulac, C. & Silvain, J.F. 2008. Host plant diversity of *Sesamia calamistis*: cytochrome *b* gene sequences reveal local genetic differentiation. *Entomologia Experimentalis et Applicata*, 128(1):154-161.

Onstad, D.W. 2013. The role of environment in insect resistance management. *(In* Onstad, D.W., *ed.* Insect resistance management: biology, economics and prediction. California: Academic Press. p. 327-372).

Oppert, B., Kramer, K.J., Beeman, R.W., Johnson, D. & McGaughey, W.H. 1997. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. *Journal of Biological Chemistry*, 272(38):23473-23476.

Oppert, B., Kramer, K.J., Johnson, D., Upton, S.J. & McGaughey, W.H. 1996. Luminal proteinases from *Plodia interpunctella* and the hydrolysis of *Bacillus thuringiensis* Cry1A(c) protoxin. *Insect Biochemistry and Molecular Biology*, 26(6):571-583.

Pace, H.C. & Brenner, C. 2001. The nitrilase superfamily: classification, structure and function. *Genome Biology*, 2(1):reviews0001.1–0001.9.

Pacheco, S., Gómez, I., Arenas, I., Saab-Rincon, G., Rodríguez-Almazán, C., Gill, S.S., Bravo, A. & Soberón, M. 2009a. Domain II loop 3 of *Bacillus thuringiensis* Cry1Ab toxin is involved in a "ping pong" binding mechanism with *Manduca sexta* aminopeptidase-N and cadherin receptors. *Journal of Biological Chemistry*, 284(47):32750-32757.

Pacheco, S., Gómez, I., Gill, S.S., Bravo, A. & Soberón, M. 2009b. Enhancement of insecticidal activity of *Bacillus thuringiensis* Cry1A toxins by fragments of a toxin-binding cadherin correlates with oligomer formation. *Peptides*, 30(3):583-588.

Pandian, G.N., Ishikawa, T., Togashi, M., Shitomi, Y., Haginoya, K., Yamamoto, S., Nishiumi, T. & Hori, H. 2008. *Bombyx mori* midgut membrane protein P252, which binds to *Bacillus*

thuringiensis Cry1A, is a chlorophyllide-binding protein, and the resulting complex has antimicrobial activity. *Applied and Environmental Microbiology*, 74(5):1324-1331.

Papadopoulou, D. & Louis, C. 2000. The glutamate dehydrogenase GENE of *Drosophila melanogaster:* molecular analysis and expression. *Journal of Neurogenetics*, 14(3):125-143.

Paramasiva, I., Sharma, H.C. & Krishnayya, P.V. 2014. Antibiotics influence the toxicity of the delta endotoxins of *Bacillus thuringiensis* towards the cotton bollworm, *Helicoverpa armigera*. *BMC Microbiology*, 14(1):200.

Pardo-López, L., Soberón, M. & Bravo, A. 2013. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. *FEMS Microbiology Reviews*, 37(1):3-22.

Park, Y., González-Martínez, R.M., Navarro-Cerrillo, G., Chakroun, M., Kim, Y., Ziarsolo, P., Blanca, J., Cañizares, J., Ferré, J. & Herrero, S. 2014. ABCC transporters mediate insect resistance to multiple Bt toxins revealed by bulk segregant analysis. *BMC Biology*, 12(1):46.

Patwardhan, A., Ray, S. & Roy, A. 2014. Molecular markers in phylogenetic studies - a review. *Phylogenetics & Evolutionary Biology*, 2:131.

Peferoen, M. 1997. Progress and prospects for field use of *Bt* genes in crops. *Trends in Biotechnology*, 15(5):173-177.

Peng, D., Xu, X., Ruan, L., Yu, Z. & Sun, M. 2010. Enhancing Cry1Ac toxicity by expression of the *Helicoverpa armigera* cadherin fragment in *Bacillus thuringiensis*. *Research in Microbiology*, 161(5):383-389.

Perera, O.P., Shelby, K.S., Popham, H.J.R., Gould, F., Adang, M.J. & Jurat-Fuentes, J.L. 2015. Generation of a transcriptome in a model lepidopteran pest, *Heliothis virescens*, using multiple sequencing strategies for profiling midgut gene expression. *PLoS One*, 10(6):e0128563.

Perera, O.P., Willis, J.D., Adang, M.J. & Jurat-Fuentes, J.L. 2009. Cloning and characterization of the Cry1Ac-binding alkaline phosphatase (HvALP) from *Heliothis virescens*. *Insect Biochemistry and Molecular Biology*, 39(4):294-302.

Peterson, B., Bezuidenhout, C.C. & Van den Berg, J. 2016. Cytochrome *c* oxidase I and cytochrome *b* gene sequences indicate low genetic diversity in South African *Busseola fusca* (Lepidoptera: Noctuidae) from maize. *African Entomology*, 24(2):518-523.

Pigott, C.R. & Ellar, D.J. 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiology and Molecular Biology Reviews*, 71(2):255-281.

Potvin, L., Laprade, R. & Schwartz, J.L. 1998. Cry1Ac, a *Bacillus thuringiensis* toxin, triggers extracellular Ca^{2+} release from intracellular stores in Cf1 cells (*Choristoneura fumiferana*, Lepidoptera). *Journal of Experimental Biology*, 201:1851-1858.

Qi, W., Ma, X., He, W., Chen, W., Zou, M., Gurr, G.M., Vasseur, L. & You, M. 2016. Characterization and expression profiling of ATP-binding cassette transporter genes in the diamondback moth, *Plutella xylostella* (L.). *BMC Genomics*, 17:760-777.

Rahman, M.M., Roberts, H.L.S. & Schmidt, O. 2007. Tolerance to *Bacillus thuringiensis* endotoxin in immune-suppressed larvae of the flour moth *Ephestia kuehniella*. *Journal of Invertebrate Pathology*, 96(2):125-132.

Rajagopal, R., Arora, N., Sivakumar, S., Rao, N.G.V., Nimbalkar, S.A. & Bhatnagar, R.K. 2009. Resistance of *Helicoverpa armigera* to Cry1Ac toxin from *Bacillus thuringiensis* is due to improper processing of the protoxin. *Biochemical Journal*, 419(2):309-316.

Rajagopal, R., Sivakumar, S., Agrawal, N., Malhotra, P. & Bhatnagar, R.K. 2002. Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. *Journal of Biological Chemistry*, 277(49):46849-46851.

Rajamohan, F., Alzate, O., Cotrill, J.A., Curtiss, A. & Dean, D.H. 1996. Protein engineering of *Bacillus thuringiensis* delta-endotoxin: mutations at domain II of Cry1Ab enhance receptor affinity and toxicity toward gypsy moth larvae. *Proceedings of the National Academy of Sciences of the United States of America*, 93:14338-14343.

Rausell, C., García-Robles, I., Sánchez, J., Muñoz-Garay, C., Martínez-Ramírez, A.C., Real, M.D. & Bravo, A. 2004. Role of toxin activation on binding and pore formation activity of the *Bacillus thuringiensis* Cry3 toxins in membranes of *Leptinotarsa decemlineata* (Say). *Biochimica et Biophysica Acta - Biomembranes*, 1660(1-2):99-105.

Raymond, B., Johnston, P.R., Wright, D.J., Ellis, R.J., Crickmore, N. & Bonsall, M.B. 2009. A mid-gut microbiota is not required for the pathogenicity of *Bacillus thuringiensis* to diamondback moth larvae. *Environmental Microbiology*, 11(10):2556-2563.

Raymond, M., Callaghan, A., Fort, P. & Pasteur, N. 1991. Worldwide migration of amplified insecticide resistance genes in mosquitoes. *Nature (London)*, 350:151-153.

Rees, D.C., Johnson, E. & Lewinson, O. 2009. ABC transporters: the power to change. *Nature Reviews Molecular Cell Biology*, 10:218-227.

Ren, X.-L., Chen, R.-R., Zhang, Y., Ma, Y., Cui, J.-J., Han, Z.-J., Mu, L.-L. & Li, G.-Q. 2013. A *Spodoptera exigua* cadherin serves as a putative receptor for *Bacillus thuringiensis* Cry1Ca toxin and shows differential enhancement of Cry1Ca and Cry1Ac toxicity. *Applied and Environmental Microbiology*, 79(18):5576-5583.

Robinson, M.D., McCarthy, D.J. & Smyth, G.K. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics Applications Note*, 26(1):139-140.

Robinson, M.D. & Oshlack, A. 2010. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology*, 11:R25.

Roe, A.D. & Sperling, F.A.H. 2007. Patterns of evolution of mitochondrial cytochrome c oxidase I and II DNA and implications for DNA barcoding. *Molecular Phylogenetics and Evolution*, 44(1):325-345.

Rohrmann, G.F. 1986. Polyhedrin structure. *Journal of General Virology*, 67:1499-1513.

Rosenbaum, E.E., Vasiljevic, E., Brehm, K.S. & Colley, N.J. 2014. Mutations in four glycosyl hydrolases reveal a highly coordinated pathway for rhodopsin biosynthesis and N-glycan trimming in *Drosophila melanogaster*. *PLoS Genetics*, 10(5):e1004349.

Roush, R.T. 1997. Bt-transgenic crops: just another pretty insecticide or a chance for a new start in resistance management? *Journal of Pesticide Science*, 51:328-334.

RStudio Team. 2015. RStudio: integrated development for R. Boston, MA: RStudio, Inc.

Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T. & Christou, P. 2011. *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnology Journal*, 9(3):283-300.

Sayyed, A.H., Gatsi, R., Kouskoura, T., Wright, D.J. & Crickmore, N. 2001. Susceptibility of a field-derived, *Bacillus thuringiensis*-resistant strain of diamondback moth to *in vitro*-activated Cry1Ac toxin. *Applied and Environmental Microbiology*, 67(9):4372-4373.

Sayyed, A.H., Haward, R., Herrero, S., Ferré, J. & Wright, D.J. 2000. Genetic and biochemical approach for characterization of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a field

population of the diamondback moth, *Plutella xylostella*. *Applied and Environmental Microbiology*, 66(4):1509-1516.

Schlötterer, C., Tobler, R., Kofler, R. & Nolte, V. 2014. Sequencing pools of individuals - mining genome-wide polymorphism data without big funding. *Nature Reviews Genetics*, 15(11):749-763.

Schnepf, H.E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J.A., Feitelson, J., Zeigler, D.R. & Dean, D.H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews*, 62(3):775-806.

Schroeder, R.J., Ahmed, S.N., Zhu, Y., London, E. & Brown, D.A. 1998. Cholesterol and sphingolipid enhance the Triton X-100 insolubility of glycosylphosphatidylinositol-anchored proteins by promoting the formation of detergent-insoluble ordered membrane domains. *Journal of Biological Chemistry*, 273(2):1150-1157.

