UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CAMPUS DE JABOTICABAL

THE INVESTIGATION OF FACTORS POTENTIALLY INVOLVED IN RESISTANCE TO *Bacillus thuringiensis* IN NATIVE *Plutella xylostella* (L.) (LEPIDOPTERA: PLUTELLIDAE) POPULATIONS

Caroline Placidi De Bortoli

Médica Veterinária

Bióloga

2018

UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CAMPUS DE JABOTICABAL

THE INVESTIGATION OF FACTORS POTENTIALLY INVOLVED IN RESISTANCE TO *Bacillus thuringiensis* IN NATIVE *Plutella xylostella* (L.) (LEPIDOPTERA: PLUTELLIDAE) POPULATIONS

Caroline Placidi De Bortoli

Orientador: Prof. Dr. Ricardo Antônio Polanczyk

Tese apresentada a Faculdade de Ciências Agrárias e Veterinárias – Unesp, Campus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Agronomia (Entomologia Agrícola)

2018

De Bortoli, Caroline Placidi

D287t **The investigation of factors potentially involved in resistance to *Bacillus thuringiensis* in native *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) populations. Caroline Placidi De Bortoli – Jaboticabal, 2018**

xi, 107 p. : il. ; 28 cm

Tese de Doutorado – Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias, 2018

Orientador: Ricardo Antônio Polanczyk

Banca examinadora: Ricardo Antônio Polanczyk, Celso Omoto, Daniel Ricardo Sosa-Gómez, Guilherme Duarte Rossi, Manoel Victor Franco Lemos

Bibliografia

1. Traça-das-crucíferas. 2. Suscetibilidade. 3. Virulência. 4. Resistência. 5. Modo de ação. 6. ABCC2. I. Título. II. Jaboticabal-Faculdade de Ciências Agrárias e Veterinárias.

CDU 595.78:632.937

Ficha catalográfica elaborada pela Seção Técnica de Aquisição e Tratamento da Informação - Serviço Técnico de Biblioteca e Documentação – UNESP, Campus de Jaboticabal.

e-mail: carubortoli@yahoo.com.br

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: THE INVESTIGATION OF FACTORS POTENTIALLY INVOLVED IN RESISTANCE TO *Bacillus thuringiensis* IN NATIVE *Plutella xylostella* (L.) (LEPIDOPTERA: PLUTELLIDAE) POPULATIONS

AUTORA: CAROLINE PLACIDI DE BORTOLI
ORIENTADOR: RICARDO ANTONIO POLANCZYK

Aprovada como parte das exigências para obtenção do Título de Doutora em AGRONOMIA (ENTOMOLOGIA AGRÍCOLA), pela Comissão Examinadora:

Prof. Dr. RICARDO ANTONIO POLANCZYK
Departamento de Fitossanidade / FCAV / UNESP - Jaboticabal

Prof. Dr. CELSO OMOTO
Departamento de Entomologia / ESALQ - USP - Piracicaba/SP

Prof. Dr. MANOEL VICTOR FRANCO LEMOS
Departamento de Biologia Aplicada à Agropecuária / FCAV / UNESP - Jaboticabal

Pesquisador Dr. DANIEL RICARDO SOSA-GOMEZ
Centro Nacional de Pesquisa de Soja / EMBRAPA - Londrina/PR

Prof. Dr. GUILHERME DUARTE ROSSI
Departamento de Fitossanidade / FCAV / UNESP - Jaboticabal

Jaboticabal, 16 de fevereiro de 2018

DADOS CURRICULARES DA AUTORA

CAROLINE PLACIDI DE BORTOLI – Nascida em 18 de maio de 1985, na cidade de Jaboticabal, São Paulo, Brasil. É Médica Veterinária graduada pelo Centro Universitário Barão de Mauá/Ribeirão Preto – SP, em dezembro de 2010, e Licenciada em Ciências Biológicas pela Faculdade de Educação São Luís de Jaboticabal – SP, em dezembro de 2015. As atividades de pesquisa em controle biológico iniciaram-se com estágio no Laboratório de Biologia e Criação de Insetos (LBCI) da Faculdade de Ciências Agrárias e Veterinárias, UNESP, Jaboticabal, SP, em janeiro de 2011. Em março de 2014 concluiu o curso de Mestrado em Agronomia (Entomologia Agrícola), sob a orientação do Prof. Dr. Ricardo Antônio Polanczyk, sendo bolsista da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) no período de Janeiro/13 a Fevereiro/14, tendo nesse período usufruído de uma Bolsa BEPE, por três meses, na "University of Sussex", Brighton, Inglaterra, sob a orientação do Prof. Dr. Neil Crickmore. Em abril de 2014 iniciou o curso de Doutorado em Agronomia (Entomologia Agrícola), sob a orientação do Prof. Dr. Ricardo Antônio Polanczyk, sendo bolsista da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) no período de Dezembro/15 a Agosto/17, tendo nesse período usufruído de uma Bolsa BEPE, por seis meses, na "University of Sussex", Brighton, Inglaterra, sob a orientação do Prof. Dr. Neil Crickmore. Sendo bolsista também do CNPQ no período de Setembro/17 a Novembro/17. Atualmente trabalha como Pesquisadora Assistente na "University of Tennessee", no laboratório de Patologia Molecular e Fisiologia de Insetos, Knoxville, Tennessee, Estados Unidos. Desde o período de iniciação científica publicou como autora/co-autora 27 (vinte e sete) trabalhos científicos em revistas indexadas, 06 (seis) capítulos de livros, 03 (três) resumos expandidos em anais de eventos científicos, 69 (sessenta e nove) resumos simples em anais de eventos científicos, tendo participado de 53 (cinquenta e três) congressos, simpósios e similares nacionais e internacionais onde apresentou 21 (vinte e uma) comunicações científicas.

“Without commitment you will never start, but without consistency you will never finish.”

Denzel Washington

For my parents, Mariangela and Sergio, and for my husband, Rafael, for the support, encouragement, companionship, affection and unconditional love.

DEDICATION

ACKNOWLEDGEMENTS

I thank God for this journey.

I acknowledge São Paulo State University, School of Agricultural and Veterinary Sciences, Jaboticabal campus, and the Department of Plant Protection for the opportunity to take my PhD course.

I also tank São Paulo Research Foundation (FAPESP) for the grants related to the following processes: 2015/05891-6 (PhD) and 2016/04868-3 (BEPE).

I show gratitude to my supervisor, Prof. Dr. Ricardo Antônio Polanczyk, for the orientation and help during my PhD.

I thank Prof. Dr. Neil Crickmore, who is an excellent scientist and a remarkably just and considerate person, a sincere thank you for all the support and various opportunities.

A very special thanks to my father, Prof. Dr. Sergio Antonio De Bortoli, for his patience, help, teaching and affection. He was and has been my hole model and he is the most honest and kind person that I have ever known.

Thanks to my LBCI colleagues and friends to whom I had the pleasure to work with. I appreciate Dagmara Gomes Ramalho, Nathália Alves dos Santos, Gustavo Oliveira de Magalhães, Matheus Moreira Dantas Pinto, Valéria Lucas de Laurentis, Ana Caroline Pires Veiga and Alessandra Marieli Vacari for the friendship, help and support.

I also thanks my lab colleagues from University of Sussex for their kindness, support and friendship.

A special thanks to my PhD colleague and husband, Rafael Ferreira dos Santos, for the love, patience, help and for being always by my side.

I acknowledge all the professors, employees from São Paulo State University.

I thank all that in some way contributed for the execution of this research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INVESTIGAÇÃO DE FATORES POTENCIALMENTE ENVOLVIDOS NA RESISTÊNCIA AO *Bacillus thuringiensis* EM POPULAÇÕES NATIVAS DE *Plutella xylostella* (L.) (LEPIDOPTERA: PLUTELLIDAE)

RESUMO - *Plutella xylostella* é uma praga de grande importância para as crucíferas em todo o mundo. Embora seja controlada com inseticidas sintéticos e biológicos, ela pode desenvolver resistência rapidamente a uma variedade de inseticidas. Os biopesticidas mais comuns utilizados para controlar *P. xylostella* são baseados na bactéria entomopatogênica *Bacillus thuringiensis*. Embora muitos estudos tenham sido realizados com Bt, o modo de ação ainda não é totalmente compreendido. Uma grande diversidade de genes é diferencialmente expressa no intestino médio de insetos resistentes, o que sugere que vários processos celulares podem estar envolvidos na resistência. Descobertas recentes mostraram que as mutações no gene que codifica o transportador ABCC2 são responsáveis pela resistência às toxinas Bt em diferentes espécies de insetos. O objetivo desta pesquisa foi testar a hipótese de que a susceptibilidade de *P. xylostella* a Bt se correlaciona com o nível de expressão dos componentes desse regulador de estresse. Os níveis de expressão dos genes ALP, APN, CDKAL 1, MAP4K4 e ABCC2 foi comparado utilizando qPCR, entre populações suscetíveis e resistentes de *P. xylostella*. A investigação da sequência de DNA do cDNA do ABCC2 foi realizada, por PCR e sequenciamento, para testar a hipótese de que a susceptibilidade de *P. xylostella* a Bt se correlaciona com mutações no gene ABCC2. Foram realizados retrocruzamentos entre populações suscetíveis e resistentes e cruzamentos de complementação entre populações resistentes. Nossa pesquisa demonstrou que não há padrões na expressão dos genes testados demonstrando nenhuma associação entre expressão e resistência/susceptibilidade. No entanto, ao investigar a sequência do gene ABCC2, encontrou-se uma mutação no gene da população brasileira resistente, que poderia ser responsável pela causa da resistência

da população estudada neste trabalho. Os ensaios de retrocruzamentos não confirmaram que a resistência foi devida à supressão de 1 pb encontrada, no entanto, os ensaios complementares indicaram que a população brasileira compartilha um alelo de resistência com a população havaiana resistente.

Palavras-chave: ABCC2, mecanismo de ação, resistência, suscetibilidade, traça-das-crucíferas, virulência.

THE INVESTIGATION OF FACTORS POTENTIALLY INVOLVED IN RESISTANCE TO *Bacillus thuringiensis* IN NATIVE *Plutella xylostella* (L.) (LEPIDOPTERA: PLUTELLIDAE) POPULATIONS

ABSTRACT - *Plutella xylostella* is a major insect pest of cruciferous crops worldwide. Although controlled with both synthetic and biological insecticides it can rapidly evolve resistance to a variety of insecticides. The most common biopesticides used to control P. xylostella are based on the entomopathogenic bacterium *Bacillus thuringiensis*. Although many studies have been performed on Bt, the mode of action is still not fully understood. A wide diversity of genes are differentially expressed in the midgut of resistant insects, this suggests that a variety of cell processes may be involved in the preservation of resistance. Recent discoveries have shown that mutations in the gene encoding an ABCC2 transporter are responsible for resistance to Bt toxins in various different insect species. The aim of this research was to test the hypothesis that the susceptibility of *P. xylostella* to Bt correlates with the level of expression of components of this putative stress-response regulon. The level of expression of ALP, APN, CDKAL 1, MAP4K4 and ABCC2 genes were compared using qPCR, between susceptible and resistant *P. xylostella* populations. The investigation of the DNA sequence of ABCC2 cDNA was performed, through PCR and sequencing, to test the hypothesis that the susceptibility of P. xylostella to Bt correlates with mutations on the ABCC2 gene. Backcrosses between susceptible and resistant populations and complementation cross between resistant populations were performed. Our research demonstrated that there were no patterns in the expression of the genes tested demonstrating no association between expression and resistance/susceptibility. However, when investigating the ABCC2 gene sequence, a mutation in the gene of the Brazilian resistant population was found, which could be responsible for the resistance of the Brazilian population studied in this research. Backcrossing assays didn't confirm that the resistance was due to the

1bp deletion found, however complementation assays indicated that the resistant Brazilian population shares a resistance allele with the resistant Hawaiian population.

Keywords: ABCC2, mode of action, resistance, susceptibility, diamondback moth, virulence.

1. INTRODUCTION

The diamondback moth, *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae) is the most destructive pest of cruciferous plants worldwide (SARFRAZ et al., 2006). Although controlled with both synthetic and biological insecticides populations of this species can rapidly evolve resistance to a variety of insecticides (SAYYED et al., 2008). Insecticidal *B. thuringiensis* toxins were thus extremely valuable as a substitute for broad-spectrum synthetic insecticides as they gave effective control of *P. xylostella* with minimal non-target effects on parasitoids and predators (SASTROSISWOJO; SASTRODIHARDJO, 1986). Also, Bt toxins pose few, if any, health hazards for producers and consumers (SCHNEPF et al., 1998). *P. xylostella* resistance to Bt toxin is now widespread in the field (FERRÉ; VAN RIE, 2002; SAYYED et al., 2005; TABASHNIK et al., 1994) and thus there is a great risk that injudicious use of insecticides will lead to the selection of multiple resistant pest populations.

Bioinsecticides based on the entomopathogenic bacterium *Bacillus thuringiensis* Berliner (Bt) (Bacillaceae) are the most common biopesticides used to control *P. xylostella* (VAN FRANKENHUYZEN, 2009; JURAT-FUENTES; JACKSON, 2012). A number of studies have observed susceptibility variations to Bt in different populations of *P. xylostella* from countries in Latin America, Europe and various Brazilian states (POLANCZYK et al., 2005; MONNERAT et al., 2006). Roux et al. (2007) used an ISSR-PCR technique to differentiate 19 *P. xylostella* populations from several countries. The authors emphasized that in the tropics the high number of generations per year may favor the emergence of mutations and thus increases the discrimination between individuals, and the high selection pressure caused by the indiscriminate application of pesticides favors the emergence of tolerant or resistant populations of this insect. *P. xylostella* was the first insect in which field resistance to Bt was identified (TABASHNIK, 1994). This increased the interest of Bt researchers about this pest (SAYYED et al.,

2005; MONNERAT et al., 2007; GONG et al., 2010; DE BORTOLI et al., 2012; FIUZA et al., 2017).

In 1901, a Japanese researcher named Ishiwata isolated a pathogenic *Bacillus* sp. from larvae of *Bombyx mori* Linnaeus, 1758 (Lepidoptera: Bombycidae) and named "sotto disease bacillus". In 1911, Berliner isolated a bacillus that killed larvae of *Ephestia kuehniella* (Zeller, 1879) (Lepidoptera: Pyralidae) and, in 1915, the same scientist described and named Bt, in honor of the province of Thuringia, in Germany, where the first infected insect was found (HABIB; ANDRADE, 1998; GLARE; O'CALLAGHAM, 2000). In 1915, Berliner mentioned the existence of crystals in spore cultures of Bt, but the activity of these crystals was only later discovered (POLANCZYK; ALVES, 2003). Hannay (1953) was the first researcher to relate the crystals, called by him as parasporal bodies, with the pathogenicity of Bt, due to the possible formation of a toxic substance that caused the death of the insects, as evidenced by Angus (1968) (HABIB, ANDRADE, 1998).

Bioinsecticides based on Bt have been available worldwide since the 1970s and has a long history of successful use in the biocontrol of insect pests in agriculture and forestry and against disease vectors (CAB, 2010; BRAVO et al., 2011; SANCHIS, 2011; JURAT-FUENTES; JACKSON, 2012; VACHON; LAPRADE; SCHWARTZ, 2012). By 2010, 300 Bt-based biopesticides (70% of them are *Bt kurstaki* based) accounted for 53% of the worldwide market for biopesticides, representing an annual turnover of 210 million dollars. The Americas concentrates 50% of the consumption, particularly the USA and Canada, with Latin America are responsible for 8–10% of the total consumption (CAB, 2010; POLANCZYK; DE BORTOLI; DE BORTOLI, 2012; POLANCZYK et al., 2017). Specifically for Brazil, until the end of 2016 there was 105 registered biological products, being 12 Bt based (POLANCZYK et al., 2017). Various transgenic crops that express insecticidal Bt toxins have been grown over a rapidly increasing area (JAMES, 2016; JURAT-FUENTES; JACKSON, 2012).

Although many studies have been carried out on Bt, the mode of action is still uncertain. After ingestion of spores/crystals by the insect, the crystals are solubilized and then to a greater or lesser extent are cleaved by the digestive proteases down to a

protease-resistant core (the so-called active toxin). These toxins can pass through the peritrophic membrane, bind to specific receptors located on the apical membrane of the midgut columnar cells and form pores in the membrane. These pores interfere with cell physiology by abolishing transmembrane ionic gradients and can lead to colloid-osmotic lysis of the cells due to the massive influx of solutes from the midgut lumen. In turn, destruction of the cells results in extensive damage to the midgut epithelial tissue and death of the intoxicated larvae. The insect can also suffer from starvation, since shortly after ingestion of the toxin the insect ceases feeding (COPPING; MENN, 2000; CRICKMORE, 2006; JURAT-FUENTES; JACKSON, 2012; VACHON; LAPRADE; SCHWARTZ, 2012).

An elaborate model involving the sequential binding of the toxins to different membrane receptors has been proposed to describe the events leading to membrane insertion and pore formation. However, it has also been proposed that, in contradiction to mechanism, Bt toxins function by activating intracellular signaling pathways which lead to the necrotic death of their target cells without the need for pore formation (BRAVO et al., 2007). Vachon et al. (2012) pointed out that the available information still supports the notion that Bt Cry toxins act by forming pores, but most events leading to their formation, following binding of the activated toxins to their receptors, remain relatively poorly understood.

Heckel (2012) and Hernández-Martínez et al. (2012) complicated the understanding of the mechanism of action of Bt toxins by the discovery that mutations in the gene encoding an ABCC2 transporter are responsible for resistance to Bt toxins in four different insect species. Baxter et al. (2011) cloned and sequenced the orthologue from *P. xylostella* using a genomic BAC library constructed from a susceptible strain Geneva88As ABCC2. Verification through PCR amplification from Bt-susceptible (Geneva88) and Bt-resistant (NO-QAGE) midgut cDNA demonstrated that the gene contains 26 exons and that the resistant strain NO-QAGE contained a 30-bp deletion in exon 20, which is predicted to remove the 12^{th} and final transmembrane domain and aberrantly position the carboxyl-terminal outside the cell. If this gene is translated and

inserted into the midgut membrane, a core ATP-binding loop is expected to be nonfunctional.

The superfamily of ABC proteins takes its name from the ATP binding cassette, an intracellular domain that binds and hydrolyzes ATP in a cycle that drives transport of molecules across a lipid bilayer membrane. The functional transporter consists of two cytosolic nucleotide-binding domains (NBDs) that bind and hydrolyze ATP and two integral transmembrane (HECKEL, 2012). The biological function of ABCC2 is unknown, but its similarity to multidrug resistance proteins suggests that it could export small hydrophobic toxins from midgut epithelial cells for eventual elimination in the feces. Heckel (2012) speculates that ABCC2 could function as one of the toxin binding proteins in the sequential binding model discussed by Bravo (2007) and further proposes that binding of the toxin to ABCC2 only occurs when the transporter is in its open configuration. When the transporter closes then the toxin is pushed into the membrane. This transient interaction between the toxin and ABCC2 may explain why no one has yet identified ABCC2 as a toxin binding protein.

Hernández-Martínez et al. (2012) suggested an alternative model in which ABCC2 is indirectly affecting the mechanism of action of the Bt toxins. Mutations in the gene encoding ABBC2 affect its function which then affects the physiology of the cell in some way that then affects the ability of the Bt toxin to form a pore. Both papers conclude that the functional role of the ABCC2 protein remains to be established, but that mutations in it clearly have significant consequences on the susceptibility of insects to Bt. What is unknown is how the mutations on ABCC2 results in a particular changes in expression of genes. One possibility is that mutations in *abcc2* in some way simulates a pathogen attack and the cells express genes in response to counter attack (AYRA-PARDO et al., 2015).

Ocelotl et al. (2017) also found that ABCC2 is associated with Bt Cry1Ac toxin oligomerization and membrane insertion in diamondback moth. Different larval gut proteins, such as cadherin, facilitates oligomerization, membrane insertion and pore formation when binding to Cry1Ac. ABCC2 facilitates Cry1Ac oligomerization and

oligomer membrane insertion in *P. xylostella*, therefore ABCC2 mutation also affects the association of Cry1Ac oligomer with the membrane.

On the other hand Guo et al. (2015) found no association between mutations in ABCC genes and resistance. They, subsequent compared levels of expression for all five ABCC genes in *BtR-1* locus in susceptible and resistant strains using qPCR.

One important gene that is involved in the early stages of the specific response to the pathogen is CDKAL1. This gene is involved in ER maintenance homeostasis. During the infection by Bt, the ER homeostasis secretory system in midgut epithelial cells is challenged. The early protected function of the CDKAL1 could derive from a role in the midgut cellular proteins ER maturation required to repair the cytotoxic damage and restore ER homeostasis (AYRA-PARDO et al., 2015). An overexpression of this gene would be beneficial so the insects would survive the Bt intoxication. Therefore, if CDKAL1 is involved in cell protein quality control, the suppression of this gene in the midgut would affect the ability of the *P. xylostella* larvae to survive ingestion of this pathogen. This gene is expected to be up-regulated in resistant *P. xylostella* populations.

Mitogen-activated protein kinase (MAPK) signaling pathway has been described to control immune defensive responses to Bt Cry toxins in insects (CANCINO-RODEZNO et al., 2010). Therefore, it is possible that the MAPK signaling pathway may be the common route that can regulate the expression of diverse receptors genes (GUO et al., 2015). If this pathway leads to the expression receptors, this gene would have an altered function or expression levels in resistant *P. xylostella* populations.

Aminopeptidase N (APN) and Alkaline Phosphatase (ALP) are known receptors and their activity were reduced in resistant strains (GUO et al., 2015). The expression of APN and ALP genes are down-regulated in resistant strains.

ABCC2 is another important gene, since it was proposed that its role is a receptor protein involved in facilitating insertion of Cry1A oligomers into the membrane (HECKEL, 2012).

Guo et al. 2015 revealed that ABCC2 was significantly down-regulated in the resistant populations as was the expression of another putative receptor ALP. They also

found that MAP4K4 was constitutively up-regulated in larvae from all resistant strains compared to the susceptible strain. RNAi silencing of ABCC2 and MAP4K4 resulted in a significant increase in larvae susceptibility to Bt. Guo et al. (2015) also speculate that a mutation that increased the expression of MAP4K4 indirectly reduced the expression of the receptors ALP and ABCC2.

The aim of this research was to test the hypothesis that the susceptibility of *P. xylostella* to Bt correlates with the level of expression of components of this putative stress-response regulon. The level of expression of ALP, APN, CDKAL 1, MAP4K4 and ABCC2 genes were compared between susceptible and resistant *P. xylostella* populations. These genes where chosen because it was proven to have a clear pattern on the susceptible and resistant (GUO et al., 2005).

In our study, we also investigated the DNA sequence of ABCC2 cDNA to test the hypothesis that the susceptibility of *P. xylostella* to Bt correlates with mutations on the ABCC2 gene.

2. LITERATURE REVIEW

2.1. *Plutella xylostella*

Diamondback moth, *P. xylostella*, is probably of Mediterranean Europe origin, the same origin of the Cruciferae Family plants. It is an cosmopolitan pest, found in various productive regions of the world independent of the climate conditions (DIAS; SOARES; MONNERAT, 2004; MAU; KESSING, 2007). Furthermore, this pest has the ability to migrate to different climate zones (CHAPMAN et al., 2002; COULSON et al., 2002).

Plutella xylostella is a short-cycle insect, in which temperature is a determining factor, because in warmer conditions the cycle can be only 12 days, while in regions with milder temperatures it is prolonged totaling 20-25 days. The number of generations varies from 5 to 10 per year, depending on the climatic conditions and the availability of food, and the population density of the pest can vary greatly from year to year (DIAS; SOARES; MONNERAT, 2004).

Females lay their eggs on the abaxial face of the leaves, usually accompanying the veins, in an isolated or grouped form (THULER, 2009). They are very fertile, laying up to 350 eggs during their life cycle. The eggs are yellow, small, elliptical, flattened and with undulating reliefs. They are deposited alone or in groups of 2 or 3, the incubation period is 2 to 4 days (DE BORTOLI et al., 2013).

After 3 or 4 days, the larvae hatch and penetrate the inside of the leaf, starting to feed on the parenchyma, for 2 or 3 days. They then leave the gallery and begin to feed on the lower leaf epidermis (DE BORTOLI et al., 2013). The larvae is initially whitish, soon acquiring light green color with a brown head; on their body are noticed dark and sparse small "hair". It presents four larval stages, in the fourth is when they begin the cocoon confection (MONNERAT, 1995). They reach maximum development after 9 to 10 days of hatching, reaching 8 to 10 mm in length (DE BORTOLI et al., 2013).

The pupae is easily recognized inside a silk cocoon, woven with interlaced threads, which helps the fixation to the plant, usually on the underside of the leaves. About 4 days after the pupae is formed, the adult emerges (DE BORTOLI et al., 2013).

Adults have a nocturnal habit and during the day they shelter between the leaves. In males, the posterior margin of the anterior wings is white and when they are resting there is a characteristic spot (diamond shape) on the dorsal surface (DE BORTOLI et al., 2013). Adults have sexual dimorphism, distinguishing males and females by observing the ventral part of the insect: the male has a slit at the end of the abdomen, while the female has two dark circular spots on both sides of the end of the abdomen.

The biological characteristics of this pest vary according to the type of Brassicaceae in which the insect feeds (DE BORTOLI et al., 2013).

2.1.1. Damage

There is a direct relationship between the phenological development of the crop and the increase of damage caused by *P. xylostella*, and it is often necessary to introduce control measures at the beginning of plant development (SILVA et al., 2003).

When in low populations, the larvae prefer younger leaves and, in high populations, the insects are distributed throughout the plant. Usually the locations of the plant where the moth is found provides protection against predators, parasitoids and even against insecticides. In cabbage plants, larvae and pupae are found in the lower part of the leaves that surround the heads. When located on external leaves, larvae and pupae are found in the lower part of the leaves (CASTELO BRANCO; FRANÇA; VILLAS BOAS, 1997).

When the larvae consumes cabbage leaves, the leaves become with a lacy appearance, and when they feed on the cabbage heads they cause holes, reducing the quantitative and the commercial value of the product (FREITAS LUZ; SABOYA; SILVA-

PEREIRA, 2002). The larvae do not feed only on leaves: in broccoli and cauliflower they can consume inflorescences and, in Brussels sprouts, shoots (TIBA, 2008).

Cabbage is attacked from the formation of the head to harvest, with damage levels close to 20% of infested plants (VACARI et al., 2008). Cabbage crop damage can be up to 95%, depending on the crop region and planting season (CZEPAK et al., 2005).

In the State of São Paulo, *P. xylostella* causes variable damage, reducing cabbage yield by up to 60% (IMENES et al., 2002). Severe attacks, mainly caused during the driest periods of the year, can cause total losses in the field production (MEDEIROS et al., 2003). The damages also cause depreciation of the product, delay plant growth and even death of plants.

In the southeastern USA, *P. xylostella* is the main lepidopteran defoliator in canola (BUNTIN, 1990; RAMACHANDRAN; BUNTIN; ALL, 2000). In Texas, it can cause annual losses of $ 40-70 million in cabbage and $ 400,000 in broccoli crops if they are not treated properly (SHELTON, 2004). Without proper management of the pest, more than $ 500 million is lost for hindering broccoli crops in California unusable and $ 80 million in cabbage in New York (SHELTON, 2004). In Canada, crucifer's moth also occurs annually in Brassicaceae, and in years of high pest outbreaks there are substantial crop losses (DOSDALL et al., 2001).

2.1.2. Control

The control of diamondback moth since the mid-twentieth century is basically done by means of chemical insecticides, due to their supposed efficacy and ease of application (TIBA, 2008). One of the difficulties in the control of this pest is related to the cultivated areas that are maintained throughout the year, with plants at different ages, providing to the insect abundant and continuous amount of food (IMENES et al., 2002).

Resistance can develop through different physiological mechanisms. For chemical products, could be a reduction in penetration by the cuticle (NOPPUN; SAITO,

MIATA, 1989), alteration at the target site for organophosphates and carbamates (LIU; TZENG; SUN, 1982; YU; NGUYEN, 1999; MAA et al., 1996), decreased nerve sensibility (HAMA; KONO; SATO, 1987) and degradation of metabolism involving enzymes such as glutathione S-transferase to parathion (KAO; HUN; SUN, 1989), a microsomal P-450 monooxygenase for pyrethroids (YAO; HUNG; SUN, 1988; HUNG; SUN, 1989) and carboxylesterase for malathion (MAA; LIAO, 2000). And the resistance of *P. xylostella* to Bt can be due to several factors, among them gut bacteria, that can influence the susceptibility of the insects to Bt; and the presence of esterases, which have the ability to promote detoxification and mutations in the ABBC2 gene (GUNNING et al., 2005; JOHNSTON; CRICKMORE, 2009; BAXTER, 2011).

In addition, frequent applications of large quantities of pesticides, without due care, cause serious environmental, animal and human health impacts. In the environment, these products accumulate and often do not reach only the target site of application, spreading and contaminating water sources and other biomes, causing ecological imbalance. In relation to human health, intoxication may occur through direct contact with the products or due to residues in the food consumed, most often "in natura".

Studies have shown alternative techniques to chemical control, such as: the use of pheromones (MICHEREFF et al., 2000), trap cultivars (CHARLESTON; KFIR, 2000; BADENAS-PEREZ; SHELTON; NAULT, 2004), resistant varieties (MELO; CASTELO BRANCO; MADEIRA, 1994; THULER; DE BORTOLI; HOFFMANN-CAMPO, 2007), insecticidal plants (TORRES; BARROS; OLIVEIRA, 2001), use of natural products extracted from plants (NEVES; NOGUEIRA, 1996), and the use of biological control agents, such as parasitoids, predators and entomopathogenic microorganisms (DIAS; SOARES; MONNERAT, 2004).

The use of entomopathogens in pest control is advantageous because it avoids ecological imbalances in the agroecossystem, since most of them are specific to the target insects and selective to the natural enemies. In addition to causing direct mortality, pathogens can affect the biological and reproductive characteristics of insects; can be associated with other compatible control methods; reduce the costs of

agricultural production; does not pollute the environment and are not toxic to humans and other animals, if correctly selected and handled (ALVES, 1998).

Among these microorganisms, the most used and studied for most agricultural pests, such as *P. xylostella*, is the bacterium Bt (MONNERAT et al., 1999; CASTELO BRANCO et al., 2003; MEDEIROS et al., 2005).

2.2. *Bacillus thuringiensis*

2.2.1. General aspects

The first commercial product based on the bacterium Bt, Sporeine, was developed and marketed in France in the 1930s for the control of flour moth larvae, *E. kuehniella* (LAMBERT; PEFEROEN, 1992; RAMOS, 2008).

The use of the entomopathogen increased in the early 1950s, in the United States, mainly Lepidoptera control (BEEGLE; YAMAMOTO, 1992). In the early 1970s, there were several products for the control of Lepidoptera, being discovered Bt var. *israelenses*, active against Diptera and used in the control of insect vectors of diseases, mainly of the genus *Aedes*, *Culex*, *Anopheles* and *Simulium*. In 1983, Bt var. *tenebrionis* was discovered, used to control coleopteran larvae (GLARE; O´CALLAGHAM, 2000).

Among the Bt Cry toxins identified as promising for insect control, 59 were tested against 71 lepidopteran species and 43 were tested for *P. xylostella* (VAN FRANKENHUYZEN, 2009).

Bacillus thuringiensis is a sporulating bacterium with rod-shaped spores that produces crystalline inclusions during sporulation (O'CALLAGHAM, 2000; CRICKMORE, 2006; GLARE; JURAT-FUENTES; JACKSON, 2012).

These crystals contain toxins-proteins, known as δ-endotoxins, which are responsible for the toxic activity of Bt against Coleoptera, Diptera, Hemiptera,

Hymenoptera, Isoptera, Lepidoptera and Orthoptera (DE MAAGD; BRAVO; CRICKMORE, 2001; VAN FRANKENHUYZEN, 2009; BRAVO et al., 2011; WU et al., 2011). Toxicity has also been reported for some species of nematodes, protozoa, mites and *Leishmania major*, the parasite that causes leishmaniasis (EDWARDS; PAYNE; SOARES, 1988; FEITELSON, 1994; GUTIÉRREZ; GONÇALVES, 2006; SILVEIRA et al., 2007; AMANCHI; HUSSAIN, 2008; EL-SADAWY et al., 2008).

Bacillus thuringiensis is a bacterium of the family Bacillaceae, gram and catalase positive, aerobic, and can also grow in anaerobiosis between 10 and 45 °C (GLARE; O´CALLAGHAM, 2000); chemoheterotrophic, whose ideal growth temperature is around 30 ± 2 °C (MORAES; CAPALBO, 1986). Its vegetative cells, as mentioned, are rod-shaped, measuring about 1.0 to 2.0 μm wide by 3.0 to 5.0 μm in length. Spores formation occurs between elliptical and cylindrical, in central position, with a sporangia not clearly extended (HABIB; ANDRADE, 1998; GLARE; O´CALLAGHAM, 2000). Bt can be found in different regions of the world and in several substrates such as soil, water, dead insects and surface of some plants (HOFTE; WHITELEY, 1989).

This bacterium develops under aerobic conditions in artificial media quite easily. Under certain constraints, such as nutrient limitation or the accumulation of undesirable metabolites, Bt begins the sporulation process and synthesizes the protein toxin containing crystal (YAMAMOTO; DEAN, 2000; JURAT-FUENTES; JACKSON, 2012). The crystal may be responsible for over 25% of the dry weight of the cell. The amount of toxin produced in the laboratory (approximately 0.5 mg protein.mL^{-1} of culture medium) and the size of the crystal indicates that each cell has to synthesize 2 x 10^{-6} δ-endotoxin molecules to form a crystal (AGAISSE; LERECLUS, 1995). These toxins are encoded by *cry* genes and different *cry* genes in a strain can direct the synthesis of related proteins that are stored in either a single or multiple crystals of distinct shape (JURAT-FUENTES; JACKSON, 2012).

The life cycle of this entomopathogen can be divided into two main phases, one of vegetative growth, in which the bacterium multiplies by bipartition, and another one of sporulation, which consists in the differentiation of the bacterium into spores. When the spore is in an environment favorable to its growth, with adequate nutrients and

temperature, it can germinate and initiate the vegetative growth (MONNERAT; PRAÇA, 2006).

This bacterium differs from the others because it produces during the sporulation process a protein inclusion called crystal. These crystals, Cry proteins, have a toxic effect and confer entomopathogenic activity against more than 300 species of insects and mites (GLARE; O'CALLAGHAM, 2000; MONNERAT; BRAVO, 2000; VAN FRANKENYUZEN, 2009; VAN FRANKENYUZEN, 2013).

Bt-based insecticides have a long history of successful use in agriculture and forestry biological control of insect pests, as well as vectors of diseases (CAB, 2010; BRAVO et al., 2011; SANCHIS, 2011; JURAT-FUENTES; JACKSON, 2012; VACHON; LAPRADE; SCHWARTZ, 2012).

More than 300 Bt-based biopesticides (70% of them with *Bt* var. *kurstaki*) account for 53% of the world market for bio-insecticides, generating around US$ 120-140,000,000 per year. The Americas account for almost 50% of this consumption, particularly the US and Canada, with Latin America accounting for only 8-10% of total consumption (CAB, 2010; POLANCZYK; DE BORTOLI; DE BORTOLI, 2012). In addition, several transgenic plants, which express Bt toxins, are grown in large areas (JAMES, 2016; JURAT-FUENTES; JACKSON, 2012).

Bt strains are isolated from soil samples, rivers, surface plants, plant remains, dead insects and small mammals, cobwebs, stored grains and uninhabited places (BRAVO et al., 1998; FORSYTH; LOGAN, 2000; SWIECICKA; FIEDORUK; BEDNARZ, 2002; BIZZARRI; BISHOP, 2007; KONECKA; KAZNOWSKI; ZIEMNICKA, 2007; THAMMASITTIRONG; ATTATHOM, 2008). Several and extensive Bt screening projects have reported that the most prolific environments for isolating strains is the dust and materials associated with grain storage (BERNHARD; JARRET; MEADOWS, 1997).

This bacterium develops easily in artificial media under aerobic conditions. With the limitation of nutrients or the accumulation of undesirable metabolites, Bt enters the sporulation process and synthesizes the protein(s) containing the crystal (; YAMAMOTO; DEAN, 2000; JURAT-FUENTES; JACKSON, 2012).

Due to the high activity of Bt toxins against a wide range of agricultural pests, extensive prospecting programs were conducted by several research groups to identify Bt strains with new/broad spectrum insecticides. Numerous publications have reported the identification of crystal-forming proteins and the cloning and sequencing of crystalline protein genes (GLARE; O'CALLAGHAM, 2000). A problem related to the lack of a uniform nomenclature for these genes arose, causing the initial literature to become quite confusing. Hofte and Whiteley (1989) presented a Cry classification based on amino acid sequences and action spectrum of toxins. In this classification Cry I toxins showed activity against insects of the order Lepidoptera, Cry II against Lepidoptera and Diptera, Cry III against Coleoptera and Cry IV against Diptera, being the Cyt class defined and separated, since it did not show homology with the Cry toxins and whose members demonstrated an overall "in vivo" cytolytic activity. After some time it was realized that this classification was not adequate, since similar toxins had different specificities, and new toxins with double activity against larvae of Lepidoptera and Coleoptera were discovered and called Cry V, creating, therefore, great confusion in the nomenclature (TAYLOR; TIPPET; GIBB, 1992). Because of this, in 1994, an International Committee was created and proposed a classification based solely on amino acid sequences. In the new classification, Roman numerals were replaced by Arabic numerals and four levels were defined (CRICKMORE et al., 1998). The constant updating of this data can be viewed on the Internet at: http://www.lifesci.sussex.ac.uk/home/NeilCrickmore/Bt/. Close to 950 different toxin genes have been cloned and classified in 74 groups of Cry, 3 groups of Cyt, and 3 groups of Vip proteins (CRICKMORE et al. 2016).

2.2.2. The toxins and its insecticidal activity

The entomopathogenic activity of Bt is mainly related to the production of crystalline inclusions called δ-endotoxins or Cry proteins in the stationary phase, which

are accumulated in the mother cell during sporulation (AGAISSE; LERECLUS, 1995; YAMAMOTO; DEAN, 2000). These crystalline inclusions may contain one or more Cry proteins and each toxin is encoded by a specific cry gene (LI et al., 1991).

In addition to the Cry proteins, Bt also produces another toxin with insecticidal activity, which can increase the toxicity of δ-endotoxins, such as α-exotoxin, β-exotoxin, hemolysins, exoenzymes and vegetative insecticidal proteins (VIPs) (HABIB; ANDRADE, 1998). Spores may also contribute to the virulence of the bacterium performing synergistic action along with the Cry proteins (JOHNSON; MCGAUGHEY, 1996).

Under unfavorable nutritional conditions, Bt disrupts cell division and initiates the sporulation process. During the sporulation phase occurs the production of δ-endotoxins or Cry proteins. δ-endotoxins accumulate in the mother cell compartment and, at the end of sporulation, a crystal is released along with the spore. There are two types of δ-endotoxins: Cry proteins and Cyt proteins. The action spectrum of δ-endotoxins is usually restricted to certain orders of insects, such as Lepidoptera, Coleoptera, Hymenoptera, Diptera or nematodes and mites (HABIB; ANDRADE, 1998; SCHNEPF et al., 1998).

B. thuringiensis can produce one or more Cry proteins with molecular mass ranging from 40 to 140 kDa (SERAFINI; BARROS; AZEVEDO, 2002). The shape of the crystal is determined by the composition and structure of the δ-endotoxins present, and can vary between bipyramidal, cuboidal, ovoid, rhomboid and spherical, or even having no definite shape (HABIB; ANDRADE, 1998; POLANCZYK; ALVES, 2003).

Cry proteins are classified into 70 groups and different subgroups, depending on the degree of similarity of their amino acids. These proteins are encoded by more than 665 already sequenced *cry* genes (CRICKMORE, 2013).

Cyt proteins show no homology with Cry proteins and have cytolytic activity, presenting affinity for unsaturated fatty acids in the lipid portion of the cell membrane (THOMAS; ELLAR, 1983). The Cyt proteins are constituted by the Cyt1 and Cyt2 groups; the Cyt1 are: Cyt1Aa, Cyt1Ab and Cyt1Ba (THIERY et al., 1997), and the Cyt2: Cyt2Aa, Cyt2Ba, Cyt2Bb, Cyt2Bc e Cyt2Ca. They have molecular mass of 27-30 kDa

and toxicity to insects of the order Diptera. In addition, the Cyt2Ca protein also exhibits activity against Coleoptera (CRICKMORE et al., 1998).

2.2.3. Mode of action

The mode of action of Cry proteins involves several steps, such as: ingestion of the spore-crystal complex by the susceptible larvae, solubilization and processing of the toxin, receptor binding, membrane insertion, pore formation and cytolysis. Cry proteins are in the form of pro-toxins and need to be activated by proteases to release toxic fragments (SCHNEPF et al.,1998; MONNERAT; BRAVO, 2000).

In order for the protoxin to be solubilized, it must come into contact with the alkaline pH of the midgut of the target insect larvae (KNOWLES, 1994). Differences in solubilization may contribute to determining changes in the degree of toxicity between Cry proteins (ARONSON et al., 1991). After the solubilization, the pro-toxins are processed by special proteases present in the insect's midgut, in order to release the toxic fragment (TOJO; AIZAWA, 1983). Proteolytic cleavage is an important factor that may contribute to determining specificity; the main type of digestive protease in insects of the orders Lepidoptera and Diptera is the serine protease type, whereas for Coleoptera they are mainly cysteine and aspartic proteases (DE MAAGD; BRAVO; CRICKMORE, 2001).

Upon activation, Cry proteins pass through the peritrophic membrane of the midgut, a protective coating, and reach the target site and bind to specific receptors present on the microvilli of the midgut columnar cells of susceptible insect larvae (ZHANG et al., 2012). Cry toxins interact with specific receptors, being an important factor for toxicity and specificity, determining the action spectrum of δ-endotoxins (MONNERAT; BRAVO, 2000; DE MAAGD; BRAVO; CRICKMORE, 2001; BRAVO; GILL; SOBERÓN, 2007). Then the formation of pores occur leading to cell lysis and extravasation of the intestinal contents into the hemocele (COPPING; MENN, 2000;

PRAÇA et al., 2004). Alternatively, the binding of Cry toxins to the cadherin protein in the midgut could activate a cell signaling pathway, leading to cell death (ZHANG et al., 2005; ZHANG et al., 2006).

The symptoms of intoxication in insects are: anorexia, intestinal paralysis, vomiting, diarrhea, total paralysis and subsequent septicemia, leading the insect to death (GUPTA et al., 1985; BRAVO; JANSENS; PEFEROEN, 1992; MONNERAT; BRAVO, 2000).

Although many insects are susceptible to Cry toxins, Bt's mode of action is not yet well defined. After ingestion of spores/crystals by the insect, the crystals are dissolved and then to a greater or lesser degree, they are cleaved by digestive proteases and transformed into active toxins. These toxins pass through the peritrophic membrane, binding to specific receptors located on the apical membrane of the midgut columnar cells, forming pores in the membrane. The formation of these pores results in the interference of cell physiology by suppression of the trans-membronic ionic gradients, which can lead to colloid-osmotic lysis of the cells due to a massive influx of solutes into the lumen of the midgut. In turn, the destruction of the cells results in great damage to the epithelial tissue of the midgut and death of the intoxicated larvae. Insects can also suffer from starvation, since, shortly after ingestion of the toxin, the insect stops feeding itself (COPPING; MENN, 2000; CRICKMORE, 2006; JURAT-FUENTES; JACKSON, 2012; VACHON; LAPRADE; SCHWARTZ, 2012).