Schumann, U., Ayliffe, M., Kazan, K. & Wang, M.-B. 2010. RNA silencing in fungi. *Frontiers in Biology*, 5(6):478-494.

Seino, A., Ogura, T., Tsubota, T., Shimomura, M., Nakakura, T., Tan, A., Mita, K., Shinoda, T., Nakagawa, Y. & Shiotsuki, T. 2010. Characterization of juvenile hormone epoxide hydrolase and related genes in the larval development of the silkworm *Bombyx mori*. *Bioscience, Biotechnology, and Biochemistry*, 74(7):1421-1429.

Sezonlin, M., Dupas, S., Le Rü, B., Faure, N., Le Gall, P. & Silvain, J.-F. 2006a. Phylogeographic pattern and regional evolutionary history of the maize stalk borer *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) in sub-Saharan Africa. *Annales de la Société Entomologique de France*, 42(3-4):339-351.

Sezonlin, M., Dupas, S., Le Rü, B., Le Gall, P., Moyal, P., Calatayud, P.A., Giffard, I., Faure, N. & Silvain, J.F. 2006b. Phylogeography and population genetics of the maize stalk borer *Busseola fusca* (Lepidoptera, Noctuidae) in sub-Saharan Africa. *Molecular Ecology*, 15(2):407-420.

Sezonlin, M., Ndemah, R., Goergen, G., Le Rü, B., Dupas, S. & Silvain, J.-F. 2012. Genetic structure and origin of *Busseola fusca* populations in Cameroon. *Entomologia Experimentalis et Applicata*, 145(2):143-152.

Shelby, K.S. & Popham, H.J. 2012. RNA-Seq study of microbially induced hemocyte transcripts from larval *Heliothis virescens* (Lepidoptera: Noctuidae). *Insects*, 3(3):743-762.

Shelton, A.M., Zhao, J.-Z. & Roush, R.T. 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. *Annual Review of Entomology*, 47:845-881.

Siegfried, B.D., Zoerb, A.C. & Spencer, T. 2001. Development of European corn borer larvae on Event 176 Bt corn: influence on survival and fitness. *Entomologia Experimentalis et Applicata*, 100(1):15-20.

Simmons, R.B. & Weller, S.J. 2001. Utility and evolution of cytochrome *b* in insects. *Molecular Phylogenetics and Evolution*, 20(2):196-210.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, 87(6):651-701.

Simpson, R.M. & Newcomb, R.D. 2000. Binding of *Bacillus thuringiensis* δ-endotoxins Cry1Ac and Cry1Ba to a 120-kDa aminopeptidase-N of *Epiphyas postvittana* purified from both brush border membrane vesicles and baculovirus-infected Sf9 cells. *Insect Biochemistry and Molecular Biology*, 30:1069-1078.

Siqueira, H.A., Gonzáles-Cabrera, J., Ferré, J., Flannagan, R. & Siegfried, B.D. 2006. Analyses of Cry1Ab binding in resistant and susceptible strains of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). *Applied and Environmental Microbiology*, 72(8):5318-5324.

Smedley, D.P., Armstrong, G. & Ellar, D.J. 1997. Channel activity caused by a *Bacillus thuringiensis* delta-endotoxin preparation depends on the method of activation. *Molecular Membrane Biology*, 14(1). (Abstract).

Smith-Unna, R.D., Boursnell, C., Patro, R., Hibberd, J.M. & Kelly, S. 2016. TransRate: reference free quality assessment of *de-novo* transcriptome assemblies *Genome Research*, 26(8):1134-1144.

Sneh, B., Schuster, S. & Broza, M. 1981. Insecticidal activity of *Bacillus thuringiensis* strains against the Egyption cotton leaf worm *Spodoptera littoralis* [Lep. : Noctuidae]. *Entomophaga*, 26(2):179-190.

Soberón, M., Gill, S.S. & Bravo, A. 2009. Signaling versus punching hole: how do *Bacillus thuringiensis* toxins kill insect midgut cells? *Cellular and Molecular Life Science*, 66(8):1337-1349.

Soberón, M., Pardo-López, L., López, I., Gómez, I., Tabashnik, B.E. & Bravo, A. 2007. Engineering modified Bt toxins to counter insect resistance. *Science*, 318:1640-1642.

Söderhäll, K. & Cerenius, L. 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. *Current Opinion in Immunology*, 10:23-28.

Storer, N.P., Babcock, J.M., Schlenz, M., Meade, T., Thompson, G.D., Bing, J.W. & Huckaba, R.M. 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. *Journal of Economic Entomology*, 103(4):1031-1038.

Tabashnik, B.E. 2008. Delaying insect resistance to transgenic crops. *Proceedings of the National Academy of Sciences*, 105(49):19029-19030.

Tabashnik, B.E., Biggs, R.W., Higginson, D.M., Unnithan, D.C., Unithan, G.C., Ellers-Kirk, C., Sisterson, M.S., Dennehy, T.J., Carrière, Y. & Morin, S. 2005. Association between resistance to Bt cotton and cadherin genotype in pink bollworm. *Journal of Economic Entomology*, 98(3):635-644.

Tabashnik, B.E., Brévault, T. & Carrière, Y. 2013. Insect resistance to Bt crops: lessons from the first billion acres. *Nature Biotechnology*, 31(6):510-521.

Tabashnik, B.E., Carriére, Y., Dennehy, T.J., Morin, S., Sisterson, M.S., Roush, R.T., Shelton, A.M. & Zhao, J. 2003. Insect resistance to transgenic Bt crops: lessons from the laboratory and field. *Journal of Economic Entomology*, 96(4):1031-1038.

Tabashnik, B.E., Cushing, N.L., Finson, N. & Johnson, M.W. 1990. Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology*, 83(5):1671-1676.

Tabashnik, B.E., Finson, N., Groeters, F.R., Moar, W.J., Johnson, M.W., Luo, K. & Adang, M.J. 1994. Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. *Proceedings of the National Academy of Sciences*, 91(10):4120-4124.

Tabashnik, B.E., Finson, N. & Johnson, M.W. 1991. Managing resistance to *Bacillus thuringiensis*: lessons from the diamondback moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology*, 84(1):49-55.

Tabashnik, B.E., Huang, F., Ghimire, M.N., Leonard, B.R., Siegfried, B.D., Rangasamy, M., Yang, Y., Wu, Y., Gahan, L.J., Heckel, D.G., Bravo, A. & Soberón, M. 2011. Efficacy of genetically modified *Bt* toxins against insects with different genetic mechanisms of resistance. *Nature Biotechnology*, 29(12):1128-1131.

Tabashnik, B.E., Johnson, K.W., Engleman, J.T. & Baum, J.A. 2000. Cross-resistance to *Bacillus thuringiensis* toxin Cry1Ja in a strain of diamondback moth adapted to artificial diet. *Journal of Invertebrate Pathology*, 76(1):81-83.

Tabashnik, B.E., Liu, Y.B., Finson, N., Masson, L. & Heckel, D.G. 1997a. One gene in diamondback moth confers resistance to four *Bacillus thuringiensis* toxins. *Proceedings of the National Academy of Sciences*, 94:1640-1644.

Tabashnik, B.E., Liu, Y.B., Malvar, T., Heckel, D.G., Masson, L., Ballester, V., Granero, F., Ménsua, J.L. & Ferré, J. 1997b. Global variation in the genetic and biochemical basis of diamondback moth resistance to *Bacillus thuringiensis*. *Proceedings of the National Academy of Sciences*, 94(24):12780-12785.

Tabashnik, B.E., Liu, Y.B., Unnithan, D.C., Carrière, Y., Dennehy, T.J. & Morin, S. 2004. Shared genetic basis of resistance to Bt toxin Cry1Ac in independent strains of pink bollworm. *Journal of Economic Entomology*, 97(3):721-726.

Tabashnik, B.E., Malvar, T., Liu, Y.B., Finson, N., Borthakur, D., Shin, B.-S., Park, S.-H., Masson, L., De Maagd, R.A. & Bosch, D. 1996. Cross-resistance of the diamondback moth indicates altered interactions with domain II of *Bacillus thuringiensis* toxins. *Applied and Environmental Microbiology*, 62:2839-2844.

Tabashnik, B.E., Schwartz, J.M., Finson, N. & Johnson, M.W. 1992. Inheritance of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology*, 85(4):1046-1055.

Tabashnik, B.E., Van Rensburg, J.B.J. & Carrière, Y. 2009. Field-evolved insect resistance to *Bt* crops: definition, theory, and data. *Journal of Economic Entomology*, 102(6):2011-2025.

Tan, Q.-Q., Liu, W., Zhu, F., Lei, C.-L. & Wang, X.-P. 2016. *Fatty acid synthase 2* contributes to diapause preparation in a beetle by regulating lipid accumulation and stress tolerance genes expression. *Nature Scientific Reports*, 7:40509.

Tanaka, S., Miyamoto, K., Noda, H., Jurat-Fuentes, J.L., Yoshizawa, Y., Endo, H. & Sato, R. 2013. The ATP-binding cassette transporter subfamily C member 2 in *Bombyx mori* larvae is a functional receptor for Cry toxins from *Bacillus thuringiensis*. *FEBS Journal*, 280(8):1782-1794.

Tanaka, S., Yoshizawa, Y. & Sato, R. 2012. Response of midgut epithelial cells to Cry1Aa is toxin-dependent and depends on the interplay between toxic action and the host apoptotic response. *FEBS Journal*, 279(6):1071-1079.

Tang, J.D., Collins, H.L., Roush, R.T., Metz, T.D., Earle, E.D. & Shelton, A.M. 1999. Survival, weight gain, and oviposition of resistant and susceptible *Plutella xylostella* (L.) on broccoli expressing Cry1Ac toxin of *Bacillus thuringiensis*. *Journal of Economic Entomology*, 92(1):47-55.

Tang, J.D., Gilboa, S., Roush, R.T. & Shelton, A.M. 1997. Inheritance, stability, and lack-of-fitness costs of field selected resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae) from Florida. *Journal of Economic Entomology*, 90:732-741.

Tang, J.D., Shelton, A.M., Van Rie, J., De Roeck, S., Moar, W.J., Roush, R.T. & Peferoen, M. 1996. Toxicity of *Bacillus thuringiensis* spore and crystal protein to resistant diamondback moth (*Plutella xylostella*). *Applied and Environmental Microbiology*, 62(2):564-569.