In recent years, an elaborate model involving the sequential attachment of toxins to different membrane receptors has been proposed to describe the events leading to membrane insertion and pore formation. However, it has also been proposed that, in contradistinction to this mechanism, Bt toxins function through the activation of intracellular signaling pathways leading to the necrotic death of their target cells, without the need for pore formation (BRAVO; GILL; SOBERÓN, 2007). Vachon, Laprade and Schwartz (2012) pointed out that the available information still supports the idea that Bt toxins act through pore formation, but most of the events that lead to its formation, after binding of the active toxins in the receptors, remain little known. The understanding of the mechanism of action of Bt toxins has been complicated recently by the discovery

that mutations in the gene encoding an ABCC2 transporter are responsible for resistance to Bt toxins in four different insect species (HECKEL, 2012; HERNÁNDEZ-MARTÍNEZ et al., 2012). The Bt Cry1Ac resistance locus (BtR-1) in the NO-QA strain of *P. xylostella* from Hawaii was originally identified using anonymous AFLP markers (HECKEL et al., 1999). A sequenced AFLP marker linked to BtR-1 contained coding sequence for the predicted gene Thyroid Hormone Receptor Interactor 12 (GenBank JN030496), which has an orthologue located on chromosome 15 of *B. mori*. Predicted proteins from *B. mori* chromosome 15 were compared using BLAST against a *P. xylostella* transcriptome (454-ESTs) to design specific primers for linkage mapping. Sixteen genes, including that encoding ABCC2, were mapped in most progeny in backcrosses to NO-QA (3 families, 184 bioassay survivors, 46 controls).

The superfamily of ABC proteins takes its name from binding with ATP, an intracellular domain that binds and hydrolyzes ATP in a cycle that carries the transport of molecules across a lipid bilayer membrane. The functional transporter consists of two cytosolic nucleotide binding domains (NBDs) that bind and hydrolyze ATP and two integral transmembrane domains (HECKEL, 2012). The biological function of the ABCC2 gene is unknown, but its resemblance to multidrug resistance proteins suggests that it could export small hydrophobic toxins from intestinal epithelial cells to eventual fecal elimination. Heckel (2012) speculates that the ABCC2 gene could function as one of the binding proteins in the sequential binding model discussed by Bravo; Gill and Soberón (2007), and further proposes that the binding of the toxin to the ABCC2 gene only occurs when the carrier is in its open configuration. When the carrier closes, the toxin is pushed into the membrane. This transient interaction between the toxin and the ABCC2 gene may explain why this gene has not yet been identified as a toxin binding protein.

Hernández-Martínez et al. (2012) suggested an alternative model in which the ABCC2 gene would be indirectly affecting the mechanism of action of Bt toxins. Mutations in the gene encoding ABBC2 affect its function, which in turn affects the physiology of the cell, so it interferes with the ability of the Bt toxin to form the pore. Both studies conclude that the functional role of ABCC2 protein has not yet been established,

but that mutations clearly have significant consequences on insect susceptibility to Bt. What is unknown is how the mutations on ABCC2 results in a particular changes in expression of genes. One possibility is that mutations in *abcc2* in some way simulates a pathogen attack and the cells express genes in response to counter attack (AYRA-PARDO et al., 2015).

As ABCC2 was correlated with Cry1Ac resistance in *Chloridea virescens* (F., 1777) (Lepidoptera: Noctuidae), the orthologue from *P. xylostella* was cloned and sequenced using a genomic BAC library constructed from a susceptible strain Geneva88 (BAXTER et al., 2011). The gene contains 26 exons, and these were verified through PCR amplification from Bt-susceptible (Geneva88) and Bt-resistant (NO-QAGE) midgut cDNA. The resistant strain NO-QAGE contained a 30-bp deletion in exon 20, which is predicted to remove the 12th and final transmembrane domain and aberrantly position the carboxyl-terminal outside the cell. If this gene is translated and inserted into the midgut membrane, a core ATP-binding loop is expected to be nonfunctional.

In two controversial studies, Broderick, Raffa and Handelsman (2006) and Broderick et al. (2009) suggested that Bt is incapable of killing *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Lymantriidae), *Manduca sexta* Linnaeus, 1763 (Lepidoptera: Sphingidae), *Pieris rapae* (Linnaeus, 1758) (Lepidoptera: Pieridae) and *Vanessa cardui* (Linnaeus, 1758) (Lepidoptera: Nymphalidae) in the absence of intestinal bacteria. Previous exposure of larvae to a combination of four antibiotics to eliminate gut bacteria severely reduced the toxicity of a commercial Bt preparation. Reinfection of larvae with an *Enterobacter* sp. rescued the toxicity of Dipel®. A subsequent study by Johnston and Crickmore (2009) showed that the loss of product activity was a result of the direct effect of the residual antibiotics on the larvae and living bacteria present in the Dipel product. Not only did they conclude that native gut bacteria were not necessary for Bt toxicity, but in fact, they showed that the presence of native intestinal bacteria helped protect the insect against Bt. In summarizing these data, Raymond et al. (2010) concluded that Bt is mainly a pathogenic agent for insects and that its main means of reproduction is insect cadaver, and that it does not require the help of other microorganisms to express its pathogenicity.

Laboratory studies with *Spodoptera exigua* (Huebner, 1808) (Lepidoptera: Noctuidae) identified a group of proteins, known as REPAT (REsponse to PAThogens) proteins were found to be up-regulated in response to not only Cry toxins, but also to Bt and other microbial pathogens (HERRERO et al., 2007). Further studies on *S. exigua* identified novel members of the *repat* gene family and showed higher expression of some members in a Bt-resistant colony in comparison with a susceptible colony (HERNÁNDEZ-MARTÍNEZ et al., 2010). Eight different *repat* genes had been described, all of them hypothetically encoding for relatively small proteins (less than 20 kDa) and their expression was restricted to the midgut cells (HERRERO et al., 2007; HERNÁNDEZ-RODRÍGUEZ; FERRÉ; HERRERO, 2009; HERNÁNDEZ-MARTÍNEZ et al., 2010; NAVARRO-CERRILLO et al., 2012). This family of genes was found exclusively in *Spodoptera* spp. and they lacked clear homologs in other insect species.

Navarro-Cerrillo et al. (2013) analysed the comprehensive larval transcriptome from *S. exigua* to verify the presence of novel repat-homolog sequences. These analyses revealed the presence of at least 46 *repat* genes in *S. exigua*, establishing a new gene superfamily in this species. These authors showed that phylogenetic analysis and studies of conserved motifs in these hypothetical proteins have allowed their classification in two main classes, αREPAT and βREPAT. Studies on the transcriptional response of *repat* genes have shown that αREPAT and βREPAT differ in their sequence but also in the pattern of regulation. The αREPAT were mainly regulated in response to the Cry1Ca toxin from Bt but not to the increase in the midgut microbiota load. In contrast, βREPAT were neither responding to Cry1Ca toxin nor to midgut microbiota. This high diversity found in their sequence and in their expression profile suggests that REPAT proteins may be involved in multiple processes that could be of relevance for the understanding of the insect gut physiology.

Genomic and proteomic approaches have begun to unravel how invertebrate defenses respond to Bt toxins on a biochemical and cellular level: a global functional analysis in the nematode *Caenorhabditis elegans* (Moupas, 1900) (Rhabditida: Rhabditidae) highlighted the importance of two MAPK pathways, p38 and JNK (KAO et al., 2011). The p38 pathway was also implicated in the protection of the lepidopteran

Manduca sexta (Linnaeus, 1763) (Lepidoptera: Sphingidae) and the dipteran *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae) against Cry toxins (CANCINO-RODEZNO et al., 2010). In general, exposure to Bt results in the altered expression of many genes including the *repat* superfamily that are induced by a variety of pathogens (NAVARRO-CERILLO et al., 2013). Various different functions have been subscribed to the insect genes that are differentially expressed upon exposure to Bt including immune defense (VAN MUNSTER et al., 2007; GUO et al., 2011; PASCUAL et al., 2012; YAO et al., 2014,); toxin activation or binding (CANDAS et al., 2003; VAN MUNSTER et al., 2007; LEI et al., 2014; YAO et al., 2014); oxidative or general metabolism (CANDAS et al., 2003; MEUNIER et al., 2006; GUO et al., 2011) and stress response (MEUNIER et al., 2006; VAN MUNSTER et al., 2007).

Several studies have also used genomic/proteomic methods to compare Bt resistant insects with their susceptible counterparts to look for differences that could account for the resistant phenotype. Candas et al. (2003) identified differences in proteinase levels, which could affect toxin activation, as well as an increased oxidative metabolism in a resistant strain of Indian Meal Moth {*Plodia interpunctella* (Hübner, 1813) (Lepidoptera: Pyralidae)}. Guo et al. (2011) compared resistant and susceptible strains of the sugarcane borer {(*Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae)} and found differences in expression of nearly 400, mainly metabolic-related genes. Lei et al. (2014) used RNA-sequencing to compare susceptible and resistant strains of *P. xylostella* and found that the majority of this insect's genes were differentially expressed between the two strains.

Hernández-Martínez et al. (2010) compared Bt resistant and susceptible strains of *S. exigua* and found that about a fifth of the genes tested were differentially expressed. Interestingly they also found that some of the genes that were induced in the susceptible insect upon exposure to Bt were constitutively overexpressed in the resistant strain. More recently a mutation in *abcc2* was also linked to resistance in this strain and RNAi silencing of *abcc2* in a susceptible strain of *S. exigua* not only decreased its susceptibility to Cry toxins but also resulted in the up-regulation of a

number of genes previously found to be induced in response to intoxication and constitutively expressed in the resistant strain (PARK et al., 2014).

Despite the large number of genes that are differentially expressed in insects exposed to Bt, or that have developed resistance to Bt, attempts have been made to validate the importance of individual genes. The role of one of the *repat* genes was tested by producing recombinant protein in a baculovirus expression system. This protein (REPAT1) was found to protect *S. exigua* from pathogen attack (HERRERO et al., 2007). RNAi is a technique that has proved successful in insects and has been used to investigate the importance of several differentially expressed genes. Yang et al. (2010) used RNAi to demonstrate that expression suppression of a putative toxin receptor (APN), that was down-regulated in a resistant strain of *D. saccharalis*, reduced the effectiveness of the Bt Cry1Ab toxin towards the susceptible strain. As well as for putative receptors RNAi has been used to show that expression suppression of the following differentially expressed genes affected susceptibility to Bt: a trypsin like protease from *S. frugiperda* (RODRÍGUEZ-CABRERA et al., 2010); Apolipoprotein-III from *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) (CONTRERAS, RAUSELL; REAL, 2013); HSP90, ATP synthase subunit B and actin from *A. aegypti* (CANCINO-RODEZNO et al., 2012). More recently it has been shown that in a resistant strain of *P. xylostella* known as Karak many genes are constitutively up regulated (AYRA-PARDO, et al., 2015). RNAi suppression of some of these genes, including a putative ER co-chaperone PxSDF2L1, resulted in an increased susceptibility to Bt. These unpublished data match those found in *S. exigua* where a single mutation (possibly in *abcc2* in both insects) resulted in the constitutive up regulation of many genes, silencing of which altered the susceptibility to Bt. It is unlikely that all of the up regulated genes are directly involved in Bt toxicity and so a likely scenario is that they form part of a complex stress-response regulon. Some aspect of that stress response (e.g. the shedding / modification / down regulation of the toxin receptor) could directly affect susceptibility to Bt, whilst suppression of other components (e.g. through RNAi) could also influence susceptibility indirectly through a feedback mechanism.

2.2.4. Resistance

Although Bt was used in leaf sprays for more than 30 years, commercialization in 1996 of the first genetically modified plants protected against insect (Bt plants) increased the importance of Bt as a source of insecticidal proteins for the control of pest insects (HERNÁNDEZ-MARTÍNEZ et al., 2012). The excessive use of Bt proteins in sprays or Bt plants led to the evolution of insect resistance in the field (FERRÉ; VAN RIE, 2002; TABASHNIK; VAN RENSBURG; CARRIÈRE, 2009). Field resistance to Bt was first described in populations of *P. xylostella* (KIRSCH; SCHMUTTERER, 1988; TABASHNIK et al., 1990; FERRÉ et al., 1991; FERRÉ; VAN RIE, 2002). One of the first resistant colonies was derived from a Hawaiian population that had been selected for resistance to Dipel® in the field and that was additionally selected in the laboratory for resistance to the same product (TABASHNIK; FINSON; JOHNSON, 1991). Insects from this population showed "mode 1" resistance, which implies strong resistance to at least one Cry1A protein (in this case Cry1Aa, Cry1Ab, Cry1Ac, all three found in Dipel®), little or no cross-resistance to Cry1C, recessive inheritance and reduced binding to at least one Cry1A protein (TABASHNIK et al., 1998).

Tabashnik and Carrière (2017) analyzed global monitoring data reported during the first two decades of transgenic crops. The cases of pest resistance to Bt crystalline (Cry) proteins produced by transgenic crops increased from 3 in 2005 to 16 in 2016. By contrast, in 17 other cases there was no decrease in pest susceptibility to Bt crops, including the recently introduced transgenic corn that produces a Bt vegetative insecticidal protein (Vip). Recessive inheritance of pest resistance has favored sustained susceptibility, but even when inheritance is not recessive, abundant refuges of non-Bt host plants have substantially delayed resistance.

The intensive use of chemicals selected resistant populations, and in these situations the farmer usually increases the frequency of applications and the dosages, in most cases, to no success. In general, a large number of applications are used per cycle of the crop, reaching 15 or 20, regardless of the presence of the pest (pre-established

applications) (GUAN-SOON, 1990; CARBALLO, 1992; SAMPSON, 1992). In Brazil, it is observed that the number of applications can vary from one to four per week, and in addition, the high biotic potential of this insect favors the manifestation of resistance, which makes it difficult to manage the pest (CASTELO BRANCO et al., 2001, THULER, 2006).

The resistance of this insect to DDT was observed in 1953 (ANKERSMIT, 1953) and to Bt in 1980 (TABASHNIK et al., 1990; SHELTON; ROBERTSON; TANG, 1993; TABASHNIK, 1994). In 1989, according to Georghiou and Lagunes-Tejada (1991), about 50 chemical insecticides were known to which populations of *P. xylostella* had resistance. In the 1990s, there were reports of insect-resistant moth populations of pyrethroid and phosphorous groups in the Federal District (CASTELO BRANCO, FRANCE, VILLAS BOAS, 1997). In addition, resistance to abamectin and deltamethrin has also been demonstrated (CASTELO BRANCO; MELO, 2002; PRAÇA et al., 2010).

Other characterizations showed that resistant insects showed cross resistance to Cry1F and Cry1J (TABASHNIK et al., 1994; TABASHNIK et al., 1996). Genetic studies indicated that resistance to Cry1A and Cry1F proteins was determined by a single autosomal "locus" (TABASHNIK et al., 1997a), and that in the offspring of that population the Cry1Ac resistance gene was genetically linked to a membrane transporter gene (ABCC2 gene) (BAXTER et al., 2011).

Any change in the complex pathway of the pathogenesis of Bt could potentially provoke resistance to Bt toxins in insects (ZHANG et al., 2012), being that resistance mechanisms can be diverse (GRIFFITTS; AROIAN, 2005; JURAT-FUENTES; ADANG, 2006; HECKEL et al., 2007), including alteration of Cry toxin solubilization and intestinal proteases, decreased peritrophic membrane permeability to Cry toxins, enhanced insect immune response, sequestering increase of toxins in the midgut by esterase production, and most importantly, reduction of toxin binding with the midgut membrane (SCHNEPF et al., 1998; TABASHNIK et al., 1998; OPPERT, 1999; HAYAKAWA et al., 2004; RAHMAN et al., 2004; GUNNING et al., 2005; MA et al., 2005). Among these resistance mechanisms, the most common is the reduction of toxin binding to midgut cells, which in different insects include mutations at Cry toxin receptors, such as cadherin (CAD),

aminopeptidase-N (APN), alkaline phosphatase (ALP) and mutations in ABCC2 transporter (PARDO-LÓPEZ; SOBERÓN; BRAVO, 2013).

Cadherin in the midgut of Lepidoptera was shown to be the functional receptor for the Cry1A toxin by demonstrating the acquired sensitivity of cells in culture after introduction and expression of a cadherin gene (NAGAMATSU et al., 1999; GOMEZ et al., 2001; DORSCH et al., 2002; HUA; JURAT-FUENTES; ADANG, 2004; FLANNAGAN et al., 2005; XIE et al., 2005; ZHANG et al., 2005). Results obtained by Zhang et al. (2012) indicated that there was no change in the amount of cadherin in *Trichoplusia ni* (Huebner, 1802) (Lepidoptera: Noctuidae) resistant to Cry1Ac, and in vitro binding by the overlap of the toxin under analysis was not different for susceptible individuals. Thus, the role of cadherin in the binding of Cry toxins in the midgut of insects is complex and still needs to be better understood.

For susceptible *P. xylostella* an integrated model was proposed for the binding sites of Cry1A and Cry1Fa proteins (BALLESTER et al., 1999). In this model, at least two binding sites are involved: one that is shared by Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa, and a second that is specific for Cry1Aa and apparently does not contribute to its toxicity. Another study with resistant larvae showed extremely reduced binding to Cry1Ab and Cry1Ac, but not to Cry1Aa (TABASHNIK et al., 1997b). It was therefore proposed that the resistance of individuals from this population was due to a mutation that altered the common binding site for Cry1Aa, Cry1Ab, Cry1Ac and Cry1F.

In another study, strong correlation was found between cross-resistance and loss of binding to Cry1Fa (HERNÁNDEZ-MARTÍNEZ et al., 2012), complementing the previous results for Cry1Aa, Cry1Ab and Cry1Ac. The lack of binding to Cry1Fa in resistant individuals supports the model proposed by Ballester et al. (1999), of common binding site for CryAb, Cry1Ac and Cry1Fa. The populations of *T. ni* and *P. xylostella* presented a different pattern of cross-resistance, although both resistance phenotypes showed a link to the same "locus" ABCC2 (HERNÁNDEZ-MARTÍNEZ et al., 2012). This may represent different mutations in the ABCC2 gene affecting susceptibility to toxins in different ways (for example, at different toxin binding sites), although the lack of current evidence indicates the functionality of the ABCC2 gene as a binding site for the toxins of

Bt. Alternatively, it is possible that mutations in this gene affect the susceptibility to toxins indirectly. Individuals of *T. ni* from a resistant colony had reduced expression of aminopeptidase N (APN1), which is a known receptor for Bt toxins (TIEWSIRI; WONG, 2011). Thus, while mutations in ABCC2 may confer insect resistance, the effect may be indirect and, as a consequence, the resistant phenotype may reflect differences in physiology between species (HERNÁNDEZ-MARTÍNEZ et al., 2012).

3. EXPERIMENTAL PROCEDURES

Bioassays took place in Dr. Neil Crickmore's laboratory, at the School of Life Sciences - University of Sussex, Falmer, Brighton, United Kingdom and in the Laboratory of Microbial Control of Arthropods Pest, at FCAV-Unesp, Jaboticabal, SP, Brazil.

3.1. Insect populations

Five *P. xylostella* populations were brought from Laboratory of Insect Rearing and Biology, UNESP, Jaboticabal, SP, Brazil to Dr. Neil Crickmore's laboratory to continue the rearing and follow through with the experiments. Also the rearing were continued in Laboratory of Microbial Control of Arthropods Pest. For the insects rearing, the methodology used was described by Barros and Vendramim (1999).

One Hawaiian population was also used for the bioassays; this population was provided by Dr. Ben Raymond from University of Exeter, Penryn Campus, Penryn, Cornwall, United Kingdom.

Description of the populations:

Population 1 (PC) - collection date: May 19, 2008 – collected in cabbage plants in the city of Alegre - ES (Brazil), in commercial area with history of insecticide application.

Population 2 (PA) - collection date: July 22, 2008 – collected in cabbage plants in the city of Alegre - ES, in commercial area with history of insecticide application.

Population 3 (Px) - collection date: January 15, 2007 – collected in cabbage plants in the city of Recife - PE (Brazil), in commercial area with history of insecticide application.

Population 4 (SBT) - collection date: July 5, 2010 – collected in cabbage plants in the city of Jaboticabal - SP, in an organic area with no history of insecticide application.

Population 5 (Bt) - collection date: May 19, 2008 – collected in cabbage plants in the city of Alegre - ES. It has been for 125 generations in the lab and selected each generation with Bt HD-1 (3 x 10^6 spores/mL).

Population 6 (NO-QA) - it is from Hawaii, collected on the year of 1989 (TABASHNIK et al., 1990). It's a highly resistant population. Selected with insecticidal Cry1Ac and has a known defect in ABCC2 (Baxter et al., 2011).

Organic cabbage was bought on a daily bases from Brighton's supermarket for the rearing in UK and for the rearing in Brazil, green houses were maintained to plant cabbages for the rearing.

3.2. Cry1Ac Preparation

The gene was cloned into the pGEM vector in *E. coli* JM109 cells. For protein expression, a loop-full of cells (from plate), were used to incubate 10 mL of LB medium containing the appropriate antibiotics at 37o C overnight. The cultures were transferred in 500 mL of 2x LB medium, containing the appropriate antibiotic and incubated at 37^o C on an orbital flatbed shaker (Gallenkamp), at 100 rev/min. Cells were harvested after 48 hours by 10 min centrifugation at 10,000 rpm at 4^o C.

The resulted pellet from the E. coli cultured cells resuspended in 30ml of 0.5M NaCl, 2% (v/v) Triton X-100 and was sonicated six 30-second periods (at 24 microns). After sonication, centrifugation followed at 13,000rpm for 15 min at 4^o C followed in a SS-34 rotor (Beckman). The pellet was washed three times with 30 ml of ice-cold buffer containing 2% Triton-X 100, 0.5M NaCl, then was washed five times with 30ml of ice cold 0.5M NaCl buffer, and two times with 100 ml of ice-cold distilled water. The final pellet was resuspended in 5ml of 50mM Tris-HCl, 50mM EDTA, 15% (w/v) sucrose, pH 8.0 buffer.