Tassone, E.E., Zastrow-Hayes, G., Mathis, J., Nelson, M.E., Wu, G., Flexner, J.L., Carrière, Y., Tabashnik, B.E. & Fabrick, J.A. 2016. Sequencing, *de novo* assembly and annotation of a pink bollworm larval midgut transcriptome. *GigaScience*, 5:28-32.

Tay, W.T., Mahon, R.J., Heckel, D.G., Walsh, T.K., Downes, S., James, W.J., Lee, S.F., Reineke, A., Williams, A.K. & Gordon, K.H.J. 2015. Insect resistance to *Bacillus thuringiensis* toxin Cry2Ab is conferred by mutations in an ABC transporter subfamily A protein. *PLoS Genetics*, 11(11):e1005534.

Teixeira, S., Cambon-Bonavita, M.-A., Serrão, E.A., Desbruyéres, D. & Arnaud-Haond, S. 2011. Recent population expansion and connectivity in the hydrothermal shrimp *Rimicaris exoculata* along the Mid-Atlantic Ridge. *Journal of Biogeography*, 38(3):564-574.

Than, W., Qin, F., Liu, W. & Wang, X. 2016. Analysis of *Sogatella furcifera* proteome that interact with P10 protein of *Southern rice black-streaked dwarf virus*. *Scientific Reports*, 6:32445.

Then, C. & Lorch, A. 2007. A simple question in a complex environment: How much Bt toxin do genetically engineered MON810 maize plants actually produce? www.greenpeace.de/fileadmin/gpd/user_upload/themen/gentechnik/greenpeace_bt_maize_engl.pdf Date of access: 10 April 2015.

Tiewsiri, K. & Wang, P. 2011. Differential alteration of two aminopeptidases N associated with resistance to *Bacillus thuringiensis* toxin Cry1Ac in cabbage looper. *Proceedings of the National Academy of Sciences*, 108(34):14037-14042.

Toussaint, E.F.A., Condamine, F.L., Kergoat, G.J., Capdevielle-Dulac, C., Barbut, J., Silvain, J.F. & Le Rü, B.P. 2012. Palaeoenvironmental shifts drove the adaptive radiation of a noctuid stemborer tribe (Lepidoptera, Noctuidae, Apameini) in the miocene. *PLoS One*, 7(7):e41377.

Trtikova, M., Wikmark, O.-G., Zemp, N., Widmer, A. & Hilbeck, A. 2015. Transgene expression and Bt protein content in transgenic Bt maize (MON810) under optimal and stressful environmental conditions. *PLoS One*, 10(4):e0123011.

Upadhyay, S.K. & Singh, P.K. 2011. Role of alkaline phosphatase in insecticidal action of Cry1Ac against *Helicoverpa armigera* larvae. *Biotechnology Letters*, 33(10):2027-2036.

USEPA. 1998. Final report of the subpanel on *Bacillus thuringiensis* (Bt) plant-pesticides and resistance management. http://www.epa.gov/scipoly/sap/meetings/1998/0298_mtg.htm Date of access: 12 July 2011.

Vachon, V., Laprade, R. & Schwartz, J.L. 2012. Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: a critical review. *Journal of Invertebrate Pathology*, 111(1):1-12.

Valaitis, A.P., Jenkins, J.L., Lee, M.K., Dean, D.H. & Garner, K.J. 2001. Isolation and partial characterization of gypsy moth BTR-270, an anionic brush border membrane glycoconjugate that binds *Bacillus thuringiensis* Cry1A toxins with high affinity. *Archives of Insect Biochemistry and Physiology*, 46(4):186-200.

Van den Berg, J. 2010. Bt-resistant target pest: quick occurrence in South Africa. (*In* European Network of Scientists for Social and Environmental Responsibility, Third World Network & Federation of German Scientists. Advancing the understanding of biosafety: latest

scientific findings, polica responses and public participation organised by Nagoya: Jutaprint. p. 58-61).

Van den Berg, J. & Campagne, P. 2015. Status of resistance to Bt cotton in China: cotton bollworm and pink bollworm. *(In* Soberón, M., Gao Y. & Bravo A., *eds*. Bt resistance: characterization and strategies for GM crops producing *Bacillus thuringiensis* toxins. UK: CAB International. p. 36-48).

Van den Berg, J., Hilbeck, A. & Bøhn, T. 2013. Pest resistance to Cry1Ab Bt maize: field resistance, contributing factors and lessons from South Africa. *Crop Protection*, 54:154-160.

Van der Hoeven, N. 2014. *Bacillus thuringiensis* toxins: their mode of action and the potential interaction between them.

Van Rensburg, J.B.J. 2007. First report of field resistance by the stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize. *South African Journal of Plant and Soil*, 24(3):147-151.

Van Rie, J., McGaughey, W.H., Johnson, D.E., Barnett, B.D. & Van Mellaert, H. 1990. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. *Science*, 247(4938):72-74.

Van Wyk, A., Van den Berg, J. & Van Hamburg, H. 2008. Diversity and comparative phenology of Lepidoptera on Bt and non-Bt maize in South Africa. *International Journal of Pest Management*, 54(1):77-87.

Vargas Jentzsch, I.M., Bagshaw, A., Buschiazzo, E., Merkel, A. & Gemmell, N.J. 2008. Evolution of microsatellite DNA. *(In* Sons, J.W., *ed.* Encyclopedia of Life Sciences (ELS). Ltd: Chichester).

Vázquez-Padrón, R.I., Gonzáles-Cabrera, J., García-Tovar, C., Neri-Bazan, L., Lopéz-Revilla, R., Hernández, M., Moreno-Fierro, L. & De la Riva, G.A. 2000. Cry1Ac protoxin from *Bacillus thuringiensis* sp. *kurstaki* HD73 binds to surface proteins in the mouse small intestine. *Biochemical and Biophysical Research Communications*, 271(1):54-58.

Vellichirammal, N.N., Wang, H., Eyun, S.-I., Moriyama, E.N., Coates, B.S., Miller, N.J. & Siegfried, B.D. 2015. Transcriptional analysis of susceptible and resistant European corn borer strains and their response to Cry1F protoxin. *BMC Genomics*, 16:558.

Vera, J.C., Wheat, C.W., Fescemyer, H.W., Frilander, M.J., Crawford, D.L., Hanski, I. & Marden, J.H. 2008. Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing. *Molecular Ecology*, 17:1636-1647.

Viljakainen, L. 2015. Evolutionary genetics of insect innate immunity. *Briefings in Functional Genomics*, 14(6):407-412.

Voua Otomo, P., Jansen van Vuuren, B. & Reinecke, S.A. 2009. Usefulness of DNA barcoding in ecotoxicological investigations: resolving taxonomic uncertainties using *Eisenia* Malm 1877 as an example. *Bulletin of Environmental Contamination and Toxicology*, 82:261-264.

Wan, P., Huang, Y., Wu, H., Huang, M., Cong, S., Tabashnik, B.E. & Wu, K. 2012. Increased frequency of pink bollworm resistance to Bt toxin Cry1Ac in China. *PLoS One*, 7(1):e29975.

Wan, P.J., Fu, K.Y., Lu, F.G., Guo, W.C. & Li, G.Q. 2014. A putative delta1-pyrroline-5-carboxylate synthetase involved in the biosynthesis of proline and arginine in *Leptinotarsa decemlineata*. *Journal of Insect Physiology*, 71:105-113.

Wang, P., Zhao, J.Z., Rodrigo-Simon, A., Kain, W., Janmaat, A.F., Shelton, A.M., Ferré, J. & Myers, J. 2007. Mechanism of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a greenhouse population of the cabbage looper, *Trichoplusia ni*. *Applied and Environmental Microbiology*, 73(4):1199-1207.

Wang, Y., Zhang, H., Li, H. & Miao, X. 2011. Second-generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control. *PLoS One*, 6(4):e18644.

Wang, Z., Gerstein, M. & Snyder, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics*, 10(1):57-63.

Will, K.W., Mishler, B.D. & Wheeler, Q.D. 2005. The perils of DNA barcoding and the need for integrative taxonomy. *Systematic Biology*, 54(5):844-851.

Williams, S., Friedrich, L., Dincher, S., Carozzi, N., Kessmann, H., Ward, E. & Ryals, J. 1992. Chemical regulation of *Bacillus thuringiensis* δ-endotoxin expression in transgenic plants. *Bio/Technology*, 10. (Abstract).

Wolfersberger, M.G., Chen, X.J. & Dean, D.H. 1996. Site-directed mutations in the third domain of *Bacillus thuringiensis* δ-endotoxin Cry1Aa affect its ability to increase the permeability

of *Bombyx mori* midgut brush border membrane vesicles. *Applied and Environmental Microbiology*, 62(1):279-282.

Wu, K. 2007. Monitoring and management strategy for *Helicoverpa armigera* resistance to Bt cotton in China. *Journal of Invertebrate Pathology*, 95(3):220-223.

Wu, K.M. & Guo, Y.Y. 2005. The evolution of cotton pest management practices in China. *Annual Review of Entomology*, 50:31-52.

Wu, X., Huang, F., Rogers Leonard, B. & Ottea, J. 2009. Inheritance of resistance to Bacillus thuringiensis Cry1Ab protein in the sugarcane borer (Lepidoptera: Crambidae). *Journal of Invertebrate Pathology*, 102(1):44-49.

Wu, Y. 2014. Detection and mechanisms of resistance evolved in insects to Cry toxins from *Bacillus thuringiensis*. *Advances in Insect Physiology*, 47:297-342.

Xiao, Y., Zhang, T., Liu, C., Heckel, D.G., Li, X., Tabashnik, B.E. & Wu, K. 2014. Mis-splicing of the ABCC2 gene linked with Bt toxin resistance in *Helicoverpa armigera*. *Scientific Reports*, 4:6184.

Xu, W., Cao, S., He, X., Luo, Y., Guo, X., Yuan, Y. & Huang, K. 2009. Safety assessment of Cry1Ab/Ac fusion protein. *Food and Chemical Toxicology*, 47(7):1459-1465.

Xu, X. & Wu, Y. 2008. Disruption of Ha_BtR alters binding of *Bacillus thuringiensis* delta-endotoxin Cry1Ac to midgut BBMVs of *Helicoverpa armigera*. *Journal of Invertebrate Pathology*, 97(1):27-32.

Xu, X., Yu, L. & Wu, Y. 2005. Disruption of a cadherin gene associated with resistance to Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. *Applied and Environmental Microbiology*, 71(2):948-954.

Yanay, C., Morpurgo, N. & Linial, M. 2008. Evolution of insect proteomes: insights into synapse organization and synaptic vesicle life cycle. *Genome Biology*, 9(2):R27.21-R27.26.

Yang, C. & Sehnal, F. 1998. Ribosomal protein L5 of *Bombyx mori*: cDNA sequence and tissue differences in the *L5* mRNA content. *Insect Molecular Biology*, 7(4):301-306.

Yang, Y.-H., Yang, Y.-J., Gao, W.-Y., Guo, J.-J., Wu, Y.-H. & Wu, Y.-D. 2009. Introgression of a disrupted cadherin gene enables susceptible *Helicoverpa armigera* to obtain resistance to *Bacillus thuringiensis* toxin Cry1Ac. *Bulletin of Entomological Research*, 99(2):175-181.

Yang, Y. 2011. Molecular mechanisms of *Bacillus thuringiensis* resistance in the sugarcane borer. Louisiana State University and Agricultural and Mechanical College. (Dissertation - PhD).

Yang, Y., Chen, H., Wu, S., Yang, Y., Xu, X. & Wu, Y. 2006. Identification and molecular detection of a deletion mutation responsible for a truncated cadherin of *Helicoverpa armigera*. *Insect Biochemistry and Molecular Biology*, 36(9):735-740.

Yang, Y., Chen, H., Wu, Y., Yang, Y. & Wu, S. 2007. Mutated cadherin alleles from a field population of *Helicoverpa armigera* confer resistance to *Bacillus thuringiensis* toxin Cry1Ac. *Applied and Environmental Microbiology*, 73(21):6939-6944.

Yang, Y., Zhu, Y.C., Ottea, J., Husseneder, C., Leonard, B.R., Abel, C. & Huang, F. 2010. Molecular characterization and RNA interference of three midgut aminopeptidase N isozymes from *Bacillus thuringiensis*-susceptible and -resistant strains of sugarcane borer, *Diatraea saccharalis*. *Insect Biochemistry and Molecular Biology*, 40(8):592-603.

Yang, Y., Zhu, Y.C., Ottea, J., Husseneder, C., Leonard, B.R., Abel, C., Luttrell, R. & Huang, F. 2011. Down regulation of a gene for cadherin, but not alkaline phosphatase, associated with Cry1Ab resistance in the sugarcane borer *Diatraea saccharalis*. *PLoS One*, 6(10):e25783.

Yang, Z.X., Wu, Q.J., Wang, S.L., Chang, X.L., Wang, J.H., Guo, Z.J., Lei, Y.Y., Xu, B.Y. & Zhang, Y.J. 2012. Expression of cadherin, aminopeptidase N and alkaline phosphatase genes in Cry1Ac-susceptible and Cry1Ac-resistant strains of *Plutella xylostella* (L.). *Journal of Applied Entomology*, 136(7):539-548.

Yonemura, N., Mita, K., Tamura, T. & Sehnal, F. 2009. Conservation of silk genes in Trichoptera and Lepidoptera. *Journal of Molecular Evolution*, 68(6):641-653.

Zago, H.B., Siqueira, H.Á.A., Pereira, E.J.G., Picanco, M.C. & Barros, R. 2014. Resistance and behavioural response of *Plutella xylostella* (Lepidoptera: Plutellidae) populations to *Bacillus thuringiensis* formulations. *Pest Management Science*, 70(3):488-495.

Zalewska, M., Kochman, A., Esteve, J.P., Lopez, F., Chaoui, K., Susini, C., Ozyhar, A. & Kochman, M. 2009. Juvenile hormone binding protein traffic - Interaction with ATP synthase and lipid transfer proteins. *Biochimica et Biophysica Acta*, 1788(9):1695-1705.

Zavala, L.E., Pardo-López, L., Cantón, P.E., Gómez, I., Soberón, M. & Bravo, A. 2011. Domains II and III of *Bacillus thuringiensis* Cry1Ab toxin remain exposed to the solvent after insertion of part of domain I into the membrane. *The Journal of Biological Chemistry*, 286(21):19109-19117.

Zhai, Y., Sun, Z., Zhang, J., Kang, K., Chen, J. & Zhang, W. 2015. Activation of the TOR signalling pathway by glutamine regulates insect fecundity. *Scientific Reports*, 5:10694.

Zhang, H., Tang, M., Yang, F., Yang, Y. & Wu, Y. 2013. DNA-based screening for an intracellular cadherin mutation conferring non-recessive Cry1Ac resistance in field populations of *Helicoverpa armigera*. *Pesticide Biochemistry and Physiology*, 107(1):148-152.

Zhang, H., Tian, W., Zhao, J., Jin, L., Yang, J., Liu, C., Yang, Y., Wu, S., Wu, K., Cui, J., Tabashnik, B.E. & Wu, Y. 2012a. Diverse genetic basis of field-evolved resistance to Bt cotton in cotton bollworm from China. *Proceedings of the National Academy of Sciences*, 109(26):10275-10280.

Zhang, H., Wu, S., Yang, Y., Tabashnik, B.E. & Wu, Y. 2012b. Non-recessive Bt toxin resistance conferred by an intracellular cadherin mutation in field-selected populations of cotton bollworm. *PLoS One*, 7(12):e53418.

Zhang, S., Cheng, H., Gao, Y., Wang, G., Liang, G. & Wu, K. 2009. Mutation of an aminopeptidase N gene is associated with *Helicoverpa armigera* resistance to *Bacillus thuringiensis* Cry1Ac toxin. *Insect Biochemistry and Molecular Biology*, 39(7):421-429.

Zhang, X., Candas, M., Griko, N.B., Rose-Young, L. & Bulla, L.A., Jr. 2005. Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R1 expressed in insect cells. *Cell Death and Differentiation*, 12(11):1407-1416.

Zhang, X., Candas, M., Griko, N.B., Taussig, R. & Bulla, L.A., Jr. 2006. A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. *Proceedings of the National Academy of Sciences*, 103(26):9897-9902.

Zhang, X., Tiewsiri, K., Kain, W., Huang, L. & Wang, P. 2012c. Resistance of *Trichoplusia ni* to *Bacillus thuringiensis* toxin Cry1Ac is independent of alteration of the cadherin-like receptor for Cry toxins. *PLoS One*, 7(5):e35991.

Zhao, J., Jin, L., Yang, Y. & Wu, Y. 2010. Diverse cadherin mutations conferring resistance to *Bacillus thuringiensis* toxin Cry1Ac in *Helicoverpa armigera*. *Insect Biochemistry and Molecular Biology*, 40(2):113-118.

Zhao, J.Z., Cao, J., Collins, H.L., Bates, S.L., Roush, R.T., Earle, E.D. & Shelton, A.M. 2005. Concurrent use of transgenic plants expressing a single and two *Bacillus thuringiensis* genes speeds insect adaptation to pyramided plants. *Proceedings of the National Academy of Sciences of the United States of America*, 102(24):8426-8430.

Zhao, J.Z., Collins, H.L., Tang, J.D., Cao, J., Earle, E.D., Roush, R.T., Herrero, S., Escriche, B., Ferré, J. & Shelton, A.M. 2000. Development and characterization of diamondback moth resistance to transgenic broccoli expressing high levels of Cry1C. *Applied and Environmental Microbiology*, 66(9):3784-3789.

Zhao, Z., Wu, G., Wang, J., Liu, C. & Qiu, L. 2013. Next-generation sequencing-based transcriptome analysis of *Helicoverpa armigera* larvae immune-primed with *Photorhabdus luminescens* TT01. *PLoS One*, 8(11):e80146.

Zhu, X., Lei, Y., Yang, Y., Baxter, S.W., Li, J., Wu, Q., Wang, S., Xie, W., Guo, Z., Fu, W. & Zhang, Y. 2015. Construction and characterisation of near-isogenic *Plutella xylostella* (Lepidoptera: Plutellidae) strains resistant to Cry1Ac toxin. *Pest Management Science*, 71(2):225-233.

Zhu, Y.-C., Kramer, K.J., Oppert, B. & Dowdy, A.K. 2000. cDNAs of aminopeptidase-like protein genes from *Plodia interpunctella* strains with different susceptibilities to *Bacillus thuringiensis* toxins. *Insect Biochemistry and Molecular Biology*, 30:215-224.

Zhuang, M., Oltean, D.I., Gómez, I., Pullikuth, A.K., Soberón, M., Bravo, A. & Gill, S.S. 2002. *Heliothis virescens* and *Manduca sexta* lipid rafts are involved in Cry1A toxin binding to the midgut epithelium and subsequent pore formation. *Journal of Biological Chemistry*, 277(16):13863-13872.

Zúñiga-Navarrete, F., Bravo, A., Soberón, M. & Gómez, I. 2012. Role of GPI-anchored membrane receptors in the mode of action of *Bacillus thuringiensis* Cry toxins. *(In* Soloneski, S., *ed.* Integrated pest management and pest control - current and future tactics. Rijeka, NM: InTech. p. 551-566).

Annexures

Annexure A

NCBI taxonomy of species from the order Lepidoptera

Common names were retrieved from the Entomological Society of America (ESA) Common Names of Insects Database (http://entsoc.org/common-names).

Scientific name (Author)	Family	Common name
Achaea janata (L.)	Erebidae	Croton caterpillar
Amyelois transitella (Walker)	Pyralidae	Navel orangeworm
Bombyx mori (L.)	Bombycidae	Silkworm
Busseola fusca (Fuller)	Noctuidae	Maize stalk/stem borer
Busseola phaia (Bowden)	Noctuidae	No common name
Diatraea saccharalis (F.)	Crambidae	Sugarcane borer
Eldana saccharina (Walker)	Pyralidae	African sugarcane borer
Ephestia kuehniella (Zeller)	Pyralidae	Mediterranean flour moth
Epiphyas postvittana (Walker)	Tortricidae	Light brown apple moth
Helicoverpa armigera (Hübner)	Noctuidae	Cotton bollworm
Helicoverpa punctigera (Wallengren)	Noctuidae	Native budworm
Helicoverpa zea (Boddie)	Noctuidae	Corn earworm / tomato fruitworm
Heliothis subflexa (Guenée)	Noctuidae	Subflexus straw moth
Heliothis virescens (F.)	Noctuidae	Tobacco budworm
Lymantria dispar (L.)	Erebidae	Gypsy moth
Manduca sexta (L.)	Sphingidae	Tobacco hornworm
Melitaea cinxia (L.)	Nymphalidae	Glanville fritillary
Ostrinia furnacalis (Guenée)	Crambidae	Asian corn borer
Ostrinia nubilalis (Hübner)	Crambidae	European corn borer
Papilio machaon (L.)	Papilionidae	Old World swallowtail
Pectinophora gossypiella (Saunders)	Gelechiidae	Pink bollworm
Plodia interpunctella (Guenée)	Pyralidae	Indian meal moth
Plutella xylostella (L.)	Plutellidae	Diamondback moth
Spodoptera exigua (Hübner)	Noctuidae	Beet armyworm
Spodoptera frugiperda (J.E. Smith)	Noctuidae	Fall armyworm
Spodoptera littoralis (Boisduval)	Noctuidae	African or Egyptian cotton leafworm
Spodoptera litura (F.)	Noctuidae	Oriental leafworm moth
Trichoplusia ni (Hübner)	Noctuidae	Cabbage looper

Annexure B

Commands and parameters used for RNA-Seq analysis

For each tool that was implemented, the usage is specified, followed by a table summarizing the options that were used. Unless specified otherwise, default values were used.