3.3. HD-1 Preparation

B. thuringiensis strain *B. thuringiensis* var. *kurstaki* HD-1 (Lepidoptera-specific) was provided by Dr. Manuel Victor Franco Lemos. The strain was obtained from *Bacillus* stock center USA. HD-1 was multiplied in Petri dishes with culture medium NA "Nutrient Agar" (meat extract 1.5 g/L, yeast extract 1.5 g/L, sodium chloride 5 g/L, bacterial peptone 5 g/L and agar 15 g/L) and incubated at 28°C during 5 days for development and sporulation of the bacteria. After this period, the bacterial contents was transferred to Falcon tube containing 10 mL of autoclaved deionized water and 0.05% Tween 20®. The suspension was homogenized and the mixture with spores, crystals and vegetative cells was subjected to three consecutive washes. The supernatant was discarded, in order to eliminate extracellular toxins, such as β-exotoxins, water was added, the pellet resupended and centrifuged at 3600 rpm for 20 minutes. After the last centrifugation, a new suspension was obtained and two serial dilutions was made for spore counting in NeuBauer's chamber (ALVES; MORAES, 1998) and standardization of the suspension used in the concentration desired.

3.4. Gut dissection

Approximately 40 guts from forth instar larvae from each population where dissected on a Petri dish containing RNAlater® (SIGMA-ALDRICH) with ice bellow. The guts where preserved in 1.5 ml Eppendorf tube with 1ml of RNAlater® (SIGMA-ALDRICH) and stored at -80^{o}C in the freezer for further experimentation.

3.5. RNA extraction

RNA was extracted from approximately 40 *P. xylostella* dissected guts using RNeasy® Plus Mini Kit (QIAGEN). The guts were put in a glass mortar (1ml tissue grinder - WHEATON® USA) with 350 µl RLT Plus (RLT plus 3.5 µl of β-mercaptoethanol). Using a glass pestle, they were ground up by moving the pestle up and down. Then the homogenized lysate was transferred to a QIAshredder (QIAGEN) spin column placed in a 2 ml collection tube by pipetting. Then the spin column was centrifuged for 2 minutes at high velocity. The column was discarded. Added 350 µl of 70% ethanol to the flow-through, and mixed well by pipetting. Up to 700 µl of the sample, including any precipitate was transferred to an RNeasy spin column placed in a 2 ml collection tube. Then centrifuged for 15 s at ≥8000 x *g*. The flow-through was discarded. Then 700 µl of Buffer RW1 was added to the RNeasy spin column. Centrifuged for 15 s at ≥8000 x *g*. The flow-through was discarded. Added 500 µl of Buffer RPE to the RNeasy spin column. Then centrifuged for 15 s at ≥8000 x *g*. The flow-through was discarded. Another 500 µl of Buffer RPE was added to the RNeasy spin column. Then centrifuged for 2 min at ≥8000 x *g*. The flow-through was discarded. The RNeasy spin column was then placed in a new 2 ml collection tube and centrifuged at full speed for 1 min. Afterwards the RNeasy spin column was then placed in a new 1.5 ml collection tube. 40 µl of RNase-free water was added directly to the spin column membrane and centrifuged for 1min at ≥8000 x *g* to elute the DNA. The RNA was stored at -80^{o}C in the freezer for further experimentation.

3.6. RNA agarose gel

Agarose (0.5 gr.) was weighted and placed in a sterile glass bottle and then 36 ml of distilled deionized water here added. The bottle was then heated in the microwave,

mixing occasionally until the agarose were completely diluted. Meanwhile, a water bath was heated up to 70^{o}C.

In another glass bottle, 5 ml of 10x MOPS (3-(*N*-Morpholino)-propanesulfonic acid) buffer were added and in a third glass bottle 2.75 ml of 36.5-38% formaldehyde solution were added.

The three bottles were put in water bath at 70^{o}C for a few minutes, after they heat up; the three contents were mixed in one bottle and added 2 µl of GelRed^{TM} nucleic acid strain, it was mixed gently and poured in to the mold with the comb, then was left to set.

While the gel was setting, the samples were prepared this way: in an Eppendorf tube were added 4 µl of distilled deionized water, 2 µl of 36.5-38% formaldehyde solution, 2 µl 10x MOPS (3-(*N*-Morpholino)-propanesulfonic acid), 9 µl of Formamide, 2 µl RNA template, 5 µl RNA loading buffer (2 µl of 0.5M EDTA, 500 µl of glycerol 50%, 498 µl of distilled deionized water and 0.4% of Bromophenol Blue). The samples were then heated for 10 minutes at 70^{o}C in a Block Thermostat, after they were placed on ice for 1 minute. 15 µl of the sample were loaded on the gel. The electrophoresis was performed in 60V/400mA during 60 min, running buffer was 1x MOPS (3-(*N*-Morpholino)-propanesulfonic acid). The results were visualized and analyzed through an ultraviolet transilluminator Gel Doc^{TM} EZ Imager (BIO-RAD®) coupled to a software image analysis (Image Lab^{TM} Software – version 4.0).

3.7. RNA quantification

Absorbance measurements were made on Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo Scientific). The ratio of absorbance at 260 nm and 280 nm was used to access the purity of RNA. A ratio of ~2.0 is generally accepted as “pure” for RNA. First the pedestal was wiped clean with a tissue, then 1 µL of RNase free water was pipetted to de pedestal and the program was blanked. After that, the pedestal was

wiped clean again and 1 µL sample was loaded and measured. The pedestal was wiped clean with a tissue between every sample.

3.8. cDNA Synthesis – Prime Script™ 1st strand cDNA Synthesis (TAKARA BIO INC)

The first mixture was prepared in individuals 0.6 ml Thin Wall Flat Cap PCR Tube with the following reagents and volume: Oligo dT Primer (50 µM) - 1 µl; dNTP Mixture (10 mM) - 1 µl; Template RNA (1µg) - 1 µl; RNase free deionized water - 7 µl.

The mixture was incubated for 5 min at 65^{o}C, the immediately cooled on ice. After that, a second mixture was prepared with the following reagents and volumes: Template RNA Primer Mixture (from step above) - 10 µl; 5X PrimeScript Buffer - 4 µl; Rnase Inhibitor (40 U/µl) - 0.5 µl; PrimeScript RTase (200 U/µ) - 1.0 µl; RNase free deionized water - 4.5 µl.

The second reaction mixture was mixed gently and incubated for 10 min at 30^{o}C, for 45 min at 42^{o}C and for 5 min at 95^{o}C to inactivate the enzyme, then cooled on ice and stored at -20^{o}C in the freezer for further experimentation.

3.9. Polymerase chain reaction (PCR)

The reaction was performed using the PfuUltra II Hotstart 2x Master Mix - Agilent Technologies. The components and volumes used were: cDNA - 1.0 µl; Forward Primer 10 pmol/µ - 1.0 µl; Reverse Primer 10 pmol/µ - 1.0 µl; Master Mix - 25.0 µl; Nuclease-free water - 22.0 µl.

The Primers along with their specifications used in the PCR for this study are listed below (Table 1).

Table 1. List of Primers along with their specifications used in the PCR

Primers	Sequence (5'->3')	Length (pb)	Product size (pb)
MAP4K4 F	CATCAACTGGCTCCGTCTG	19	188
MAP4K4 R	TCATCTTCGGTGACATCCATC	21	
ABCC2 F	AGTCTTGGCACGCAAACGG	19	103
ABCC2 R	CGAACAGACGCATGAAGGACAT	22	
ALP F	GCACACACCATGACCGTAGCAG	22	169
ALP R	GGCTCTTCGTGACATCG	17	
CDKAL1 F	GTCGGACACAACGAGTTCTACG	22	148
CDKAL1 R	CAGTTAAACCGGGCATCTTTGGC	23	
L32 F	CCAATTTACCGCCCTACC	18	120
L32 R	TACCCTGTTGTCAATACCTCT	21	
PxABCC2 Full length F	CCATTGTATGGGTATTAGGTG	21	4119
PxABCC2 Full length R	CTAAGSTACAAGCTATCATTGAG	23	

The mixture was prepared in 0.6 ml Thin Wall Flat Cap PCR individual's tube, briefly centrifuged to spin down the contents and eliminate any air bubbles. The thermal sequence and amplification timing were: an initial denaturation at 94 °C for 2 min followed by 35 cycles of: denaturation at 95°C for 45 sec.; annealing at 50°C for 45 sec. and elongation at 72°C for 30 sec., followed by final extension at 72°C for 3 min. For the ABCC2 full length gene the thermal sequence and amplification timing were: an initial denaturation at 95°C for 2 min followed by 30 cycles of: denaturation at 95°C for 1 min.; annealing at 59 °C for 20 sec. and elongation at 72 °C for 2 min., followed by final extension at 72 °C for 3 min.

3.10. Agarose gel

Agarose (0.6 g for 2% agarose gel or 0.3 g 1% agarose gel) was weighted and placed in a sterile glass bottle and then 30 ml of 1 x TBE distilled. The bottle was then heated in the microwave, mixing occasionally until the agarose were completely dissolved. After, the mixture was left to cool down for about 15 minutes. When it had already cooled down, 1.5 µl of GelRed™ nucleic acid was added then it was mixed gently and poured in to the mold with the comb, then left to dry.

3.11. DNA electrophoresis on agarose gel

The amplified products were submitted to horizontal electrophoresis on 1 or 2% agarose gels stained with GelRed™ Nucleic Acid strain (Biotium®) (1.5 µl/30ml) in running buffer TBE 1X pH 8.0 (89 mM Tris; 89 mM Boric Acid; 2 mM EDTA). 5 µl of the amplified product plus 2 µl of DNA loading buffer were load up in the gel wells. The electrophoresis was performed at 120V/400mA for 50 min. For the determination of the amplified products, we used a molecular weight marker (100 pb DNA Ladder, BioLabs®). The results were visualized and analyzed through an ultraviolet transilluminator Gel Doc™ EZ Imager (BIO-RAD®) coupled to a software image analysis (Image Lab™ Software – version 4.0).

3.12. PCR purification

The PCR products were purified using the QIAprep® Spin Miniprep Kit. 250 µl of PB buffer was added to 50 µl of the PCR products, the mixture were transferred to a

spin column in a collection tube, centrifuged and the flow-through discarded. 750 µl of PE buffer was added to the spin column, centrifuged and the flow-through discarded. The spin column was centrifuged again to dry, the collection tube with the flow-through was discarded and the spin column placed in a new Eppendorf tube. 30 µl of buffer EB was added in the middle of the cotton ring; it was left to set for 1 min. and centrifuged for 1 min. The Eppendorf tube containing the purified PCR product was stored at -20^{o}C for sequencing.

3.13. PCR purification from agarose gel band

After the PCR reactions, the band relative to the amplicons were cut from the agarose gel with a scalpel blade, and placed in Eppendorfs® tubes properly identified. Further, the extraction of the PCR products from the agarose gel was performed using the QIAquick® Gel Extraction Kit (250) (Qiagen®)

QG buffer (600 µl) were added to the Eppendorfs® and placed in a Block Thermostat to heat at 50-55^{o} C, mixed occasionally until the band had completely dissolved. The Eppendorfs® were centrifuged for 5 sec. and with pipettes all the contents from the Eppendorfs® were transferred to a spin column in a collection tube provided on the Kit. After transferred, the mixtures were centrifuged for 30 sec. and the flow-through discarded. The membrane was then washed with 500 µl of QG buffer, centrifuged for 30 sec. and the flow-through discarded. 750 µl of PE Buffer was added, centrifuged for 30 sec. and the flow-through discarded. Another centrifuged for 30 seconds was performed to dry the membrane. The spin column was transferred to a new Eppendorf tube, 30 µl of EB buffer was added to the middle of the cotton ring in the membrane, incubated for 1 min. at room temperature and centrifuged for 1 min. The Eppendorf tube containing the purified PCR product was stored at -20^{o}C for further sequencing.

3.14. Sequencing

Samples from all 6 populations were sent for sequencing to Eurofins MWG Operon- Europe and tested against the primers listed on Table 2.

Table 2. List of Primers along with their specifications used for sequencing

Primers	Sequence (5'->3')	Length (pb)
PxABCC2 Full length F	CCATTGTATGGGTATTAGGTG	21
PxABCC2 Full length R	CTAAGSTACAAGCTATCATTGAG	23
PxABCC2N1F	GCCTTCMTGTTCCTGCACTACTTC	24
PxABCC2N1R	CGCAGCGTGGCCGAGAACAGAACA	24
PxABCC2N2F	GGGCGGGCAGGCGGACAC	18
PxABCC2N2R	GATAATATAGACGCGAGGTAGACG	24
PxABCC2N3F	TGCCTGCAGCCCGACTTCAA	20
PxABCC2N3R	GCCCGTTTGCGTGCCGAGACTTG	23
PxABCC2N4F	CATCGTGGGCCTGTTCTCTGTCA	23
PxABCC2N4R	TGCTGGTCCTTGTCCCCCTCAC	22
PxABCC2N5F	CGGAGCTGGTGGAACGCAAATC	22
PxABCC2N5R	GGCAGCACGCGTCGCACACCTT	22
PxABCC2Cterm	CATGGACAAGGGTGAAGTGGTG	22
PxABCC2Nterm	GGTATGGCGTTCCCGAGCAG	20

3.15. Quantitative real-time PCR (qRT-PCR)

The reaction were performed using the Power SYBR® Green PCR Master mix (Applied Biosystems by Thermo Fisher Scientific). The components and volumes used were: cDNA - 1.0 µl; Forward Primer (200 nM) - 1.0 µl; Reverse Primer (200 nM) - 1.0 µl; Master Mix - 10.0 µl; Nuclease-free water - 7.0 µl.

The Primers along with their specifications used in the qPCR for this study are listed below on Table 3.

Table 3. List of Primers along with their specifications used in the qPCR

Primers	Sequence (5'->3')	Length (pb)	Product size (pb)
MAP4K4 F	CATCAACTGGCTCCGTCTG	19	188
MAP4K4 R	TCATCTTCGGTGACATCCATC	21	
ABCC2 F	AGTCTTGGCACGCAAACGG	19	103
ABCC2 R	CGAACAGACGCATGAAGGACAT	22	
ALP F	GCACACACCATGACCGTAGCAG	22	169
ALP R	GGCTCTTCGTGACATCG	17	
CDKAL1 F	GTCGGACACAACGAGTTCTACG	22	148
CDKAL1 R	CAGTTAAACCGGGCATCTTTGGC	23	
L32 F	CCAATTTACCGCCCTACC	18	120
L32 R	TACCCTGTTGTCAATACCTCT	21	
APN F	TCACTGAGTCCCATCCCA	19	157
APN R	TGCCAGACGGCACATTTT	18	

Negative control reactions included ddH_20 instead of cDNA template, which resulted in no amplified products. The mixtures were pipetted in each well of a 96-well

reaction plate, the plate was sealed, briefly centrifuged to spin down the contents and eliminate any air bubbles. The amplification reaction was performed on ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The qPCR program included Holding Stage at 95°C for 10 min., 40 cycling Stage at 95°C for 15 sec. and 60°C for 1 min., Melt Curve Stage at 95°C for 15 sec., 60°C for 1 min. and 95°C for 15 sec.. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method, expression levels were normalized to the ribosomal protein L32 gene (GenBank accession no. AB180441) and the expression ratio was estimated as described previously (HERRERO, 2007). Three technical replicates and three biological replicates were used for each treatment.

3.16. Lethal concentration (LC_{50}) bioassays with Cry1Ac toxin

For this bioassay, leaf discs of Chinese cabbage 4 cm diameter were immersed for 10 sec. in 30 ml Cry1Ac toxin suspensions (toxin, distilled deionized water, 50 µl/ml Triton-X 100®); the control treated only with water plus Triton-X 100®. After that, the leaves were left to dry at room temperature, the discs were individualized in 4.5 cm diameter Petri dishes, with moistened filter paper below. 20 sec. instar larvae of each population were placed on a leaf disc and assayed against different Cry1Ac toxin concentrations, each disc was considered 1 repetition, and 3 repetitions per treatment were observed, and repeated for each population. Mortality was recorded 3 days after treatment and 50% lethal concentration (LC_{50}) was estimated by Probit analyses (SAS INSTITUTE, 2002).

The concentrations of the Cry1Ac tested were: 0.001 µg/ml, 0.006 µg/ml, 0.025 µg/ml, 0.05 µg/ml, 0.1 µg/ml, 0.2 µg/ml, 0.5 µg/ml, 0.75 µg/ml; 1.0 µg/ml; 1.5 µg/ml, for populations PX, PC, SBT, PA; 0.05 µg/ml, 0.2 µg/ml, 0.75 µg/ml, 1.0 µg/ml, 1.5 µg/ml, 2.0 µg/ml, 2.5 µg/ml, 3.0 µg/ml, 3.5 µg/ml, 4.5 µg/ml, 5.5 µg/ml, for population Bt and 0.75 µg/ml, 1.0 µg/ml, 1.5 µg/ml, 2.5 µg/ml, 3.0 µg/ml, 3.5 µg/ml, 4.5 µg/ml, 5.5 µg/ml, and 7.5 µg/ml for population NO-QA.

The Petri dishes were wrapped with PVC cling film and kept in a test chamber with temperature 25 ± 1 °C, relative humidity of 70 ± 10% and photoperiod of 12L: 12D hour. After three days were carried out assessments and recorded the number of dead larvae in each treatment. Those larvae that did not move when touched with a fine bristles brush were considered dead.

3.17. Backcrossing the resistant population with the lab susceptible population

Bioassays were performed at the Laboratory of Microbial Control of Arthropods Pest (UNESP, Jaboticabal).

In order that meaningful comparisons could be made between the resistant and susceptible strains of *P. xylostella* near isogenic populations of the two needed to be established. This was achieved by repeatedly backcrossing the resistant with the susceptible lines and selecting for resistant offspring.

There is no meiotic recombination in lepidopteran females, so genes that are linked in females will remain linked in chromosomes handed down by the mother (WANG, 2011). Since we wanted to move the resistance allele to the susceptible background this is best done by mating resistant males with susceptible females. Therefore, Bt resistant males were crossed with susceptible females from PX population, and the offspring was reared on fresh kale leaves without toxin. Individuals from this F1 generation were randomly mated, and second-instar larvae of the F2 progeny were treated with a discriminating dose of HD-1 Bt strain, that is known to kill heterozygous (but not homozygous resistant) individuals, resulting in 40-70 % mortality. Bt HD-1 strain was diluted in sterile deionized distilled water containing 50 µl/ml Triton-X 100®. The surviving larvae were reared to pupation, and then males were backcrossed with females from the susceptible PX population. This process was repeated for eight generations. In every even generation (F2, F4, F6 and F8), after 3 days of the HD-1 spraying, survived larvae were collected and stored individually in Eppendorfs with 75%

ethanol solutions for further testing in Dr. Neil Crickmore's lab. A DNasy prep were carried out and then amplified the region around the mutation from genomic DNA. New primers where designed to avoid any intron/exon boundaries. These are:

BtMutF (21mer):

5'- GAG CTG GTG GAA CGC AAA TCC - 3'

BtMutR (18mer):

5'- CTT CCC GGA TCC CAC GGG - 3'

The F primer was then used for sequencing at Eurofins.

3.18. Complementation tests between Bt and NO-QAGE

The genetic basis of resistance in Brazilian Bt population is unknown whereas in NO-QAGE it has been mapped to the *abcc2* gene (BAXTER, 2011). To establish whether resistance in Bt population is also in *abcc2* reciprocal male and female, crosses was performed between the two strains. Offspring were then assayed against discriminatory doses of HD-1 and Cry1Ac toxin. Leaf discs of Kale 4 cm diameter were immersed for 10 sec. in 30 ml HD-1 and Cry1Ac suspensions (HD-1 or Cry1Ac, distilled deionized water, 50 µl/ml Triton-X 100®); the control treated only with water plus Triton-X 100®. After that, the leaves were left to dry at room temperature, the discs were individualized in 4.5 cm diameter Petri dishes, with moistened filter paper below. 20 sec. instar larvae of each population were placed on a leaf disc and assayed against different HD-1 and Cry1Ac concentrations, each disc was considered 1 repetition, and 3 repetitions per treatment were observed, and repeated for each population. Mortality was recorded 3 days after treatment and 50% lethal concentration (LC_{50}) was estimated by Probit analyses (SAS INSTITUTE, 2002).

The concentrations of the Cry1Ac tested were: 0.5 µg/ml, 0.75 µg/ml, 1.0 µg/ml, 1.5 µg/ml, 2.5 µg/ml, 3.5 µg/ml, 4.5 µg/ml, 5.5 µg/ml and 7.5 µg/ml. And of the HD-1 were: $1.2x10^5$ spores/ml, $1.6x10^5$ spores/ml, $2.4x10^5$ spores/ml, $1.2x10^6$ spores/ml,

$2.4x10^6$ spores/ml, $3x10^6$ spores/ml, $4x10^6$ spores/ml, $6x10^6$ spores/ml and $9x10^6$ spores/ml.

The Petri dishes were wrapped with PVC cling film and kept in a test chamber with temperature 25 ± 1 °C, relative humidity of 70 ± 10% and photoperiod of 12L: 12D hour. After three days were carried out assessments and recorded the number of dead larvae in each treatment. Those larvae that did not move when touched with a fine bristles brush were considered dead.

Concentration-mortality response was performed with Cry1Ac protein of Bt against second-instar *P. xylostella* larvae of crossing between NO-QA resistant male population X Bt Brazilian resistant female population and crossing between NO-QA resistant female population X Bt Brazilian resistant male population on the first, second, sixth, seventh and eighth generations.

Also concentration-mortality response was performed with HD1 protein of Bt against second-instar *P. xylostella* larvae of crossing between NO-QA resistant male population X Bt Brazilian resistant female population and crossing between NO-QA resistant female population X Bt Brazilian resistant male population on the first, second, sixth, and seventh generations.