Quality analysis of reads using FastQC:

$fastqc *.fastq

Command	Description
fastqc	Perform quality control checks on raw sequence data (in fastq format)

***De novo* assembly of RNA-Seq data using Trinity:**

$TRINITY_HOME/trinity --seqType <*options*> --max_memory <*options*> --SS_lib_type <*options*> --CPU <*options*> --left <*input_file*> --right <*output_file* > --trimmomatic --normalize_reads --output <*options*>

Command	Option	Description
--seqType		Type of reads
	fa	Indicates FASTA files
	fq	Indicates FASTQ files
--max_memory		Suggested maximum memory to use by Trinity (provided in Gb of RAM)
--SS_lib_type		Strand-specific RNA-Seq read orientation. For paired-end reads, specify RF or FR.
--CPU		Number of CPUs to use (default: 2)
--left*		Left reads, one or more file names (separated by commas, no spaces)
--right**		Right reads, one or more file names (separated by commas, no spaces)
--trimmomatic***		Run Trimmomatic to quality trim reads
--normalize_reads		Run *in silico* normalization of reads (default: 50 reads coverage)
--output		Name of directory for output

* All forward reads of the four sample groups (S0-Bt, S0-nBt, S1-Bt and S1-nBt) combined.
** All reverse reads of the four sample groups (S0-Bt, S0-nBt, S1-Bt and S1-nBt) combined.
*** Commands and options for Trimmomatic are specified in the following table.

Trimming of reads using Trimmomatic:

java -jar $TRIMMOMATIC_HOME/Trimmomatic.jar PE:<*options*> ILLUMINACLIP:<*options*> SLIDINGWINDOW:<*options*> LEADING:<*options*> TRAILING:<*options*> MINLEN:<*options*>

Command	Option	Description
PE		Indicates paired-end data
	-threads	Indicates the number of threads to use
	-phred33	Specifies the base quality encoding
	input_file.F.fq* / input_file.R.fq**	Paired-end mode requires 2 input files (for forward (F) and reverse (R) reads) in Fastq format
	output_file.P.qtrim	Name of output file for paired forward/reverse reads
	output_file.U.qtrim	Name of output file for unpaired forward/reverse reads
ILLUMINACLIP		Cut adapter and other Illumina-specific sequences from the read
	adapters.fa***	Specifies the FASTA file containing all the adapters, PCR sequences, etc.
	seed mismatches	Specifies the maximum mismatch count which will still allow a full match to be performed
	palindrome clip threshold	Specifies how accurate the match between the two ‘adapter ligated’ reads must be for PE palindrome read alignment
	simple clip threshold	Specifies how accurate the match between the sequence of any adapter or other sequencing artefact must be against the read
SLIDINGWINDOW		Performs a sliding window trimming approach: it starts scanning at the 5' end and clips the read once the average quality within the window falls below the specified threshold
	windowSize	Specifies the number of bases to average across (set to 4)
	requiredQuality	Specifies the average quality required (set to 5)
LEADING		Cut bases off the start of a read if it is below the specified threshold quality (set to 5)
TRAILING		Cut bases off the end of a read if it is below the specified threshold quality (set to 5)
MINLEN		Drop the read if it is below the specified length (set to 25)

* All forward reads of the four sample groups (S0-Bt, S0-nBt, S1-Bt and S1-nBt) combined.

** All reverse reads of the four sample groups (S0-Bt, S0-nBt, S1-Bt and S1-nBt) combined.

*** Adapters are specified in the following table.

List of adapters and primers to be trimmed according to FASTQC reports:

Name	Sequence
PrefixPE/1 / PCR_Primer 1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
PCR_Primer 1_rc	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT
TruSeq Adapter, Index 6	GATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCGTCTTCTGCTTG
TruSeq Adapter, Index 6_rc	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

Quality assessment of assembly using Trinity:

perl $TRINITY_HOME/util/TrinityStats.pl <*options*>

Option	Description
input_file.fasta	FASTA file containing assembled sequences

Note: TrinityStats.pl is an embedded PERL script in Trinity to assess the quality and coverage of the obtained assembly.

Quality assessment of assembly using Transrate:

$TRANSRATE_HOME/transrate --assembly=<*options*> --lef=<*options*> --right=<*options*> --threads=<*options*> --output=<*options*>

Command	Description
--assembly	Assembly file(s) in FASTA format, comma-separated
--left	Left reads file(s) in FASTQ format, comma-separated
--right	Right reads file(s) in FASTQ format, comma-separated
--threads	Number of threads to use (default: 8)
--output	Specifies name of directory for output

Identification of transcripts using Wise2:

$HOME_WISE2/estwisedb -pfam <*options*> input_file.fasta -codon <*options*> -alg <*options*> -aalg <*options*> -quiet -para -sum > output_file.wise

Command	Description
-pfam	Protein HMM library
input_file.fasta	FASTA file with query sequences for identification
-codon	Codon file with codons necessary to translate the FASTA sequences before comparing them to HMM profiles (set to codon.table.5)
-alg	Algorithm used for searching (set to 333)
-aalg	Algorithm used for alignment (set to 333)
-quiet	No report on stderr
-para	Show parameters in output file
-output_file.wise	Specify output file whereto results will be written

Identification of transcripts using BLAST (Basic Local Alignment Search Tool):

Library creation:

makeblastdb -input_type <*options*> -dbtype <*options*> -parse_seqids -in <*options*> -out <*options*> -title <*options*>

Command	Description
-input_type	Input file type (set to FASTA)
-dbtype	Specifies the molecular type of the database (set to nucl or prot)
-parse_seqids	Parse bar delimited sequence identifiers in FASTA input
-in	Input file / database name
-out	Name of BLAST database to be created
-title	Title for BLAST database

Blastn:

blastn -db <*options*> -query <*options*> -out <*options*> -best_hit_overhang <*options*> -best_hit_score_edge <*options*> -num_threads <*options*> -outfmt <*options*> -evalue <*options*>

Command	Option	Description
-db		File name of the BLAST database
-query		Input FASTA file name
-out		Output file name
-best_hit_overhang		Best hit algorithm overhang value (set to 0.1)
-best_hit_score_edge		Best hit algorithm score edge value (set to 0.1)
-num_threads		Number of threads (CPUs) to use in blast search
-outfmt		Alignment view options (set to 7: tabular with comment lines)
	qacc	Query accession number/name
	sseqid	Subject sequence ID
	qstart	Start of alignment in query
	qend	End of alignment in query
	sstart	Start of alignment in subject
	send	End of alignment in subject
	evalue	Calculated expect value
	bitscore	Calculated bit score
	pident	Percentage of identical matches
-evalue		Expect (E) value for saving hits (set to 1 e-10)

Blastx:

blastn -db <*options*> -query <*options*> -out <*options*> -best_hit_overhang <*options*> -best_hit_score_edge <*options*> -num_threads <*options*> -outfmt <*options*> -evalue <*options*> -matrix <*options*> -word_size <*options*> -threshold <*options*> -query_gencode <*options*>

Command	Option	Description
-db		File name of the BLAST database
-query		Input FASTA file name
-out		Output file name
-best_hit_overhang		Best hit algorithm overhang value (set to 0.1)
-best_hit_score_edge		Best hit algorithm score edge value (set to 0.1)
-num_threads		Number of threads (CPUs) to use in blast search
-outfmt		Alignment view options (set to 7: tabular with comment lines)
	qacc	Query accession number/name
	sseqid	Subject sequence ID
	qstart	Start of alignment in query
	qend	End of alignment in query
	sstart	Start of alignment in subject
	send	End of alignment in subject
	evalue	Calculated expect value
	bitscore	Calculated bit score
	pident	Percentage of identical matches
-evalue		expect (E) value for saving hits (set to 1 e-10)
-matrix		Scoring matrix name (set to PAM250)
-word_size		Word size for initial match (set to 4)
-threshold		Minimum score to add a word to the BLAST lookup table (set to 15)
-query_gencode		Genetic code to translate query (set to codon.table.1)

Estimating expression levels of transcripts using RSEM (RNA-Seq by Expectation-Maximization):

Abundance estimation for each group individually:

$TRINITY_HOME/util/align_and_estimate_abundance.pl --transcripts <*options*> --seqType <*options*> --left <*options*> --right <*options*> --SS_lib_type <*options*> --output_prefix <*options*> --est_method <*options*> --aln_method <*options*> --trinity_mode --prep_reference --output_dir <*options*>

Command	Description
--transcripts	Transcript FASTA file containing assembly results
--seqType	Type of reads (‘fa’for FASTA or ‘fq’ for FASTQ files)
--left	Left reads file name
--right	Right reads file name
--SS_lib_type	Strand-specific RNA-Seq read orientation (for paired-end: RF or FR)
--output_prefix	Prefix for output files
--est_method	Abundance estimation method (set to RSEM)
--aln_method	Alignment method (set to Bowtie*)
--trinity_mode	Automatically generates the gene_trans_map file (a file containing ‘gene(tab)transcript’ identifiers per line) and uses it
--prep_reference	Builds target reference
--output_dir	Write all files to output directory

* RSEM is currently only compatible with gap-free alignments, thus Bowtie was used as an aligner.