3.19. Lethal concentration (LC_{50}) bioassays with HD-1

For this bioassay, leaf discs of Chinese cabbage 4 cm diameter were immersed for 10 seconds in 30 ml HD-1 suspensions (HD-1, distilled deionized water, 50 µl/ml Triton-X 100®); the control treated only with water plus Triton-X 100®. After that, the leaves were left to dry at room temperature, the discs were individualized in 4.5 cm diameter Petri dishes, with moistened filter paper below. Twenty second instar larvae of each population were placed on a leaf disc and assayed against different HD-1 concentrations, each disc was considered 1 replication, and 3 replications per treatment were observed, and repeated for each population. Mortality was recorded 3 days after

treatment and 50% lethal concentration (LC_{50}) was estimated by Probit analyses (SAS INSTITUTE, 2002).

The concentrations of the HD-1 tested were: $2.4x10^2$ spores/ml, $1.6x10^3$ spores/ml, $2.4x10^3$ spores/ml, $1.6x10^4$ spores/ml, $2.4x10^4$ spores/ml, $1.6x10^5$ spores/ml, $2.4x10^5$ spores/ml, $1.6x10^6$ spores/ml and $2.4x10^6$ spores/ml for the PX susceptible population and $1.2x10^5$ spores/ml, $1.6x10^5$ spores/ml, $2.4x10^5$ spores/ml, $1.2x10^6$ spores/ml, $2.4x10^6$ spores/ml, $3x10^6$ spores/ml, $4x10^6$ spores/ml, $6x10^6$ spores/ml and $9x10^6$ spores/ml for the Bt resistant population.

The Petri dishes were wrapped with PVC cling film and kept in a test chamber with temperature 25 ± 1 °C, relative humidity of 70 ± 10% and photoperiod of 12L: 12D hour. After three days were carried out assessments and recorded the number of dead larvae in each treatment. Those larvae that did not move when touched with a fine bristles brush were considered dead.

3.20. Statistical analyses

The mortality concentrations data were taken to regression Probit analysis (FINNEY, 1971) and obtained the median lethal concentration values (LC_{50}) using the SAS software (SAS INSTITUTE, 2002). Differences between the LC_{50} values were significant when 95% of confidence limit of one treatment did not overlap with the confidence limit of the other treatment.

4. RESULTS

4.1. Lethal concentration (LC_{50}) bioassays with Cry1Ac toxin

The relative toxicity of the Bt Cry1Ac protein presented different effect for the populations of *P. xylostella* used, being more effective for the populations PA, PC, PX and SBT, which is demonstrated by the lower values found for LC_{50}. The toxicity was significantly higher for populations Bt and NO-QA. The LC_{50} was 78.7 times higher for Bt population then the LC_{50} estimated for population PX and 94.4 higher for NO-QA (Table 4). Biologically, the resistance ratio must be greater than 200 times in relation to the susceptible population for a given population to be considered resistant. Another important point to be highlighted is that the mortality of the control was less than 5% until the fifth day of evaluation. Both resistant populations showed high levels of resistance to Cry1Ac (>50 fold).

Table 4. Probit analysis concentration-mortality response with Cry1Ac protein of *Bacillus thuringiensis* against second-instar *Plutella xylostella* larvae of different strains from Brazil and Hawaii.

Populations	n[a]	LC_{50} (95% C.I.) (µg/mL)	Slope ± SE	χ^2	DF	P-value	RR[b]
Bt	60	1.26 (0.94-1.75)	1.09 ± 0.20	2.35	4	0.6707	78.7
PA	60	0.022 (0.016-0.031)	0.98 ± 0.09	8.92	5	0.1124	1.37
PC	60	0.001 (0.0007-0.0028)	0.65 ± 0.06	5.97	4	0.2016	0.062
PX	60	0.016 (0.0028-0.0728)	0.67 ± 0.12	6.96	3	0.0731	
SBT	60	0.058 (0.0329-0.1090)	0.61 ± 0.09	3.96	3	0.2654	3.62
NO-QA	60	1.51 (1.25-1.78)	1.94 ± 0.21	3.95	5	0.5569	94.4

[a] n = number of insects per concentration;
[b] RR (resistance ratio) = LC_{50} test population/LC_{50} PX population.

4.2. Lethal concentration (LC_{50}) bioassays with HD-1

The LC_{50} estimated for the HD1 strain was 1054 fold higher for the resistant population than susceptible population. The relative toxicity of the Bt HD1 strain presented different biological activity for the populations of *P. xylostella* used, being more effective for the population Px, which is demonstrated by the lower values found for LC_{50}. The HD1 strain showed LC_{50} values of 9.8×10^5 spores/mL to resistant population and 0.93×10^3 to susceptible population (Table 5). Biologically, the resistance ratio must be greater than 10 times in relation to the susceptible population for a given population to be considered resistant, so this prove that our Bt population is indeed resistant.

Table 5. Probit analysis concentration-mortality response with HD1 of *Bacillus thuringiensis* against second-instar *Plutella xylostella* larvae from resistant and susceptible Brazilian populations.

Populations	n[a]	CL_{50} (95% C.I.) (spores/mL)	Slope ± SE	χ^2	DF	P-value	RR[b]
Bt	60	9.8×10^5 (4.8×10^5- 2.2×10^6)	0.44 ± 0.10	1.60	4	0.8093	1054
PX		0.93×10^3 (1.8×10^3 - 2.8×10^3)	0.35 ± 0.05	2.51	4	0.6428	

[a]n = number of insects;
[b]RR(resistance ratio) = CL_{50} resistance population/CL_{50} test population.

4.3. RNA extraction

To start with, we extracted RNA from different amount of guts, to establish how many guts would be enough to get sufficient amount of RNA for further experimentation. To see if the RNA extraction using the RNeasy® Plus Mini Kit (QIAGEN) did work and

how efficient it was, electrophoresis was performed on a RNA agarose gel (Figures 1, 2 and 3).

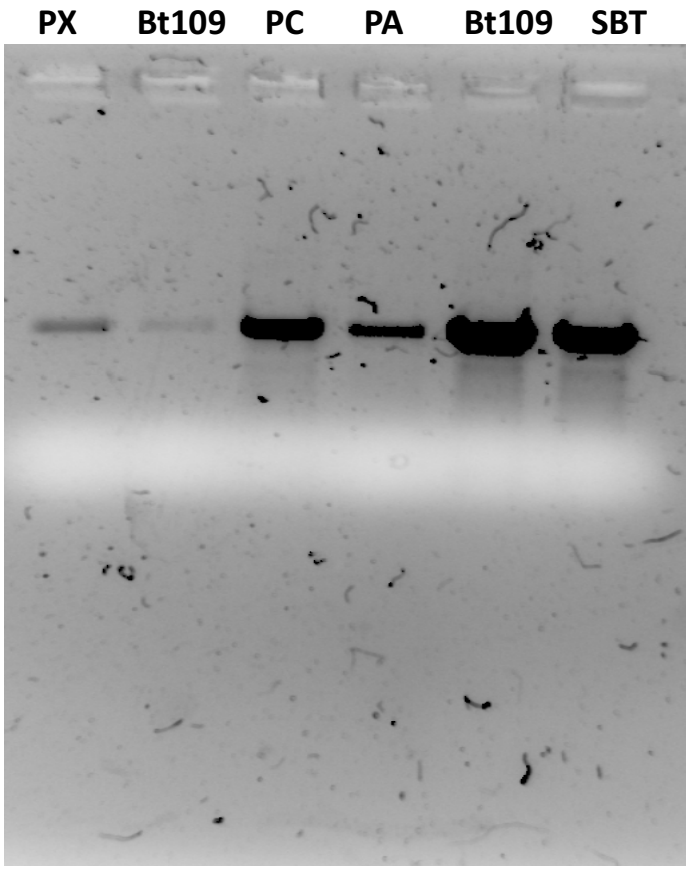

Figure 1: Gel red agarose electrophoresis. Lane 1: population PX, RNA from 2 guts; Lane 2: population Bt generation 109, RNA from 1 gut; Lane 3: population PC, RNA from 7 guts; Lane 4: population PA, RNA from 5 guts; Lane 5: population Bt generation 109, RNA from 13 gut; Lane 6: population SBT, RNA from 10 guts.

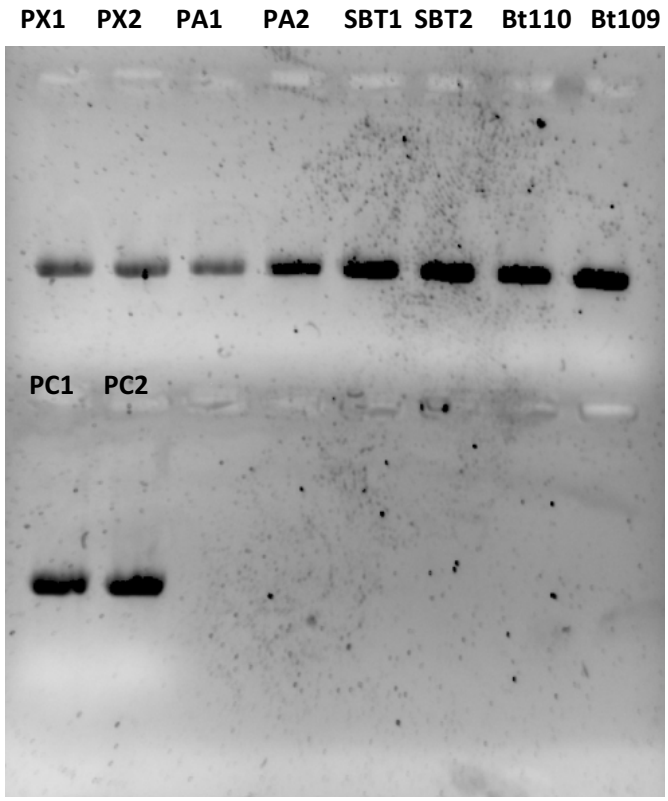

Figure 2: Gel red agarose electrophoresis. Lane 1: population PX sample 1; Lane 2: population PX sample 2; Lane 3: population PA, sample 1; Lane 4: population PA, sample 2; Lane 5: population SBT, sample 1; Lane 6: population SBT, sample 2; Lane 7: population Bt, generation 110; Lane 8: population Bt, generation 109; Lane 9: population PC, sample 1; Lane 10: population PC, sample 2. All of the RNA samples were extracted from 10 guts.

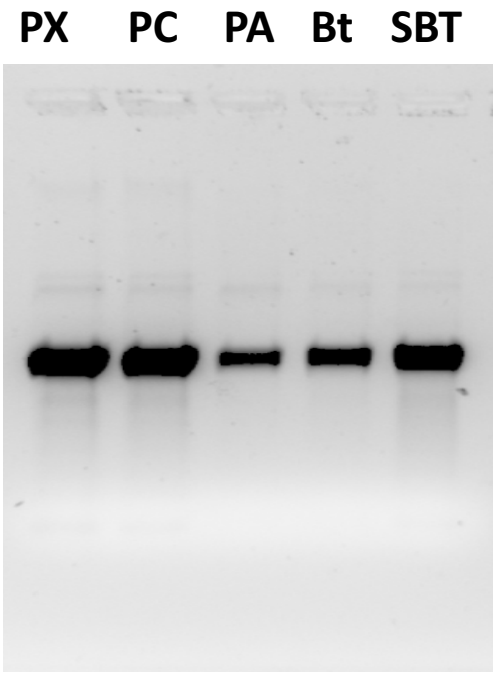

Figure 3: Gel red agarose electrophoresis. Lane 1: population PX; Lane 2: population PC; Lane 3: population PA; Lane 4: population Bt generation 111; Lane 5: population SBT. All of the RNA samples were extracted from 40 guts

RNA extracted from over 7 guts gave us sufficient amount, but we established that a good amount of RNA for further experimentation would be extracted from 40 guts.

4.4. RNA quantification

For a more accurate measurement of the RNA concentration and its purity, absorbance measurements were made on Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo Scientific). The ratio of absorbance at 260 nm and 280 nm is used to access the purity of RNA. A ratio of ~2.0 is generally accepted as “pure” for RNA. Total RNA concentration (ng/µl) and ratio of absorbance at 260 nm and 280 nm of

3 different samples of PX, PC, PA, SBT, Bt and NO-QA populations are shown on Table 6.

Table 6. Total RNA concentration (ng/µl) and ratio of absorbance at 260 nm and 280 nm of 3 different samples of PX, PC, PA, SBT, Bt and NO-QA populations.

Population sample	Concentration (ng/µl)	260 / 280 Ratio
PX sample 1	1357	2.1
PX sample 2	785	2.1
PX sample 3	1000	2.13
PA sample 1	602	2.1
PA sample 2	413	2.1
PA sample 3	600	2.14
PC sample 1	1660	2.1
PC sample 2	826	2.1
PC sample 3	830	2.14
SBT sample 1	1024	2.09
SBT sample 2	884	2.1
SBT sample 3	900	2.14
Bt109 sample 1	973.5	2.1
Bt110 sample 2	935	2.1
Bt111 sample 3	470	2.1
NO-QA sample 1	1128	2.1
NO-QA1 sample 2	1179	2.1
NO-QA2 sample 3	1065.2	2.09

Knowing the concentration of the RNA samples, we were able to calculate the amount to use for the cDNA synthesis. The Prime Script™ 1st strand cDNA Synthesis Kit (TAKARA BIO INC) required 1 µg - 100 ng/µl of total RNA per reaction.

4.5. cDNA Synthesis

The cDNA was synthetized using the Prime Script™ 1st strand cDNA Synthesis (TAKARA BIO INC) kit according to the manufacture instructions.

4.6. Polymerase chain reaction (PCR) to confirm successful production of cDNA

Since the qPCR, with the cDNA template extracted with the High Capacity cDNA Reverse Transciption Kit, gave poor expression data, we decided to perform a PCR before performing another qPCR, using the cDNA sample extracted using the TAKARA Kit from populations SBT and PC. Figures 4 and 5 show that the cDNA synthesis using the Prime Script™ 1st strand cDNA Synthesis (TAKARA BIO INC) did work, and gave products of the expected size, so we went ahead and performed the qPCR with the new cDNA.

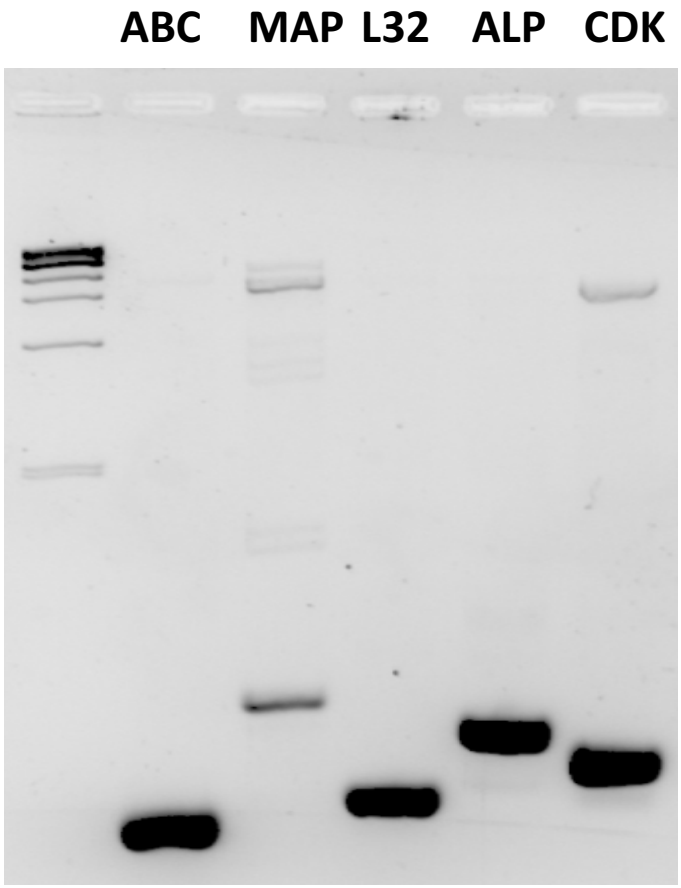

Figure 4: Gel red agarose electrophoresis. Lane 1: DNA ladder 1Kb; Lane 2: ABCC2 gene; Lane 3: MAP4K4 gene; Lane 4: L32 gene; Lane 5: ALP gene and Lane 6 CDKAL1. All the genes were tested against population SBT.

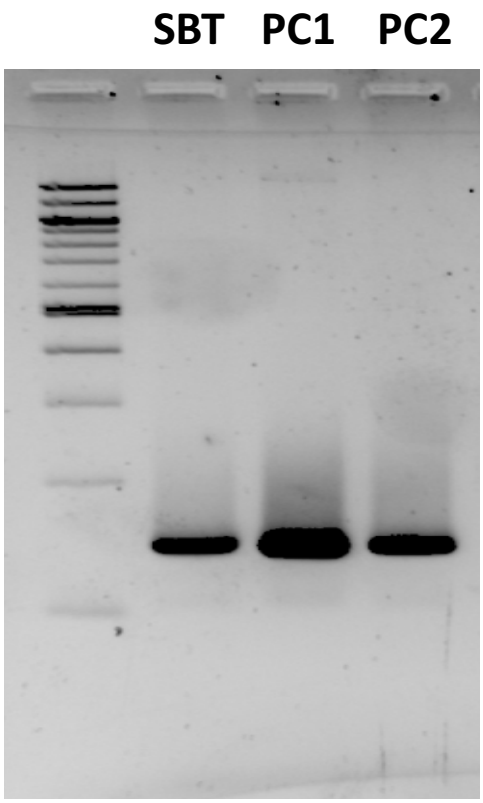

Figure 5: Gel red agarose electrophoresis. Lane 1: DNA ladder 100 bp; Lane 2: SBT population; Lane 3: PC population, sample 1; Lane 4: PC population, sample 2. All the populations were tested against gene CDKAL1.

4.7. Quantitative real-time PCR (qRT-PCR)

Figures 6 to 11 shows the relative expression levels of ABCC2, ALP, APN, MAP4K4, and CDKAL1 by qPCR in midguts of fourth-instar larvae from susceptible and resistant *P. xylostella* strains. Expression of the ribosomal protein L32 gene was used as the internal reference gene to normalize datasets and calculate relative expression levels, which were calculated assigning a value of 1 to the expression levels in SBT samples. Gene expression was observed, the results are shown bellow.

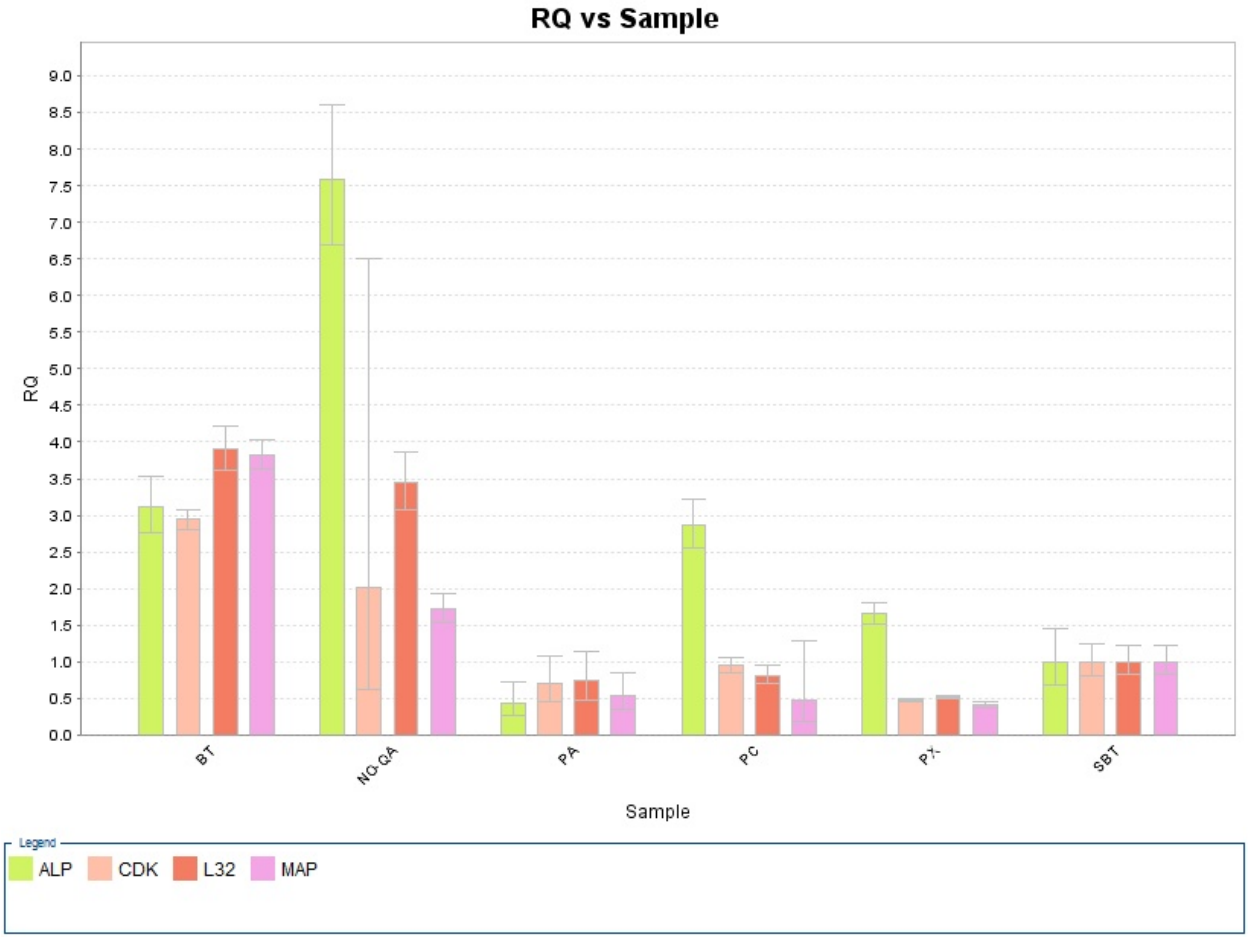

Figure 6. Relative expression levels of CDKAL1, L32, MAP and ALP as determined by qPCR in midguts of fourth-instar larvae from susceptible and resistant *Plutella xylostella* strains. Expression of the ribosomal protein L32 gene was used as the internal reference gene to normalize datasets and calculate relative expression levels, which were reached assigning a value of 1 to the expression levels in SBT samples.

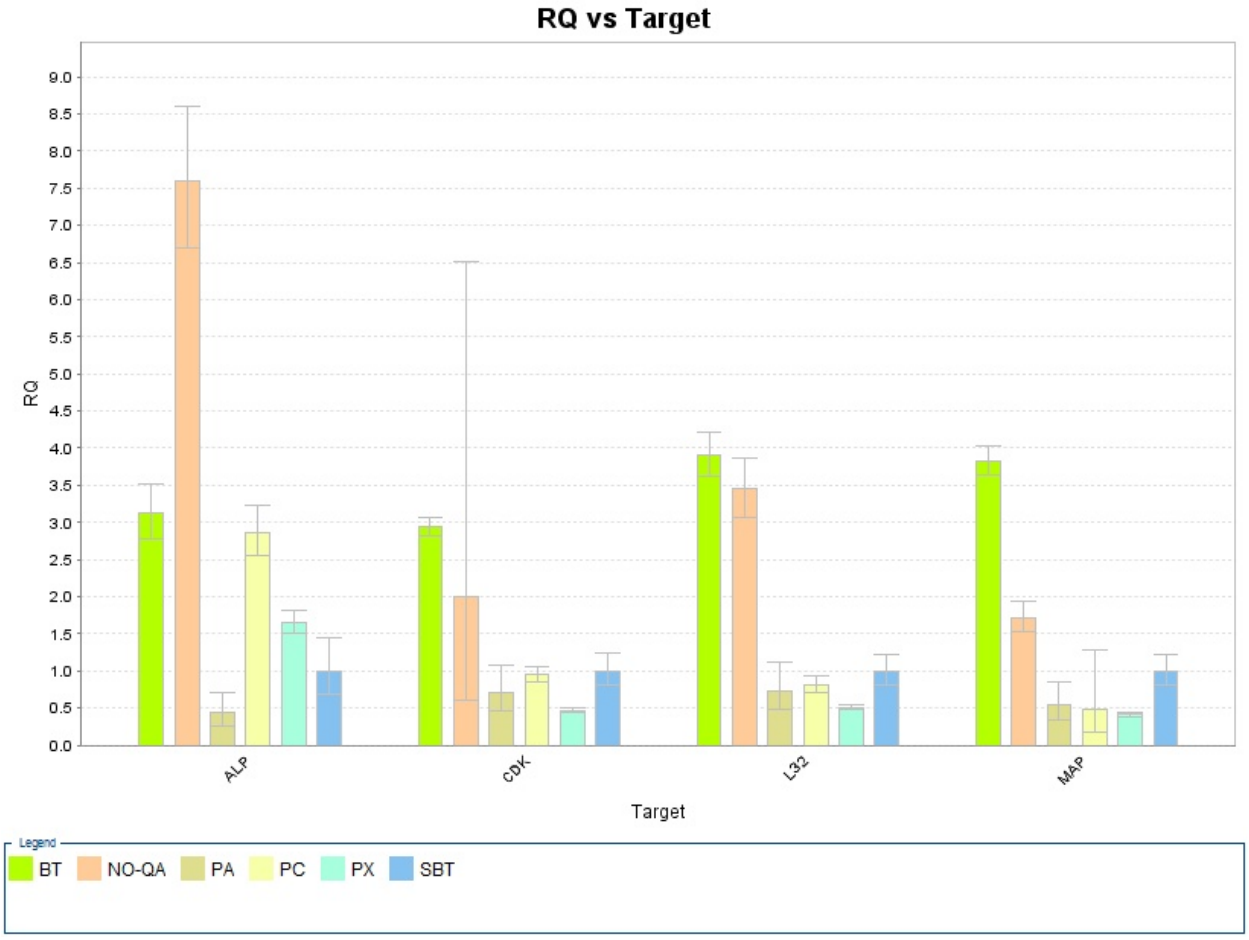

Figure 7. Relative expression levels of CDKAL1, L32, MAP and ALP as determined by qPCR in midguts of fourth-instar larvae from susceptible and resistant *Plutella xylostella* strains. Expression of the ribosomal protein L32 gene was used as the internal reference gene to normalize datasets and calculate relative expression levels, which were reached assigning a value of 1 to the expression levels in SBT samples.

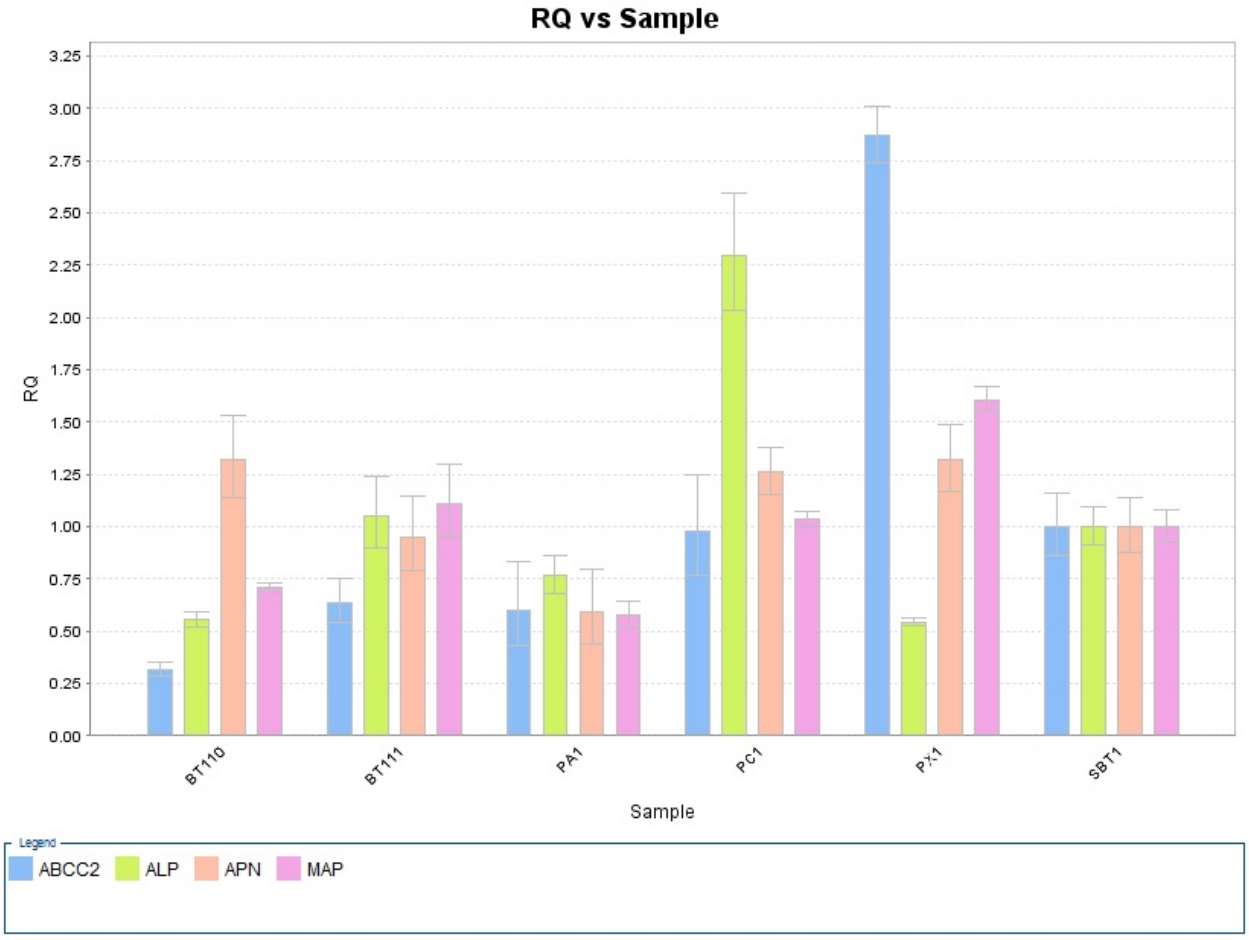

Figure 8. Relative expression levels of ABCC2, APN, MAP and ALP as determined by qPCR in midguts of fourth-instar larvae from susceptible and resistant *Plutella xylostella* strains. Expression of the ribosomal protein L32 gene was used as the internal reference gene to normalize datasets and calculate relative expression levels, which were reached assigning a value of 1 to the expression levels in SBT samples.

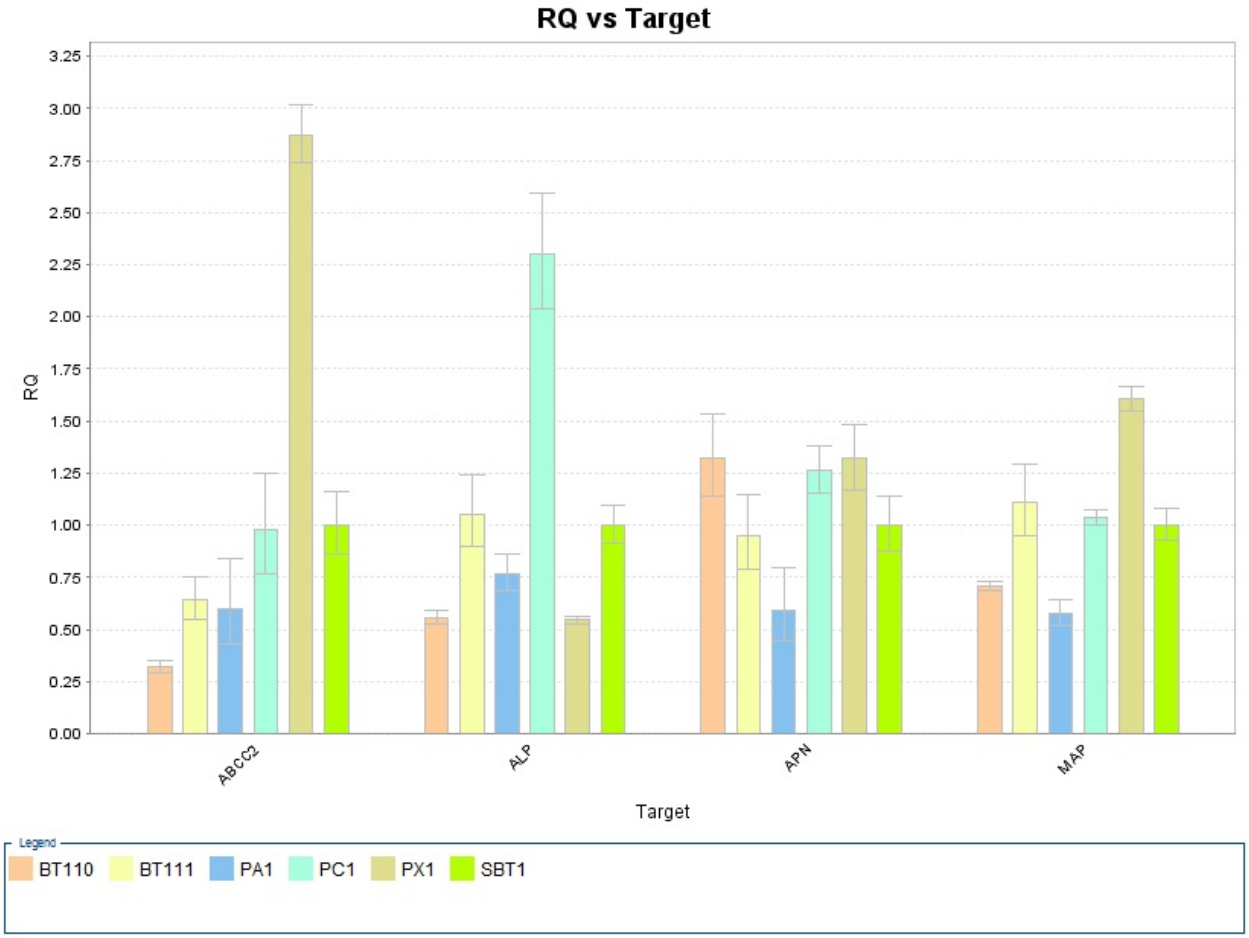

Figure 9. Relative expression levels of ABCC2, APN, MAP and ALP as determined by qPCR in midguts of fourth-instar larvae from susceptible and resistant *Plutella xylostella* strains. Expression of the ribosomal protein L32 gene was used as the internal reference gene to normalize datasets and calculate relative expression levels, which were reached assigning a value of 1 to the expression levels in SBT samples.

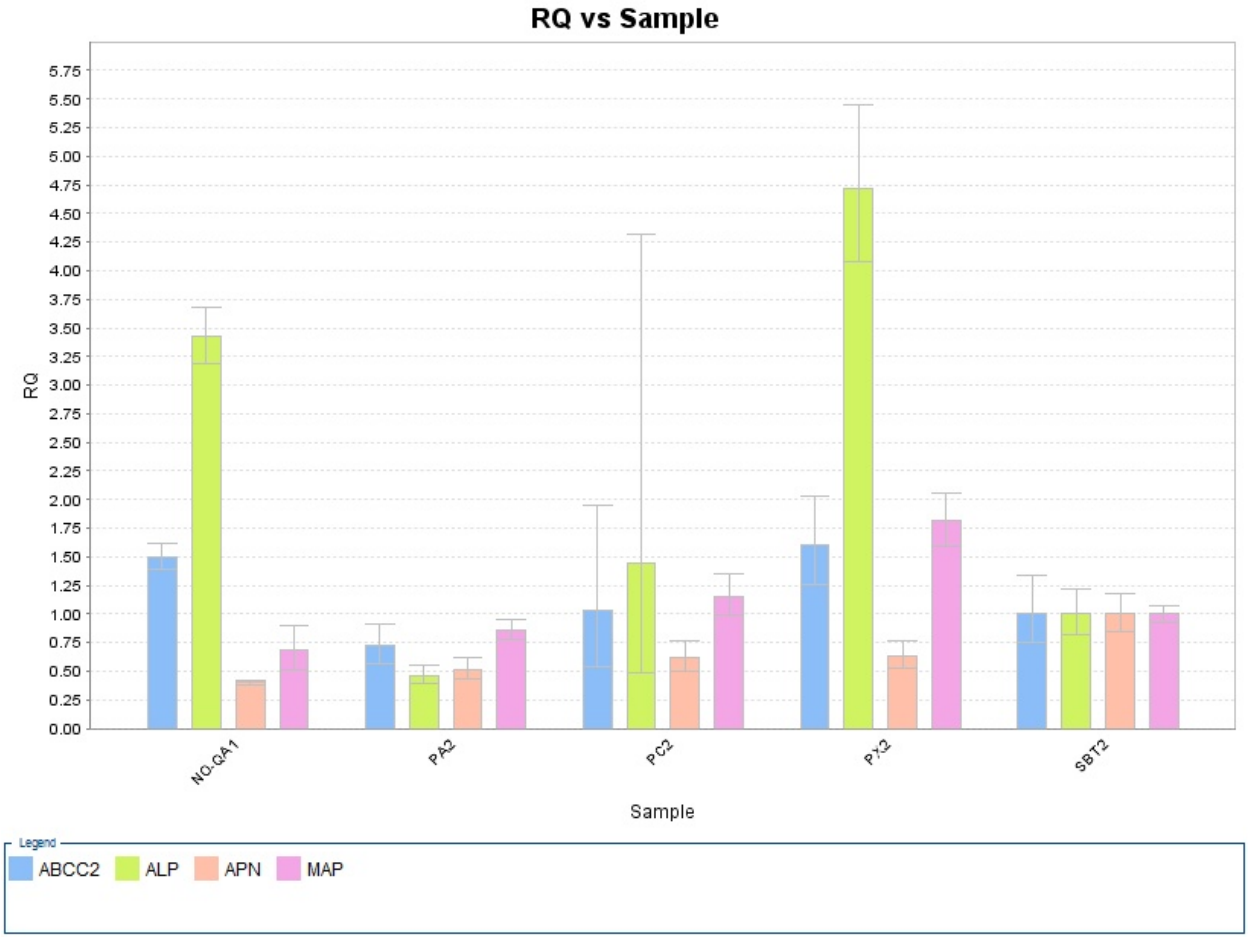

Figure 10. Relative expression levels of ABCC2, APN, MAP and ALP as determined by qPCR in midguts of fourth-instar larvae from susceptible and resistant *Plutella xylostella* strains. Expression of the ribosomal protein L32 gene was used as the internal reference gene to normalize datasets and calculate relative expression levels, which were reached assigning a value of 1 to the expression levels in SBT samples.

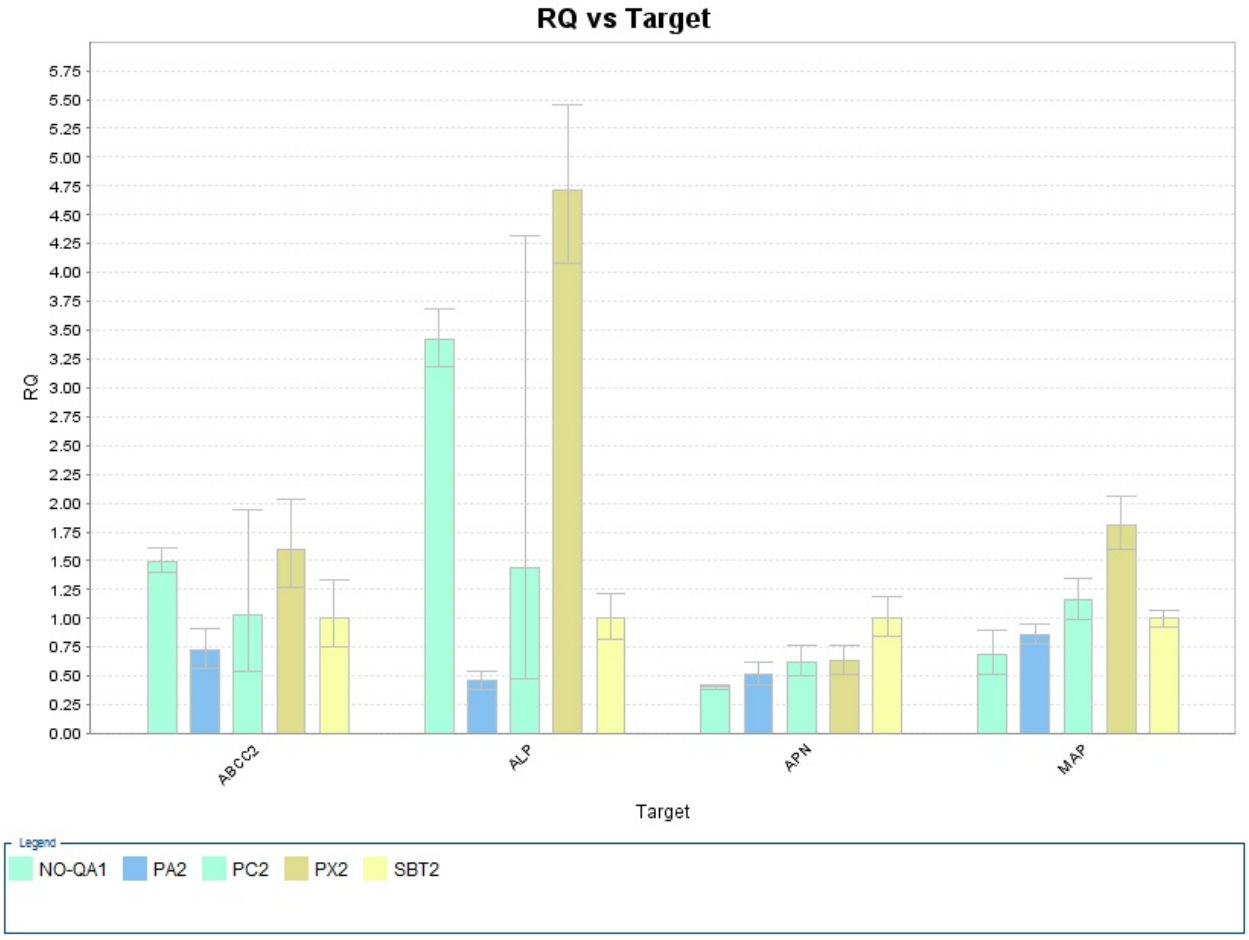

Figure 11. Relative expression levels of ABCC2, APN, MAP and ALP as determined by qPCR in midguts of fourth-instar larvae from susceptible and resistant *Plutella xylostella* strains. Expression of the ribosomal protein L32 gene was used as the internal reference gene to normalize datasets and calculate relative expression levels, which were reached assigning a value of 1 to the expression levels in SBT samples.

The relative expression levels of all genes tested in all of the *P. xylostella* populations failed to show any pattern. The experiment where performed in triplicates and even so, each time, the level of expression where different and non-consistent.

4.8. Polymerase chain reaction (PCR) – ABCC2 gene

The band was very clear on the 1% agarose gel (Figures 12, 13 and 14), so PCR purification was performed with ABCC2 full length gene from the PCR product and the samples were sent for sequencing.

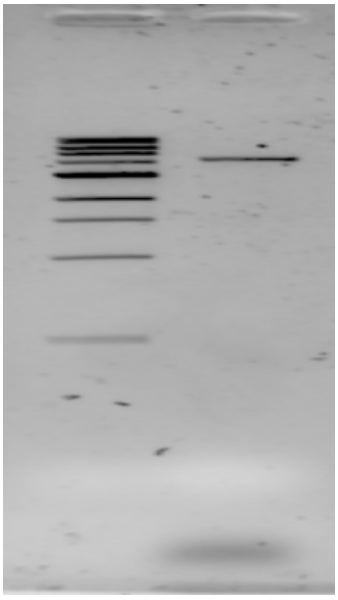

Figure 12: Gel red agarose electrophoresis. Lane 1: DNA ladder 1 Kb; Lane 2: SBT population PCR ABCC2 Full Length.