Combination of abundance estimation results for counts matrix generation and cross-sample normalization:

$TRINITY_HOME/util/abundance_estimates_to_matrix.pl --est_method <*options*> --output_prefix <*options*><isoforms_results>

Command	Description
--est_method	Abundance estimation method used (set to RSEM)
--out_prefix	Prefix for output files (default: ‘matrix’)
isoforms_results	Path to isoforms table results files for each group

Differential expression analysis using edgeR (Empirical analysis of Digital Gene Expression data) in R:

$TRINITY_HOME/Analysis/DifferentialExpression/run_DE_analysis.pl --matrix <*options*> --method <*options*> --dispersion <*options*> --output <*options*>

Command	Description
--matrix	Matrix of raw read counts
--method	Method for assessing differentially expressed transcripts (set to edgeR)
--dispersion	edgeR dispersion value (set to 0.5)
--output	Name of directory for output files

Extraction of differentially expressed transcripts and heatmap generation using R:

$TRINITY_HOME/Analysis/DifferentialExpression/analyze_diff_expr.pl --matrix <*options*> --max_genes_clust <*options*> -P <*options*> -C <*options*>

Command	Description
--matrix	Matrix of raw read counts
--max_genes_clust	Maximal number of clustered genes/transcripts (set to 250 000)
-P	P-value cutoff for FDR (set to 0.001 <=> 1e-3)
-C	Minimum absolute number of fold change

Clusters of transcripts with similar expression profiles were extracted by cutting the transcript cluster dendrogram at a given percentage of its height (e.g. 60%). This was done using a Trinity subprogram with the following parameters:

$TRINITY_HOME/Analysis/DifferentialExpression/define_clusters_by_cutting_tree.pl --Ptree 60 -R diffExpr.P1e-3_C10.matrix.RData

Command	Description
--Ptree	Cut tree based on this percent of max (height) of tree
-R	Specifies a filename to store R output data

Annexure C

Complete list of mapped KEGG pathways in the assembled transcriptome of *Busseola fusca*

Rank	Pathway	#Enzs in Pathway	Enzyme	#Seqs of Enzyme	Sequences
1	Biosynthesis of antibiotics	18	ec:2.6.1.52 - transaminase, ec:4.1.1.17 - decarboxylase, ec:2.6.1.5 - transaminase, ec:4.2.1.11 - hydratase, ec:5.3.1.1 - isomerase, ec:2.7.4.6 - kinase, ec:1.2.4.2 - dehydrogenase (succinyl-transferring), ec:2.7.2.11 - 5-kinase, ec:2.7.1.40 - kinase, ec:1.1.1.35 - dehydrogenase, ec:1.2.1.41 - dehydrogenase, ec:1.4.1.14 - synthase (NADH), ec:1.3.5.1 - dehydrogenase, ec:1.4.3.3 - oxidase, ec:4.3.1.19 - ammonia-lyase, ec:1.1.1.42 - dehydrogenase (NADP+), ec:4.2.1.3 - hydratase, ec:3.5.1.14 - acid amidohydrolase	1, 2, 1, 2, 1, 2, 1, 1, 4, 2, 1, 1, 2, 1, 2, 1, 1, 1	DN75733.0.1.2, DN68748.0.1.1, DN72861.3.1.1, DN61168.0.1.2, DN36032.0.3.1, DN36032.0.2.1, DN62798.0.1.4, DN64433.0.3.3, DN70488.0.2.2, DN67850.0.1.4, DN80083.0.6.1, DN70271.0.1.4, DN53684.0.1.1, DN70271.0.1.2, DN70271.0.1.3, DN72435.0.6.2, DN72435.0.6.3, DN80083.0.6.1, DN84080.1.3.1, DN64813.0.3.3, DN64813.0.3.7, DN67844.0.1.4, DN50616.0.1.1, DN61974.0.1.1, DN75277.3.2.1, DN82451.0.3.4, DN78729.2.1.8
2	Purine metabolism	9	ec:2.7.4.6 - kinase, ec:3.1.4.17 - phosphodiesterase, ec:2.7.1.40 - kinase, ec:3.6.1.3 - adenylpyrophosphatase, ec:3.5.3.4 - ec:3.5.3.4 allantoicase, ec:2.7.7.6 - RNA polymerase, ec:2.7.7.7 - DNA polymerase, ec:2.4.2.1 - phosphorylase, ec:2.7.1.20 - kinase	2, 1, 4, 2, 1, 1, 1, 1, 5	DN64433.0.3.3, DN70488.0.2.2, DN66982.0.1.1, DN70271.0.1.4, DN53684.0.1.1, DN70271.0.1.2, DN70271.0.1.3, DN80895.0.1.4, DN71299.0.2.1, DN83565.1.4.2, DN83380.0.2.1, DN78053.0.2.2, DN77822.1.2.5, DN82750.0.1.6, DN82750.0.1.2, DN82750.0.1.8, DN82750.0.1.10, DN82750.0.1.7
3	Carbon fixation pathways in prokaryotes	7	ec:1.5.1.5 - dehydrogenase (NADP+), ec:3.5.4.9 - cyclohydrolase, ec:6.3.4.3 - ligase, ec:1.1.1.35 - dehydrogenase, ec:1.3.5.1 - dehydrogenase, ec:1.1.1.42 - dehydrogenase (NADP+), ec:4.2.1.3 - hydratase	1, 1, 1, 2, 2, 1, 1	DN82263.0.5.10, DN82263.0.5.10, DN82263.0.5.10, DN72435.0.6.2, DN72435.0.6.3, DN64813.0.3.3, DN64813.0.3.7, DN75277.3.2.1, DN82451.0.3.4
4	Tyrosine metabolism	6	ec:2.6.1.5 - transaminase, ec:3.7.1.2 - beta-diketonase, ec:1.13.11.27 - dioxygenase, ec:1.14.16.2 - 3-monooxygenase, ec:4.1.1.25 - decarboxylase, ec:4.1.1.28 - decarboxylase	1, 1, 1, 1, 1, 3	DN61168.0.1.2, DN75999.1.4.2, DN77224.1.3.2, DN67852.0.3.6, DN72221.0.1.3, DN75332.4.1.1, DN75332.4.1.4, DN75332.4.1.3
5	Alanine, aspartate and glutamate metabolism	5	ec:4.1.1.15 - decarboxylase, ec:6.3.1.2 - synthetase, ec:1.2.1.24 - dehydrogenase (NAD+), ec:1.4.1.14 - synthase (NADH), ec:1.4.1.2 - dehydrogenase	1, 3, 1, 1, 1	DN76013.0.2.2, DN74250.0.2.1, DN54305.0.1.1, DN69409.0.2.8, DN73836.0.1.1, DN84080.1.3.1, DN75447.0.1.2

Rank	Pathway	#Enzs in Pathway	Enzyme	#Seqs of Enzyme	Sequences
6	Fatty acid biosynthesis	5	ec:1.1.1.100 - reductase, ec:2.3.1.39 - S-malonyltransferase, ec:1.3.1.10 - reductase (NADPH, Si-specific), ec:3.1.2.14 - hydrolase, ec:2.3.1.41 - synthase I	1, 1, 1, 1, 1	DN51665.0.1.3, DN51665.0.1.3, DN51665.0.1.3, DN51665.0.1.3, DN51665.0.1.3
7	Arginine and proline metabolism	4	ec:4.1.1.17 - decarboxylase, ec:2.7.2.11 - 5-kinase, ec:1.2.1.41 - dehydrogenase, ec:1.4.3.3 - oxidase	2, 1, 1, 1	DN68748.0.1.1, DN72861.3.1.1, DN80083.0.6.1, DN80083.0.6.1, DN67844.0.1.4
8	Butanoate metabolism	4	ec:4.1.1.15 - decarboxylase, ec:1.2.1.24 - dehydrogenase (NAD+), ec:1.1.1.35 - dehydrogenase, ec:1.3.5.1 - dehydrogenase	1, 1, 2, 2	DN76013.0.2.2, DN73836.0.1.1, DN72435.0.6.2, DN72435.0.6.3, DN64813.0.3.3, DN64813.0.3.7
9	Citrate cycle (TCA cycle)	4	ec:1.2.4.2 - dehydrogenase (succinyl-transferring), ec:1.3.5.1 - dehydrogenase, ec:1.1.1.42 - dehydrogenase (NADP+), ec:4.2.1.3 - hydratase	1, 2, 1, 1	DN67850.0.1.4, DN64813.0.3.3, DN64813.0.3.7, DN75277.3.2.1, DN82451.0.3.4
10	Glutathione metabolism	4	ec:4.1.1.17 - decarboxylase, ec:2.5.1.18 - transferase, ec:1.1.1.42 - dehydrogenase (NADP+), ec:2.3.2.2 - glutamyl transpeptidase	2, 2, 1, 3	DN68748.0.1.1, DN72861.3.1.1, DN36217.0.1.4, DN36217.0.1.2, DN75277.3.2.1, DN73895.2.1.7, DN83724.0.1.1, DN83724.0.1.2
11	Isoquinoline alkaloid biosynthesis	4	ec:2.6.1.5 - transaminase, ec:1.14.16.2 - 3-monooxygenase, ec:4.1.1.25 - decarboxylase, ec:4.1.1.28 - decarboxylase	1, 1, 1, 3	DN61168.0.1.2, DN67852.0.3.6, DN72221.0.1.3, DN75332.4.1.1, DN75332.4.1.4, DN75332.4.1.3
12	One carbon pool by folate	4	ec:6.3.3.2 - cyclo-ligase, ec:1.5.1.5 - dehydrogenase (NADP+), ec:3.5.4.9 - cyclohydrolase, ec:6.3.4.3 - ligase	5, 1, 1, 1	DN74868.0.2.6, DN74868.0.2.5, DN74868.0.2.8, DN74868.0.2.2, DN74868.0.2.9, DN82263.0.5.10, DN82263.0.5.10, DN82263.0.5.10
13	Oxidative phosphorylation	4	ec:1.10.2.2 - reductase, ec:1.9.3.1 - oxidase, ec:1.6.5.3 - reductase (H+-translocating), ec:1.3.5.1 - dehydrogenase	1, 1, 2, 2	DN72875.1.2.2, DN33841.0.1.1, DN56251.0.1.1, DN64250.0.1.1, DN64813.0.3.3, DN64813.0.3.7
14	Pyrimidine metabolism	4	ec:2.7.4.6 - kinase, ec:2.7.7.6 - RNA polymerase, ec:2.7.7.7 - DNA polymerase, ec:2.4.2.1 - phosphorylase	2, 1, 1, 1	DN64433.0.3.3, DN70488.0.2.2, DN83380.0.2.1, DN78053.0.2.2, DN77822.1.2.5
15	Amino sugar and nucleotide sugar metabolism	3	ec:3.2.1.52 - hexosaminidase, ec:1.6.2.2 - reductase, ec:3.2.1.14 - chitodextrinase	2, 1, 8	DN71426.0.1.1, DN83325.2.2.6, DN74048.0.1.7, DN73727.0.2.2, DN73727.0.3.1, DN73727.0.2.4, DN73727.0.3.3, DN79883.0.4.1, DN79883.0.3.1, DN79883.0.8.1, DN79883.0.11.1