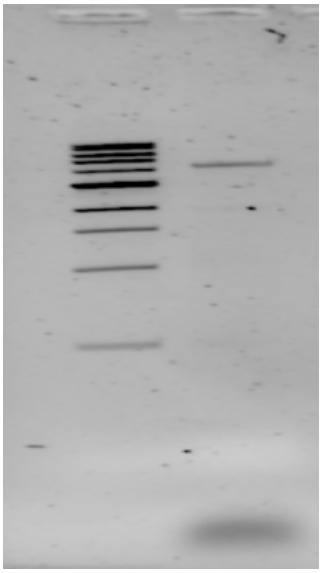

Figure 13: Gel red agarose electrophoresis. Lane 1: DNA ladder 1 Kb; Lane 2: NO-QA population PCR ABCC2 Full Length.

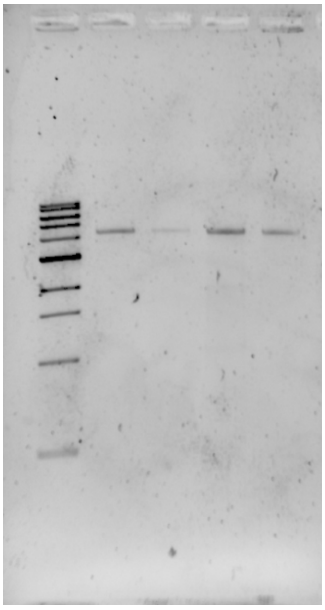

Figure 14: Gel red agarose electrophoresis. Lane 1: DNA ladder 1 Kb; Lane 2: PA population; Lane 3: Bt population; Lane 4: Px population; Lane 5: PC population; PCR ABCC2 Full Length.

Nested PCRs were performed to improve the amount of the DNA to get better results. In this case we had different bands on the gel (Figures 15, 16 and 17) so we purified the samples from the gel before sending for sequencing. The purified samples are shown in Figures 18, 19 and 20.

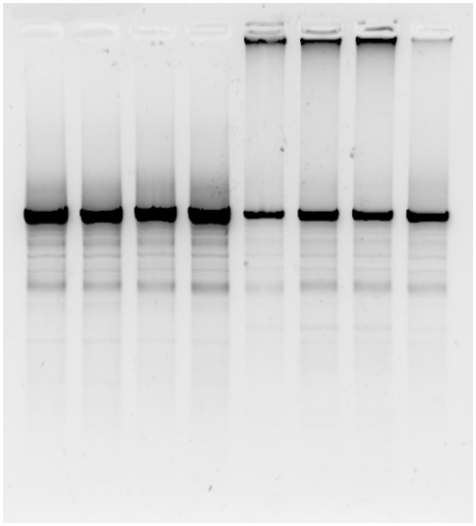

Figure 15: Gel red agarose electrophoresis. Lanes 1, 2, 3 and 4: PX population; Lanes 5, 6, 7 and 8: Bt population; nested PCR ABCC2 Full Length.

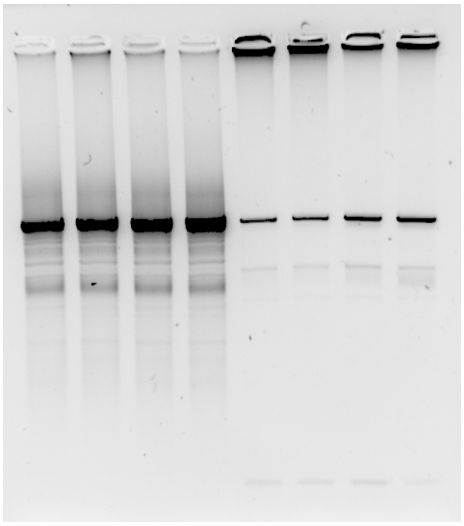

Figure 16: Gel red agarose electrophoresis. Lanes 1, 2, 3 and 4: PA population; Lanes 5, 6, 7 and 8: PC population; nested PCR ABCC2 Full Length.

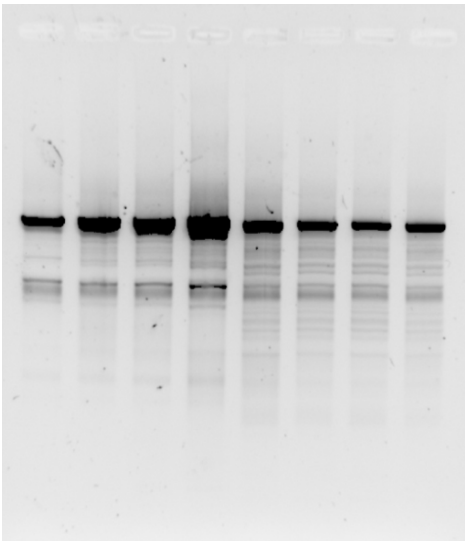

Figure 17: Gel red agarose electrophoresis. Lanes 1, 2, 3 and 4: SBT population; Lanes 5, 6, 7 and 8: NO-QA population; nested PCR ABCC2 Full Length.

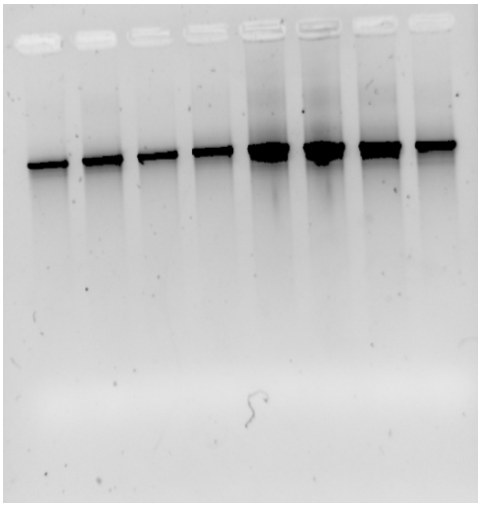

Figure 18: Gel red agarose electrophoresis. Lanes 1, 2, 3 and 4: Bt population; Lanes 5, 6, 7 and 8: PA population; nested PCR Purified products from agarose gel.

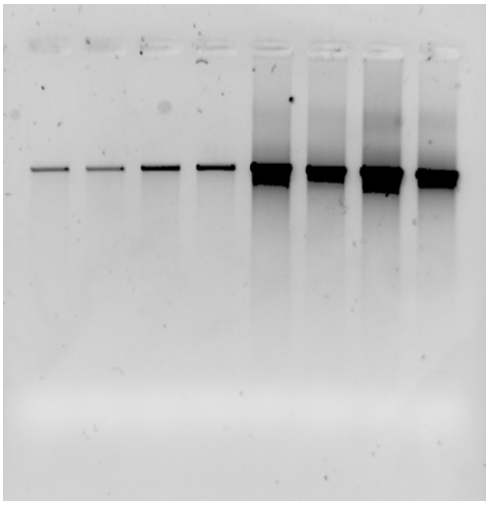

Figure 19: Gel red agarose electrophoresis. Lanes 1, 2, 3 and 4: PC population; Lanes 5, 6, 7 and 8: Px population; nested PCR Purified products from agarose gel.

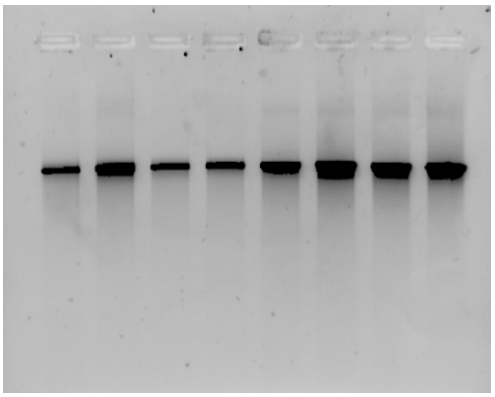

Figure 20: Gel red agarose electrophoresis. Lanes 1, 2, 3 and 4: NO-QA population; Lanes 5, 6, 7 and 8: SBT population; nestedPCR Purified products from agarose gel.

Absorbance measurements were also made on Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo Scientific) for all the samples before sending for sequencing (Table 7).

Table 7. Total DNA concentration (ng/µl) and ratio of absorbance at 260 nm and 280 nm of samples of PX, PC, PA, SBT, Bt and NO-QA populations, samples combined.

Population sample	Concentration (ng/µl)	260 / 280 Ratio
PX	10.2	1.89
PA	9.2	2.53
PC	4.2	3.9
SBT	7.9	2.33
Bt	21.4	1.55
NO-QA	8.7	1.95

Samples from all 6 populations were sent for sequencing to Eurofins MWG Operon- Europe and tested against the following primers: PxABCC2 Full length F, PxABCC2 Full length R, PxABCC2N1F, PxABCC2N1R, PxABCC2N2F, PxABCC2N2R, PxABCC2N3F, PxABCC2N3R, PxABCC2N4F, PxABCC2N4R, PxABCC2N5F, PxABCC2N5R, PxABCC2Cterm and PxABCC2Nterm (Table 2).

4.9. ABCC2 Sequencing

ABBC2 mutations in this gene have been shown to cause resistance to Bt in several insects and in particular a deletion was identified in a resistant population of *P. xylostella* (BAXTER, 2011). Therefore we decided to check whether or not these same deletions, or other mutations in this gene, were present in our strains.

The whole ABCC2 gene from populations Px, PA, SBT, Bt and NO-QA was sequenced and then the sequences were aligned as shown on Appendix 1.

There was one region on the Bt population that was problematic, the primers designed to give us data across that region had all failed to give a clear sequence.

However when we went back to the actual data the peaks were very clear and we were able to work out that at a point in that region there were two sequences. One is the expected sequence (i.e. same as Px) the other contained a 1 base pair deletion. We excluded the overlapping parts and aligned the gene sequences. The deletion is highlighted on the alignment bellow. Clearly this deletion could be responsible for the resistant phenotype.