Rank	Pathway	#Enzs in Pathway	Enzyme	#Seqs of Enzyme	Sequences
16	Arginine biosynthesis	3	ec:6.3.1.2 - synthetase, ec:1.4.1.2 - dehydrogenase, ec:3.5.1.14 - acid amidohydrolase	3, 1, 1	DN74250.0.2.1, DN54305.0.1.1, DN69409.0.2.8, DN75447.0.1.2, DN78729.2.1.8
17	Biotin metabolism	3	ec:1.1.1.100 - reductase, ec:1.3.1.10 - reductase (NADPH, Si-specific), ec:2.3.1.41 - synthase I	1, 1, 1	DN51665.0.1.3, DN51665.0.1.3, DN51665.0.1.3
18	Cysteine and methionine metabolism	3	ec:2.6.1.5 - transaminase, ec:1.13.11.54 - dioxygenase [iron(II)-requiring], ec:5.3.1.23 - isomerase	1, 2, 1	DN61168.0.1.2, DN71632.0.1.2, DN71632.0.1.4, DN71218.0.8.2
19	Glycine, serine and threonine metabolism	3	ec:2.6.1.52 - transaminase, ec:1.4.3.3 - oxidase, ec:4.3.1.19 - ammonia-lyase	1, 1, 2	DN75733.0.1.2, DN67844.0.1.4, DN50616.0.1.1, DN61974.0.1.1
20	Glycolysis / Gluconeogenesis	3	ec:4.2.1.11 - hydratase, ec:5.3.1.1 - isomerase, ec:2.7.1.40 - kinase	2, 1, 4	DN36032.0.3.1, DN36032.0.2.1, DN62798.0.1.4, DN70271.0.1.4, DN53684.0.1.1, DN70271.0.1.2, DN70271.0.1.3
21	Methane metabolism	3	ec:2.6.1.52 - transaminase, ec:4.2.1.11 - hydratase, ec:4.1.1.25 - decarboxylase	1, 2, 1	DN75733.0.1.2, DN36032.0.3.1, DN36032.0.2.1, DN72221.0.1.3
22	Nicotinate and nicotinamide metabolism	3	ec:1.2.1.24 - dehydrogenase (NAD+), ec:2.7.1.23 - kinase, ec:2.4.2.1 - phosphorylase	1, 2, 1	DN73836.0.1.1, DN83662.4.3.2, DN83662.4.3.6, DN77822.1.2.5
23	Nitrogen metabolism	3	ec:6.3.1.2 - synthetase, ec:1.4.1.14 - synthase (NADH), ec:1.4.1.2 - dehydrogenase	3, 1, 1	DN74250.0.2.1, DN54305.0.1.1, DN69409.0.2.8, DN84080.1.3.1, DN75447.0.1.2
24	Phenylalanine metabolism	3	ec:2.6.1.5 - transaminase, ec:1.13.11.27 - dioxygenase, ec:4.1.1.28 - decarboxylase	1, 1, 3	DN61168.0.1.2, DN77224.1.3.2, DN75332.4.1.1, DN75332.4.1.4, DN75332.4.1.3
25	Taurine and hypotaurine metabolism	3	ec:4.1.1.15 - decarboxylase, ec:1.4.1.2 - dehydrogenase, ec:2.3.2.2 - glutamyl transpeptidase	1, 1, 3	DN76013.0.2.2, DN75447.0.1.2, DN73895.2.1.7, DN83724.0.1.1, DN83724.0.1.2
26	Tryptophan metabolism	3	ec:1.2.4.2 - dehydrogenase (succinyl-transferring), ec:1.1.1.35 - dehydrogenase, ec:4.1.1.28 - decarboxylase	1, 2, 3	DN67850.0.1.4, DN72435.0.6.2, DN72435.0.6.3, DN75332.4.1.1, DN75332.4.1.4, DN75332.4.1.3
27	Aminobenzoate degradation	2	ec:3.1.3.2 - phosphatase, ec:3.1.3.1 - phosphatase	3, 1	DN75118.0.1.5, DN75118.0.1.4, DN66881.0.1.3, DN71954.0.2.10
28	Biosynthesis of unsaturated fatty acids	2	ec:1.1.1.100 - reductase, ec:1.14.19.1 - 9-desaturase	1, 1	DN51665.0.1.3, DN81287.1.8.2

Rank	Pathway	#Enzs in Pathway	Enzyme	#Seqs of Enzyme	Sequences
29	Carbapenem biosynthesis	2	ec:2.7.2.11 - 5-kinase, ec:1.2.1.41 - dehydrogenase	1, 1	DN80083.0.6.1, DN80083.0.6.1
30	Drug metabolism - cytochrome P450	2	ec:2.5.1.18 - transferase, ec:2.4.1.17 - 1-naphthol glucuronyltransferase	2, 2	DN36217.0.1.4, DN36217.0.1.2, DN71186.2.2.5, DN77350.0.2.2
31	Drug metabolism - other enzymes	2	ec:3.1.1.1 - ali-esterase, ec:2.4.1.17 - 1-naphthol glucuronyltransferase	9, 2	DN73070.4.2.3, DN73070.4.2.2, DN83344.9.4.1, DN70534.0.2.2, DN83344.9.3.5, DN70534.0.2.1, DN77628.2.2.7, DN83040.2.1.6, DN74294.0.8.2, DN71186.2.2.5, DN77350.0.2.2
32	Fatty acid elongation	2	ec:3.1.2.22 - hydrolase, ec:1.1.1.35 - dehydrogenase	1, 2	DN74871.1.2.1, DN72435.0.6.2, DN72435.0.6.3
33	Glyoxylate and dicarboxylate metabolism	2	ec:6.3.1.2 - synthetase, ec:4.2.1.3 - hydratase	3, 1	DN74250.0.2.1, DN54305.0.1.1, DN69409.0.2.8, DN82451.0.3.4
34	Indole alkaloid biosynthesis	2	ec:4.1.1.28 - decarboxylase, ec:4.3.3.2 - synthase	3, 1	DN75332.4.1.1, DN75332.4.1.4, DN75332.4.1.3, DN77268.0.1.5
35	Lysine degradation	2	ec:1.2.4.2 - dehydrogenase (succinyl-transferring), ec:1.1.1.35 - dehydrogenase	1, 2	DN67850.0.1.4, DN72435.0.6.2, DN72435.0.6.3
36	Metabolism of xenobiotics by cytochrome P450	2	ec:2.5.1.18 - transferase, ec:2.4.1.17 - 1-naphthol glucuronyltransferase	2, 2	DN36217.0.1.4, DN36217.0.1.2, DN71186.2.2.5, DN77350.0.2.2
37	Pyruvate metabolism	2	ec:2.7.1.40 - kinase, ec:3.1.2.6 - hydrolase	4, 1	DN70271.0.1.4, DN53684.0.1.1, DN70271.0.1.2, DN70271.0.1.3, DN75416.0.3.2
38	T cell receptor signalling pathway	2	ec:3.1.3.16 - phosphatase, ec:2.7.10.2 - protein-tyrosine kinase	3, 1	DN81327.0.1.4, DN81327.0.1.10, DN80886.0.1.9, DN81649.0.6.9
39	Ubiquinone and other terpenoid-quinone biosynthesis	2	ec:2.6.1.5 - transaminase, ec:1.13.11.27 - dioxygenase	1, 1	DN61168.0.1.2, DN77224.1.3.2
40	Various types of N-glycan biosynthesis	2	ec:3.2.1.113 - 1,2-alpha-mannosidase, ec:3.2.1.52 - hexosaminidase	1, 2	DN67626.0.1.2, DN71426.0.1.1, DN83325.2.2.6
41	Aminoacyl-tRNA biosynthesis	1	ec:6.1.1.16 - ligase	1	DN78089.1.1.3

Rank	Pathway	#Enzs in Pathway	Enzyme	#Seqs of Enzyme	Sequences
42	Ascorbate and aldarate metabolism	1	ec:2.4.1.17 - 1-naphthol glucuronyltransferase	2	DN71186.2.2.5, DN77350.0.2.2
43	Benzoate degradation	1	ec:1.1.1.35 - dehydrogenase	2	DN72435.0.6.2, DN72435.0.6.3
44	beta-Alanine metabolism	1	ec:4.1.1.15 - decarboxylase	1	DN76013.0.2.2
45	Betalain biosynthesis	1	ec:4.1.1.28 - decarboxylase	3	DN75332.4.1.1, DN75332.4.1.4, DN75332.4.1.3
46	Caprolactam degradation	1	ec:1.1.1.35 - dehydrogenase	2	DN72435.0.6.2, DN72435.0.6.3
47	Carbon fixation in photosynthetic organisms	1	ec:5.3.1.1 - isomerase	1	DN62798.0.1.4
48	Cyanoamino acid metabolism	1	ec:2.3.2.2 - glutamyl transpeptidase	3	DN73895.2.1.7, DN83724.0.1.1, DN83724.0.1.2
49	D-Arginine and D-ornithine metabolism	1	ec:1.4.3.3 - oxidase	1	DN67844.0.1.4
50	Fatty acid degradation	1	ec:1.1.1.35 - dehydrogenase	2	DN72435.0.6.2, DN72435.0.6.3
51	Folate biosynthesis	1	ec:3.1.3.1 - phosphatase	1	DN71954.0.2.10
52	Fructose and mannose metabolism	1	ec:5.3.1.1 - isomerase	1	DN62798.0.1.4
53	Geraniol degradation	1	ec:1.1.1.35 - dehydrogenase	2	DN72435.0.6.2, DN72435.0.6.3
54	Glycerolipid metabolism	1	ec:3.1.1.3 - lipase	2	DN77628.3.5.6, DN77628.3.5.4
55	Glycosaminoglycan degradation	1	ec:3.2.1.52 - hexosaminidase	2	DN71426.0.1.1, DN83325.2.2.6
56	Glycosphingolipid biosynthesis - ganglio series	1	ec:3.2.1.52 - hexosaminidase	2	DN71426.0.1.1, DN83325.2.2.6

Rank	Pathway	#Enzs in Pathway	Enzyme	#Seqs of Enzyme	Sequences
57	Glycosphingolipid biosynthesis - globo and isoglobo series	1	ec:3.2.1.52 - hexosaminidase	2	DN71426.0.1.1, DN83325.2.2.6
58	Histidine metabolism	1	ec:4.1.1.28 - decarboxylase	3	DN75332.4.1.1, DN75332.4.1.4, DN75332.4.1.3
59	Inositol phosphate metabolism	1	ec:5.3.1.1 - isomerase	1	DN62798.0.1.4
60	N-Glycan biosynthesis	1	ec:3.2.1.113 - 1,2-alpha-mannosidase	1	DN67626.0.1.2
61	Novobiocin biosynthesis	1	ec:2.6.1.5 - transaminase	1	DN61168.0.1.2
62	Other glycan degradation	1	ec:3.2.1.52 - hexosaminidase	2	DN71426.0.1.1, DN83325.2.2.6
63	Penicillin and cephalosporin biosynthesis	1	ec:1.4.3.3 - oxidase	1	DN67844.0.1.4
64	Pentose and glucuronate interconversions	1	ec:2.4.1.17 - 1-naphthol glucuronyltransferase	2	DN71186.2.2.5, DN77350.0.2.2
65	Pentose phosphate pathway	1	ec:1.1.3.4 - oxidase	1	DN74918.0.1.3
66	Phenylalanine, tyrosine and tryptophan biosynthesis	1	ec:2.6.1.5 - transaminase	1	DN61168.0.1.2
67	Phenylpropanoid biosynthesis	1	ec:1.11.1.7 - lactoperoxidase	1	DN81252.0.1.2
68	Porphyrin and chlorophyll metabolism	1	ec:2.4.1.17 - 1-naphthol glucuronyltransferase	2	DN71186.2.2.5, DN77350.0.2.2
69	Primary bile acid biosynthesis	1	ec:1.1.1.35 - dehydrogenase	2	DN72435.0.6.2, DN72435.0.6.3
70	Retinol metabolism	1	ec:2.4.1.17 - 1-naphthol glucuronyltransferase	2	DN71186.2.2.5, DN77350.0.2.2

Rank	Pathway	#Enzs in Pathway	Enzyme	#Seqs of Enzyme	Sequences
71	Riboflavin metabolism	1	ec:3.1.3.2 - phosphatase	3	DN75118.0.1.5, DN75118.0.1.4, DN66881.0.1.3
72	Starch and sucrose metabolism	1	ec:3.2.1.1 - glycogenase	1	DN69097.0.1.3
73	Steroid hormone biosynthesis	1	ec:2.4.1.17 - 1-naphthol glucuronyltransferase	2	DN71186.2.2.5, DN77350.0.2.2
74	Styrene degradation	1	ec:3.7.1.2 - beta-diketonase	1	DN75999.1.4.2
75	Th1 and Th2 cell differentiation	1	ec:3.1.3.16 - phosphatase	3	DN81327.0.1.4, DN81327.0.1.10, DN80886.0.1.9
76	Toluene degradation	1	ec:1.1.1.35 - dehydrogenase	2	DN72435.0.6.2, DN72435.0.6.3
77	Tropane, piperidine and pyridine alkaloid biosynthesis	1	ec:2.6.1.5 - transaminase	1	DN61168.0.1.2
78	Valine, leucine and isoleucine biosynthesis	1	ec:4.3.1.19 - ammonia-lyase	2	DN50616.0.1.1, DN61974.0.1.1
79	Valine, leucine and isoleucine degradation	1	ec:1.1.1.35 - dehydrogenase	2	DN72435.0.6.2, DN72435.0.6.3
80	Vitamin B6 metabolism	1	ec:2.6.1.52 - transaminase	1	DN75733.0.1.2

#Enzs: number of enzymes
#Seqs: number of sequences

Annexure D

Title pages of published manuscripts

Short communications

Cytochrome *c* oxidase I and cytochrome *b* gene sequences indicate low genetic diversity in South African *Busseola fusca* (Lepidoptera: Noctuidae) from maize

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The African maize stem borer, *Busseola fusca*, feeds on a limited number of host plant species (Calatayud *et al.* 2014), which include crops such as maize, pearl millet and sorghum. This pest occurs throughout sub-Saharan Africa where it causes economic damage to maize and sorghum crops (Kfir *et al.* 2002). In an attempt to combat lepidopteran stem borers, genetically modified Bt maize that express insecticidal Cry toxins were developed to kill larvae that feed on these plants (George *et al.* 2011). Bt maize was introduced into South Africa during 1998 and within the first decade after release, field-evolved resistance of *B. fusca* to Bt maize was reported (Van Rensburg 2007). Since this first report, resistant populations have been reported from several parts of the maize-production region of South Africa (Kruger *et al.* 2011; Van den Berg *et al.* 2013). Explanations provided for this rapid resistance development were confined to agronomical reasons (Van Rensburg 2007; Kruger *et al.* 2009, 2011), non-compliance to refuge requirements (Kruger *et al.* 2009) and non-recessive inheritance of resistance (Campagne *et al.* 2013). Not much is however understood about the molecular genetics of *B. fusca*. Previously, Sezonlin *et al.* (2006b) noted that limited population genetic and phylogeography data are available for *B. fusca*.

Sequences available for *B. fusca* are limited to some cytochrome *b* (cyt*b*) (Sezonlin *et al.* 2006b, 2012) and some microsatellite data (Faure & Silvain 2005). Through the use of DNA-sequence data from the mitochondrial genome, phylogenetic relationships among species and/or populations can be estimated (Assefa *et al.* 2006; Simon *et al.* 1994). Sezonlin *et al.* (2006a) used cyt*b* sequences of *B. fusca* from West, Central, South and East Africa to demonstrate that three clades (*W*, *KI* and *KII*) were dominant, and that these were associated with geographic differences and ecological preferences. Based on genetic differentiation in cyt*b* sequences they concluded that *B. fusca* populations could be divided into three major groups, comprising 108 haplotypes. This included a homogeneous and geographically isolated population from West Africa (*W*), and two populations from East, Central and southern Africa (*KI* and *KII*). The latter two had overlapping distributions, although clade *KI* was restricted to East Africa (Sezonlin *et al.* 2006a). This clade distribution corresponded to the distribution patterns of various plant and animal species and climatic conditions and could be linked to the paleogeography of the region. However, in that study, *B. fusca* individuals were only collected at six localities in South Africa.

In studies on related species it has been hypothesised that understanding the exact genetic relationship of the pest species as well as the genetic diversity is important in the development of integrated pest management approaches (Zhang *et al.* 2012). Thus the aim of this study was to determine the genetic diversity of South African *B. fusca* populations from maize, sampled widely across the maize-production region, using cytochrome *c* oxidase I (COI) and cyt*b* sequences.

Seventy-two *B. fusca* larvae were collected from maize at 25 localities in South Africa (Fig. 1, Table 1). DNA was isolated using a NucleoSpin® Tissue Kit (Macherey-Nagel, Germany) following the instructions of the manufacturer. A 710 bp fragment of the *COI* barcoding gene was amplified using the LCO1490 (5′-GGTCAACAAATCATAAAGATAT TGG-3′) and HCO2198 (5′-TAAACTTCAGG GTGACCAAAAAATCA-3′) primer set from Folmer *et al.* (1994). The CP1 (5′-GATGATGAAATTTT

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Review

Biological and Microbial Control

An Overview of Mechanisms of Cry Toxin Resistance in Lepidopteran Insects

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Abstract

Arthropods have the capacity to evolve resistance to insecticides and insecticidal traits in genetically modified crops. Resistance development among Lepidoptera is a common phenomenon, and a repertoire of resistance mechanisms to various Cry toxins have been identified from laboratory, greenhouse, and field studies in this insect order. Elucidation of such resistance mechanisms is crucial for developing IRM (insect resistance management) strategies to ensure sustainable use of genetically modified crops. This mini review provides a comprehensive overview of mechanisms of resistance that have been reported for lepidopteran pests. This study demonstrated that resistance mechanisms are highly complex, and the most common mechanism of resistance is altered binding sites. It is yet to be established whether all these altered binding sites are regulated by an MAPK signaling pathway, which might suggest a universal mechanism of resistance in lepidopterans.

Key words: resistance, receptor, signaling, Cry, toxin

Genetically modified crops with insecticidal traits from *Bacillus thuringiensis*, known as Bt crops, have been cultivated since 1996 for the management of insect pests (James 2015). Although no resistance to Bt crops were reported in target pests during the first few years of cultivation, evolution of target pest resistance to Bt crops was a concern since the development of these crops (Roush 1997). As a result, insect resistance management programs were imposed to prevent or delay resistance evolution (Bates et al. 2005). However, various factors (social, economic, biological, and environmental) hampered the efficacy of these programs (Bates et al. 2005, Kruger et al. 2009), which enabled several insect species to develop Bt resistance.

Although *Plodia interpunctella* was the first lepidopteran pest reported to have developed resistance to a sprayable Bt product under laboratory conditions in 1985 (McGaughey 1985), the first field-evolved resistance to commercial Bt formulations was observed in a *Plutella xylostella* population in 1986 (Tabashnik et al. 1990). According to Tabashnik et al. (2013), *Helicoverpa zea* was the first species reported to have evolved field resistance to Bt crops (cotton). This was reported in 2002, a mere 6 yr after the introduction of Bt crops. Since then, several reports of field-evolved resistance to Bt crops have been published. Some examples are discussed in recent reviews (Pardo-López et al. 2013, Tabashnik et al. 2013, Kumar and Kumari 2015). The presumption that resistance was unlikely to evolve in the field was ascribed to a lack of resistance reports, as well as inaccurate reflections of potential for resistance evolution in laboratory selection experiments compared to the field (Tabashnik et al. 1990, Wang et al. 2007, Yang et al. 2007).

Field-evolved resistance to Bt crops in lepidopterans has been reported for *Busseola fusca* in South Africa (Kruger et al. 2009), *Diatraea saccharalis* in the United States (Huang et al. 2012), *Helicoverpa armigera* in China and Australia (Downes et al. 2007, Liu et al. 2010), *Helicoverpa punctigera* in Australia (Downes et al. 2010a), *H. zea* in the United States (Luttrell et al. 2004), *Ostrinia furnacalis* in the Philippines (Alcantara et al. 2011), *Pectinophora gossypiella* in China and India (Dhurua and Gujar 2011, Wan et al. 2012), and *Spodoptera frugiperda* in Brazil and the United States (Storer et al. 2010, Farias et al. 2014). It is evident that evolution of resistance in these pests is a major threat to the sustainable use of these transgenic crops (Yang et al. 2007, Tabashnik et al. 2013).

Studies into resistance mechanisms commenced mainly during the early 2000s. These studies have indicated that resistance can be ascribed to several factors, including variations in any one of the steps of the Cry toxin mode of action (Jurat-Fuentes et al. 2004). A general overview of potential mechanisms of resistance is indicated in Fig. 1. The most reported mechanism of resistance seems to be altered binding of Cry toxins to receptors (Ferré and Van Rie 2002). It is also notable that a single species can evolve a repertoire of resistance mechanisms to the same or different Cry toxins (Table 1). Numerous studies have been published on the topic of Bt toxin resistance in lepidopteran insects, but these studies are invariably restricted to specific species and strains, geographical regions, or certain mechanisms of resistance. The aim of this study was thus to compose an overview on all the mechanisms of Cry toxin resistance that have been reported for lepidopteran pests from laboratory,