Below the consensus sequence alignment off all the populations without the sequences overlapping. In bold and underlined is the T that was found deleted in the Bt population and the bold region is normally found deleted in the ABCC2 gene of the NO-QA population:

```
NOQA            ATGGAAAACGGAAGCGGAGCCCGGAAAGAGTCGGAAGAGAAGAAAGAGGTGAAGAAGGGC  60
BTDELETED       ATGGAAAACGGAAGCGGAGCCCGGAAAGAGTCGGAAGAGAAGAAAGAGGTGAAGAAGGGC  60
BT              ATGGAAAACGGAAGCGGAGCCCGGAAAGAGTCGGAAGAGAAGAAAGAGGTGAAGAAGGGC  60
SBT             ATGGAAAACGGAAGCGGAGCCCGGAAAGAGTCGGAAGAGAAGAAAGAGGTGAAGAAGGGC  60
PA              ATGGAAAACGGAAGCGGAGCCCGGAAAGAGTCGGAAGAGAAGAAAGAGGTGAAGAAGGGC  60
PX              ATGGAAAACGGAAGCGGAGCCCGGAAAGAGTCGGAAGAGAAGAAAGAGGTGAAGAAGGGC  60
                ************************************************************

NOQA            AAACCCAACGTGCTGTCCCGGCTGTTCATGTGCTGGGTGTGCCCCGTGCTGGTGGGCGGC  120
BTDELETED       AAACCCAACGTGCTGTACCGGCTGTTCATGTGCTGGGTGTGCCCCGTGCTGGTGGGCGGC  120
BT              AAACCCAACGTGCTGTACCGGCTGTTCATGTGCTGGGTGTGCCCCGTGCTGGTGGGCGGC  120
SBT             AAACCCAACGTGCTGTACCGGCTGTTCATGTGCTGGGTGTGCCCCGTGCTGGTGGGCGGC  120
PA              AAACCCAACGTGCTGTACCGGCTGTTCATGTGCTGGGTGTGCCCCGTGCTGGTGGGCGGC  120
PX              AAACCCAACGTGCTGTACCGGCTGTTCATGTGCTGGGTGTGCCCCGTGCTGGTGGGCGGC  120
                *************** ****************************************

NOQA            AACCGGCGCGACGTGGAGGAGCGGGACCTCATACCGCCGCCCAGCGCCAAGTACAAGTCC  180
BTDELETED       AACCGGCGCGACGTGGAGGAGCGGGACCTCATACCGCCGCCCAGCGCCAAGTACAAGTCC  180
BT              AACCGGCGCGACGTGGAGGAGCGGGACCTCATACCGCCGCCCAGCGCCAAGTACAAGTCC  180
SBT             AACCGGCGCGACGTGGAGGAGCGGGACCTCATACCGCCGCCCAGCGCCAAGTACAAGTCC  180
PA              AACCGGCGCGACGTGGAGGAGCGGGACCTCATACCGCCGCCCAGCGCCAAGTACAAGTCC  180
PX              AACCGGCGCGACGTGGAGGAGCGGGACCTCATACCGCCGCCCAGCGCCAAGTACAAGTCC  180
                ************************************************************

NOQA            GAGTCTCTCGGAGACAAGTTTGAAAGGTACTGGCTAGAAGAGCTGGGTCTGGCGACGCAG  240
BTDELETED       GAGTCTCTCGGAGACAAGTTTGAAAGGTACTGGCTAGAAGAGCTGGGTCTGGCGACGCAG  240
BT              GAGTCTCTCGGAGACAAGTTTGAAAGGTACTGGCTAGAAGAGCTGGGTCTGGCGACGCAG  240
SBT             GAGTCTCTCGGAGACAAGTTTGAAAGGTACTGGCTAGAAGAGCTGGGTCTGGCGACGCAG  240
PA              GAGTCTCTCGGAGACAAGTTTGAAAGGTACTGGCTAGAAGAGCTGGGTCTGGCGACGCAG  240
PX              GAGTCTCTCGGAGACAAGTTTGAAAGGTACTGGCTAGAAGAGCTGGGTCTGGCGACGCAG  240
                ************************************************************
```

```
NOQA            CGCGGGGTGGCGCCGTCGCTGTGGCGGGCGCTGCGCCGCGCCTTCTGGCTGTCCTACATG  300
BTDELETED       CGCGGGGTGGCGCCGTCGCTGTGGCGGGCGCTGCGCCGCGCCTTCTGGCTGTCCTACATG  300
BT              CGCGGGGTGGCGCCGTCGCTGTGGCGGGCGCTGCGCCGCGCCTTCTGGCTGTCCTACATG  300
SBT             CGCGGGGTGGCGCCGTCGCTGTGGCGGGCGCTGCGCCGCGCCTTCTGGCTGTCCTACATG  300
PA              CGCGGGGTGGCGCCGTCGCTGTGGCGGGCGCTGCGCCGCGCCTTCTGGCTGTCCTACATG  300
PX              CGCGGGGTGGCGCCGTCGCTGTGGCGGGCGCTGCGCCGCGCCTTCTGGCTGTCCTACATG  300
                ************************************************************

NOQA            CCGGGCGCGCTGCTGCTGCTCGGGAACGCCATACCCAGGACCATCCAGCCGCTGCTATTC  360
BTDELETED       CCGGGCGCGCTGCTGCTGCTCGGGAACGCCATACCCAGGACCATCCAGCCGCTGCTATTC  360
BT              CCGGGCGCGCTGCTGCTGCTCGGGAACGCCATACCCAGGACCATCCAGCCGCTGCTATTC  360
SBT             CCGGGCGCGCTGCTGCTGCTCGGGAACGCCATACCCAGGACCATCCAGCCGCTGCTATTC  360
PA              CCGGGCGCGCTGCTGCTGCTCGGGAACGCCATACCCAGGACCATCCAGCCGCTGCTATTC  360
PX              CCGGGCGCGCTGCTGCTGCTCGGGAACGCCATACCCAGGACCATCCAGCCGCTGCTATTC  360
                ************************************************************

NOQA            ACGCGGCTGCTCTCCTATTGGTCGGCGGACAGCACCATGACCCGACTGGAGGCCGGATAT  420
BTDELETED       ACGCGGCTGCTCTCCTATTGGTCGGCGGACAGCACCATGACCCGACTGGAGGCCGGATAT  420
BT              ACGCGGCTGCTCTCCTATTGGTCGGCGGACAGCACCATGACCCGACTGGAGGCCGGATAT  420
SBT             ACGCGGCTGCTCTCCTATTGGTCGGCGGACAGCACCATGACCCGACTGGAGGCCGGATAT  420
PA              ACGCGGCTGCTCTCCTATTGGTCGGCGGACAGCACCATGACCCGACTGGAGGCCGGATAT  420
PX              ACGCGGCTGCTCTCCTATTGGTCGGCGGACAGCACCATGACCCGACTGGAGGCCGGATAT  420
                ************************************************************

NOQA            TGGGCCATGGGCATGCTGCTGTGCAACTTCCTGGCCATGGTGTGCCACCACCACAACACG  480
BTDELETED       TGGGCCATGGGCATGCTGCTGTGCAACTTCCTGGCCATGGTGTGCCACCACCACAACACG  480
BT              TGGGCCATGGGCATGCTGCTGTGCAACTTCCTGGCCATGGTGTGCCACCACCACAACACG  480
SBT             TGGGCCATGGGCATGCTGCTGTGCAACTTCCTGGCCATGGTGTGCCACCACCACAACACG  480
PA              TGGGCCATGGGCATGCTGCTGTGCAACTTCCTGGCCATGGTGTGCCACCACCACAACACG  480
PX              TGGGCCATGGGCATGCTGCTGTGCAACTTCCTGGCCATGGTGTGCCACCACCACAACACG  480
                ************************************************************

NOQA            CTGTTCGTCGGCCGCTTTGGCATGAAAGTCAGGATTGCCTGTTGCTCGCTGCTCTATCGG  540
BTDELETED       CTGTTCGTCGGCCGCTTTGGCATGAAAGTCAGGATTGCCTGTTGCTCGCTGCTCTATCGG  540
BT              CTGTTCGTCGGCCGCTTTGGCATGAAAGTCAGGATTGCCTGTTGCTCGCTGCTCTATCGG  540
SBT             CTGTTCGTCGGCCGCTTTGGCATGAAAGTCAGGATTGCCTGTTGCTCGCTGCTCTATCGG  540
PA              CTGTTCGTCGGCCGCTTTGGCATGAAAGTCAGGATTGCCTGTTGCTCGCTGCTCTATCGG  540
PX              CTGTTCGTCGGCCGCTTTGGCATGAAAGTCAGGATTGCCTGTTGCTCGCTGCTCTATCGG  540
                ************************************************************

NOQA            AAGCTGCTCCGTCTAAACCAGAGGTCCCTGCAAAGCACGGCGGCGGGCAAGCTGGTGAAC  600
BTDELETED       AAGCTGCTCCGTCTAAACCAGAGGTCCCTGCAAAGCACGGCGGCGGGCAAGCTGGTGAAC  600
BT              AAGCTGCTCCGTCTAAACCAGAGGTCCCTGCAAAGCACGGCGGCGGGCAAGCTGGTGAAC  600
SBT             AAGCTGCTCCGTCTAAACCAGAGGTCCCTGCAAAGCACGGCGGCGGGCAAGCTGGTGAAC  600
PA              AAGCTGCTCCGTCTAAACCAGAGGTCCCTGCAAAGCACGGCGGCGGGCAAGCTGGTGAAC  600
PX              AAGCTGCTCCGTCTAAACCAGAGGTCCCTGCAAAGCACGGCGGCGGGCAAGCTGGTGAAC  600
                ************************************************************

NOQA            CTGATGTCGAACGACGTGGCGCGCTTCGACTACGCCTTCATGTTCCTGCACTACTTCTGG  660
BTDELETED       CTGATGTCGAACGACGTGGCGCGCTTCGACTACGCCTTCATGTTCCTGCACTACTTCTGG  660
BT              CTGATGTCGAACGACGTGGCGCGCTTCGACTACGCCTTCATGTTCCTGCACTACTTCTGG  660
SBT             CTGATGTCGAACGACGTGGCGCGCTTCGACTACGCCTTCATGTTCCTGCACTACTTCTGG  660
PA              CTGATGTCGAACGACGTGGCGCGCTTCGACTACGCCTTCATGTTCCTGCACTACTTCTGG  660
PX              CTGATGTCGAACGACGTGGCGCGCTTCGACTACGCCTTCATGTTCCTGCACTACTTCTGG  660
                ************************************************************
```

```
NOQA            ATGATCCCGCTGCAGTCGGCCGCCGTGCTGTACTTCATGTTCCGCGCCGCCGGCTGGGCG  720
BTDELETED       ATGATCCCGCTGCAGTCGGCCGCCGTGCTGTACTTCATGTTCCGCGCCGCCGGCTGGGCG  720
BT              ATGATCCCGCTGCAGTCGGCCGCCGTGCTGTACTTCATGTTCCGCGCCGCCGGCTGGGCG  720
SBT             ATGATCCCGCTGCAGTCGGCCGCCGTGCTGTACTTCATGTTCCGCGCCGCCGGCTGGGCG  720
PA              ATGATCCCGCTGCAGTCGGCCGCCGTGCTGTACTTCATGTTCCGCGCCGCCGGCTGGGCG  720
PX              ATGATCCCGCTGCAGTCGGCCGCCGTGCTGTACTTCATGTTCCGCGCCGCCGGCTGGGCG  720
                ************************************************************

NOQA            CCCATCGTGGGCCTGTTCTCTGTCATGCTGCTCATACTGCCTATACAAGCCGGCCTCACC  780
BTDELETED       CCCATCGTGGGCCTGTTCTCTGTCATGCTGCTCATACTGCCTATACAAGCCGGCCTCACC  780
BT              CCCATCGTGGGCCTGTTCTCTGTCATGCTGCTCATACTGCCTATACAAGCCGGCCTCACC  780
SBT             CCCATCGTGGGCCTGTTCTCTGTCATGCTGCTCATACTGCCTATACAAGCCGGCCTCACC  780
PA              CCCATCGTGGGCCTGTTCTCTGTCATGCTGCTCATACTGCCTATACAAGCCGGCCTCACC  780
PX              CCCATCGTGGGCCTGTTCTCTGTCATGCTGCTCATACTGCCTATACAAGCCGGCCTCACC  780
                ************************************************************

NOQA            AAGCTCACAGCTGTCTACAGGAGGGAGACTGCACAAAGGACAGATAAGAGAATTAAACTA  840
BTDELETED       AAGCTCACAGCTGTCTACAGGAGGGAGACTGCACAAAGGACAGATAAGAGAATTAAACTA  840
BT              AAGCTCACAGCTGTCTACAGGAGGGAGACTGCACAAAGGACAGATAAGAGAATTAAACTA  840
SBT             AAGCTCACAGCTGTCTACAGGAGGGAGACTGCACAAAGGACAGATAAGAGAATTAAACTA  840
PA              AAGCTCACAGCTGTCTACAGGAGGGAGACTGCACAAAGGACAGATAAGAGAATTAAACTA  840
PX              AAGCTCACAGCTGTCTACAGGAGGGAGACTGCACAAAGGACAGATAAGAGAATTAAACTA  840
                ************************************************************

NOQA            ATGAGCGAAATTATCAATGGTATTCAGGTGATAAAGATGTACGCGTGGGAGGTCCCCTTC  900
BTDELETED       ATGAGCGAAATTATCAATGGTATTCAGGTGATAAAGATGTACGCGTGGGAGGTCCCCTTC  900
BT              ATGAGCGAAATTATCAATGGTATTCAGGTGATAAAGATGTACGCGTGGGAGGTCCCCTTC  900
SBT             ATGAGCGAAATTATCAATGGTATTCAGGTGATAAAGATGTACGCGTGGGAGGTCCCCTTC  900
PA              ATGAGCGAAATTATCAATGGTATTCAGGTGATAAAGATGTACGCGTGGGAGGTCCCCTTC  900
PX              ATGAGCGAAATTATCAATGGTATTCAGGTGATAAAGATGTACGCGTGGGAGGTCCCCTTC  900
                ************************************************************

NOQA            CAGAAGGTGGTGGGGTCCTCCCGCGCGCACGAGGTGGAGGCGTTGAAGAGGGCGTCCTTC  960
BTDELETED       CAGAAGGTGGTGGGGTCCTCCCGCGCGCACGAGGTGGAGGCGTTGAAGAGGGCGTCCTTC  960
BT              CAGAAGGTGGTGGGGTCCTCCCGCGCGCACGAGGTGGAGGCGTTGAAGAGGGCGTCCTTC  960
SBT             CAGAAGGTGGTGGGGTCCTCCCGCGCGCACGAGGTGGAGGCGTTGAAGAGGGCGTCCTTC  960
PA              CAGAAGGTGGTGGGGTCCTCCCGCGCGCACGAGGTGGAGGCGTTGAAGAGGGCGTCCTTC  960
PX              CAGAAGGTGGTGGGGTCCTCCCGCGCGCACGAGGTGGAGGCGTTGAAGAGGGCGTCCTTC  960
                ************************************************************

NOQA            GTGCAGGGCACCTTCGGCGGGTTCATGCTGTTCACGGAGCGCACCTCGCTCTTCCTCACC 1020
BTDELETED       GTGCAGGGCACCTTCGGCGGGTTCATGCTGTTCACGGAGCGCACCTCGCTCTTCCTCACC 1020
BT              GTGCAGGGCACCTTCGGCGGGTTCATGCTGTTCACGGAGCGCACCTCGCTCTTCCTCACC 1020
SBT             GTGCAGGGCACCTTCGGCGGGTTCATGCTGTTCACGGAGCGCACCTCGCTCTTCCTCACC 1020
PA              GTGCAGGGCACCTTCGGCGGGTTCATGCTGTTCACGGAGCGCACCTCGCTCTTCCTCACC 1020
PX              GTGCAGGGCACCTTCGGCGGGTTCATGCTGTTCACGGAGCGCACCTCGCTCTTCCTCACC 1020
                ************************************************************

NOQA            GTCATGACGCTGGTTCTGACTGGGAGCATGGCGACTGCTACTACGGTGTATCCAATCCAA 1080
BTDELETED       GTCATGACGCTGGTTCTGACTGGGAGCATGGCGACTGCTACTACGGTGTATCCAATCCAA 1080
BT              GTCATGACGCTGGTTCTGACTGGGAGCATGGCGACTGCTACTACGGTGTATCCAATCCAA 1080
SBT             GTCATGACGCTGGTTCTGACTGGGAGCATGGCGACTGCTACTACGGTGTATCCAATCCAA 1080
PA              GTCATGACGCTGGTTCTGACTGGGAGCATGGCGACTGCTACTACGGTGTATCCAATCCAA 1080
PX              GTCATGACGCTGGTTCTGACTGGGAGCATGGCGACTGCTACTACGGTGTATCCAATCCAA 1080
                ************************************************************
```

```
NOQA          CAATACTTTAGTATAATCCAATCCAATTTGGCGCTCATCCTCCCTATCGCCATCGCTCAA 1140
BTDELETED     CAATACTTTAGTATAATCCAATCCAATTTGGCGCTCATCCTCCCTATCGCCATCGCTCAA 1140
BT            CAATACTTTAGTATAATCCAATCCAATTTGGCGCTCATCCTCCCTATCGCCATCGCTCAA 1140
SBT           CAATACTTTAGTATAATCCAATCCAATTTGGCGCTCATCCTCCCTATCGCCATCGCTCAA 1140
PA            CAATACTTTAGTATAATCCAATCCAATTTGGCGCTCATCCTCCCTATCGCCATCGCTCAA 1140
PX            CAATACTTTAGTATAATCCAATCCAATTTGGCGCTCATCCTCCCTATCGCCATCGCTCAA 1140
              ************************************************************

NOQA          CTCACTGAGATGTTGGTCTCATTGGAAAGACTTCAGGAGTTTCTGATGTTAGACGAGAGG 1200
BTDELETED     CTCACTGAGATGTTGGTCTCATTGGAAAGACTTCAGGAGTTTCTGATGTTAGACGAGAGG 1200
BT            CTCACTGAGATGTTGGTCTCATTGGAAAGACTTCAGGAGTTTCTGATGTTAGACGAGAGG 1200
SBT           CTCACTGAGATGTTGGTCTCATTGGAAAGACTTCAGGAGTTTCTGATGTTAGACGAGAGG 1200
PA            CTCACTGAGATGTTGGTCTCATTGGAAAGACTTCAGGAGTTTCTGATGTTAGACGAGAGG 1200
PX            CTCACTGAGATGTTGGTCTCATTGGAAAGACTTCAGGAGTTTCTGATGTTAGACGAGAGG 1200
              ************************************************************

NOQA          GAAGACCTGTCGGTGATGCCGGGCGGGCAGGCGGACTCCGCGCCCGTGGCCTTCAAGTAC 1260
BTDELETED     GAAGACCTGTCGGTGATGCCGGGCGGGCAGGCGGACTCCGCGCCCGTGGCCTTCAAGTAC 1260
BT            GAAGACCTGTCGGTGATGCCGGGCGGGCAGGCGGACTCCGCGCCCGTGGCCTTCAAGTAC 1260
SBT           GAAGACCTGTCGGTGATGCCGGGCGGGCAGGCGGACTCCGCGCCCGTGGCCTTCAAGTAC 1260
PA            GAAGACCTGTCGGTGATGCCGGGCGGGCAGGCGGACTCCGCGCCCGTGGCCTTCAAGTAC 1260
PX            GAAGACCTGTCGGTGATGCCGGGCGGGCAGGCGGACTCCGCGCCCGTGGCCTTCAAGTAC 1260
              ************************************************************

NOQA          ACGAAGGAGACCACGGCGCCCGCCTACATCGTGTCCAAGCGGTACTCCAAAAAGGAGGAC 1320
BTDELETED     ACGAAGGAGACCACGGCGCCCGCCTACATCGTGTCCAAGCGGTACTCCAAAAAGGAGGAC 1320
BT            ACGAAGGAGACCACGGCGCCCGCCTACATCGTGTCCAAGCGGTACTCCAAAAAGGAGGAC 1320
SBT           ACGAAGGAGACCACGGCGCCCGCCTACATCGTGTCCAAGCGGTACTCCAAAAAGGAGGAC 1320
PA            ACGAAGGAGACCACGGCGCCCGCCTACATCGTGTCCAAGCGGTACTCCAAAAAGGAGGAC 1320
PX            ACGAAGGAGACCACGGCGCCCGCCTACATCGTGTCCAAGCGGTACTCCAAAAAGGAGGAC 1320
              ************************************************************

NOQA          GACACCGGCCTGGCTGCGGAGCTGGTGGAACGCAAATCCACGAGCGAGTTTGCGGTCGAG 1380
BTDELETED     GACACCGGCCTGGCTGCGGAGCTGGTGGAACGCAAATCCACGAGCGAGTTTGCGGTCGAG 1380
BT            GACACCGGCCTGGCTGCGGAGCTGGTGGAACGCAAATCCACGAGCGAGTTTGCGGTCGAG 1380
SBT           GACACCGGCCTGGCTGCGGAGCTGGTGGAACGCAAATCCACGAGCGAGTTTGCGGTCGAG 1380
PA            GACACCGGCCTGGCTGCGGAGCTGGTGGAACGCAAATCCACGAGCGAGTTTGCGGTCGAG 1380
PX            GACACCGGCCTGGCTGCGGAGCTGGTGGAACGCAAATCCACGAGCGAGTTTGCGGTCGAG 1380
              ************************************************************

NOQA          CTGAACGACGTGAGTGCGTCGTGGGGGGGGTGAGGGGGACAAGGACCAGCACACGCTGCGC 1440
BTDELETED     CTGAACGACGTGAGTGCGTCGTGGGGGGGGTGAGGGGGACAAGGACCAGCACACGCTGCGC 1440
BT            CTGAACGACGTGAGTGCGTCGTGGGGGGGGTGAGGGGGACAAGGACCAGCACACGCTGCGC 1440
SBT           CTGAACGACGTGAGTGCGTCGTGGGGGGGGTGAGGGGGACAAGGACCAGCACACGCTGCGC 1440
PA            CTGAACGACGTGAGTGCGTCGTGGGGGGGGTGAGGGGGACAAGGACCAGCACACGCTGCGC 1440
PX            CTGAACGACGTGAGTGCGTCGTGGGGGGGGTGAGGGGGACAAGGACCAGCACACGCTGCGC 1440
              ************************************************************

NOQA          GGGGTGTCGATGCGCGTGCGCCGCGGCAAGCTGGCCGCCATCATCGGACCCGTGGGATCC 1500
BTDELETED     GGGGTGTCGATGCGCGTGCGCCGCGGCAAGCTGGCCGCCATCA-CGGACCCGTGGGATCC 1499
BT            GGGGTGTCGATGCGCGTGCGCCGCGGCAAGCTGGCCGCCATCATCGGACCCGTGGGATCC 1500
SBT           GGGGTGTCGATGCGCGTGCGCCGCGGCAAGCTGGCCGCCATCATCGGACCCGTGGGATCC 1500
PA            GGGGTGTCGATGCGCGTGCGCCGCGGCAAGCTGGCCGCCATCATCGGACCCGTGGGATCC 1500
PX            GGGGTGTCGATGCGCGTGCGCCGCGGCAAGCTGGCCGCCATCATCGGACCCGTGGGATCC 1500
              ****************************************** ****************
```

```
NOQA            GGGAAGTCGTCGCTGCTCCAAGTGTTGCTGAAAGAGCTTCCGGTGTCGTCCGGAACCGTG 1560
BTDELETED       GGGAAGTCGTCGCTGCTCCAAGTGTTGCTGAAGGAGCTTCCGGTGTCGTCCGGAACCGTG 1559
BT              GGGAAGTCGTCGCTGCTCCAAGTGTTGCTGAAGGAGCTTCCGGTGTCGTCCGGAACCGTG 1560
SBT             GGGAAGTCGTCGCTGCTCCAAGTGTTGCTGAAGGAGCTTCCGGTGTCGTCCGGAACCGTG 1560
PA              GGGAAGTCGTCGCTGCTCCAAGTGTTGCTGAAGGAGCTTCCGGTGTCGTCCGGAACCGTG 1560
PX              GGGAAGTCGTCGCTGCTCCAAGTGTTGCTGAAGGAGCTTCCGGTGTCGTCCGGAACCGTG 1560
                ******************************** *************************

NOQA            GGCGTACACGGACAGATCTCGTACGCGTGCCAGGAGTCCTGGCTCTTCTCCGCCACGGTG 1620
BTDELETED       GGCGTACACGGACAGATCTCGTACGCGTGCCAGGAGTCCTGGCTCTTCTCCGCCACGGTG 1619
BT              GGCGTACACGGACAGATCTCGTACGCGTGCCAGGAGTCCTGGCTCTTCTCCGCCACGGTG 1620
SBT             GGCGTACACGGACAGATCTCGTACGCGTGCCAGGAGTCCTGGCTCTTCTCCGCCACGGTG 1620
PA              GGCGTACACGGACAGATCTCGTACGCGTGCCAGGAGTCCTGGCTCTTCTCCGCCACGGTG 1620
PX              GGCGTACACGGACAGATCTCGTACGCGTGCCAGGAGTCCTGGCTCTTCTCCGCCACGGTG 1620
                ************************************************************

NOQA            CGGGACAACATCCTCTTCGGGCTGCCGTATGACTCCAAGAAATATAAGAAGGTGTGCGAC 1680
BTDELETED       CGGGACAACATCCTCTTCGGGCTGCCGTATGACTCCAAGAAATATAAGAAGGTGTGCGAC 1679
BT              CGGGACAACATCCTCTTCGGGCTGCCGTATGACTCCAAGAAATATAAGAAGGTGTGCGAC 1680
SBT             CGGGACAACATCCTCTTCGGGCTGCCGTATGACTCCAAGAAATATAAGAAGGTGTGCGAC 1680
PA              CGGGACAACATCCTCTTCGGGCTGCCGTATGACTCCAAGAAATATAAGAAGGTGTGCGAC 1680
PX              CGGGACAACATCCTCTTCGGGCTGCCGTATGACTCCAAGAAATATAAGAAGGTGTGCGAC 1680
                ************************************************************

NOQA            GCGTGCTGCCTGCAGCCTGACTTCAAGCAGTTCCCGTACGGAGACCTGTCGCTGGTGGGC 1740
BTDELETED       GCGTGCTGCCTGCAGCCTGACTTCAAGCAGTTCCCGTACGGAGACCTGTCGCTGGTGGGC 1739
BT              GCGTGCTGCCTGCAGCCTGACTTCAAGCAGTTCCCGTACGGAGACCTGTCGCTGGTGGGC 1740
SBT             GCGTGCTGCCTGCAGCCTGACTTCAAGCAGTTCCCGTACGGAGACCTGTCGCTGGTGGGC 1740
PA              GCGTGCTGCCTGCAGCCTGACTTCAAGCAGTTCCCGTACGGAGACCTGTCGCTGGTGGGC 1740
PX              GCGTGCTGCCTGCAGCCTGACTTCAAGCAGTTCCCGTACGGAGACCTGTCGCTGGTGGGC 1740
                ************************************************************

NOQA            GAGCGCGGCGTATCGCTGTCCGGGGGGCAGCGCGCACGGATCAACCTGGCCCGCGCCGTG 1800
BTDELETED       GAGCGCGGCGTATCGCTGTCCGGGGGGCAGCGCGCACGGATCAACCTGGCCCGCGCCGTG 1799
BT              GAGCGCGGCGTATCGCTGTCCGGGGGGCAGCGCGCACGGATCAACCTGGCCCGCGCCGTG 1800
SBT             GAGCGCGGCGTATCGCTGTCCGGGGGGCAGCGCGCACGGATCAACCTGGCCCGCGCCGTG 1800
PA              GAGCGCGGCGTATCGCTGTCCGGGGGGCAGCGCGCACGGATCAACCTGGCCCGCGCCGTG 1800
PX              GAGCGCGGCGTATCGCTGTCCGGGGGGCAGCGCGCACGGATCAACCTGGCCCGCGCCGTG 1800
                ************************************************************

NOQA            TATCGGGACGCCGACATCTACATATTCGACGACCCGCTGTCGGCGGTGGACGCGAATGTG 1860
BTDELETED       TATCGGGACGCCGACATCTACATATTCGACGACCCGCTGTCGGCGGTGGACGCGAATGTG 1859
BT              TATCGGGACGCCGACATCTACATATTCGACGACCCGCTGTCGGCGGTGGACGCGAATGTG 1860
SBT             TATCGGGACGCCGACATCTACATATTCGACGACCCGCTGTCGGCGGTGGACGCGAATGTG 1860
PA              TATCGGGACGCCGACATCTACATATTCGACGACCCGCTGTCGGCGGTGGACGCGAATGTG 1860
PX              TATCGGGACGCCGACATCTACATATTCGACGACCCGCTGTCGGCGGTGGACGCGAATGTG 1860
                ************************************************************

NOQA            GGTCGGCAGCTGTTCGAGGGCTGCATCAACGGCTACCTGCGCGGCCGCACGCGCGTGCTC 1920
BTDELETED       GGTCGGCAGCTGTTCGAGGGCTGCATCAACGGCTACCTGCGCGGCCGCACGCGCGTGCTC 1919
BT              GGTCGGCAGCTGTTCGAGGGCTGCATCAACGGCTACCTGCGCGGCCGCACGCGCGTGCTC 1920
SBT             GGTCGGCAGCTGTTCGAGGGCTGCATCAACGGCTACCTGCGCGGCCGCACGCGCGTGCTC 1920
PA              GGTCGGCAGCTGTTCGAGGGCTGCATCAACGGCTACCTGCGCGGCCGCACGCGCGTGCTC 1920
PX              GGTCGGCAGCTGTTCGAGGGCTGCATCAACGGCTACCTGCGCGGCCGCACGCGCGTGCTC 1920
                ************************************************************
```

```
NOQA          GTCACGCATCAGATACACTTCCTCAAGGCCGCAGACTACATAGTCATACTCAACGAGGGT 1980
BTDELETED     GTCACGCATCAGATACACTTCCTCAAGGCCGCAGACTACATAGTCATACTCAACGAGGGT 1979
BT            GTCACGCATCAGATACACTTCCTCAAGGCCGCAGACTACATAGTCATACTCAACGAGGGT 1980
SBT           GTCACGCATCAGATACACTTCCTCAAGGCCGCAGACTACATAGTCATACTCAACGAGGGT 1980
PA            GTCACGCATCAGATACACTTCCTCAAGGCCGCAGACTACATAGTCATACTCAACGAGGGT 1980
PX            GTCACGCATCAGATACACTTCCTCAAGGCCGCAGACTACATAGTCATACTCAACGAGGGT 1980
              ************************************************************

NOQA          GCCATTGAAAATATGGGCACGTACGATGATTTGACGAAGCTGGAGAATTCGCTGCTGCTG 2040
BTDELETED     GCCATTGAAAATATGGGCACGTACGATGATTTGACGAAGCTGGAGAATTCGCTGCTGCTG 2039
BT            GCCATTGAAAATATGGGCACGTACGATGATTTGACGAAGCTGGAGAATTCGCTGCTGCTG 2040
SBT           GCCATTGAAAATATGGGCACGTACGATGATTTGACGAAGCTGGAGAATTCGCTGCTGCTG 2040
PA            GCCATTGAAAATATGGGCACGTACGATGATTTGACGAAGCTGGAGAATTCGCTGCTGCTG 2040
PX            GCCATTGAAAATATGGGCACGTACGATGATTTGACGAAGCTGGAGAATTCGCTGCTGCTG 2040
              ************************************************************

NOQA          CCCAAGCAACAGGAGGGTTCCGGCGATGACAGTAAGGATGAACTAGCTATTCCAAATGCA 2100
BTDELETED     CCCAAGCAACAGGAGGGTTCCGGCGATGACAGTAAGGATGAACTAGCTATTCCAAATGCA 2099
BT            CCCAAGCAACAGGAGGGTTCCGGCGATGACAGTAAGGATGAACTAGCTATTCCAAATGCA 2100
SBT           CCCAAGCAACAGGAGGGTTCCGGCGATGACAGTAAGGATGAACTAGCTATTCCAAATGCA 2100
PA            CCCAAGCAACAGGAGGGTTCCGGCGATGACAGTAAGGATGAACTAGCTATTCCAAATGCA 2100
PX            CCCAAGCAACAGGAGGGTTCCGGCGATGACAGTAAGGATGAACTAGCTATTCCAAATGCA 2100
              ************************************************************

NOQA          GCAAAGAAGCCAATAATGGAACGGGGGATATCAGTGATCTCAGTGAAGAGTGAAGACAAC 2160
BTDELETED     GCAAAGAAGCCAATAATGGAACGGGGGATATCAGTGATCTCAGTGAAGAGTGAAGACAAC 2159
BT            GCAAAGAAGCCAATAATGGAACGGGGGATATCAGTGATCTCAGTGAAGAGTGAAGACAAC 2160
SBT           GCAAAGAAGCCAATAATGGAACGGGGGATATCAGTGATCTCAGTGAAGAGTGAAGACAAC 2160
PA            GCAAAGAAGCCAATAATGGAACGGGGGATATCAGTGATCTCAGTGAAGAGTGAAGACAAC 2160
PX            GCAAAGAAGCCAATAATGGAACGGGGGATATCAGTGATCTCAGTGAAGAGTGAAGACAAC 2160
              ************************************************************

NOQA          GGTGAGGCGCGCAAGGAGCAGGTGCAGGCGGCGGAGGAGCGCGCCTCCGGGAACCTCAAG 2220
BTDELETED     GGTGAGGCGCGCAAGGAGCAGGTGCAGGCGGCGGAGGAGCGCGCCTCCGGGAACCTCAAG 2219
BT            GGTGAGGCGCGCAAGGAGCAGGTGCAGGCGGCGGAGGAGCGCGCCTCCGGGAACCTCAAG 2220
SBT           GGTGAGGCGCGCAAGGAGCAGGTGCAGGCGGCGGAGGAGCGCGCCTCCGGGAACCTCAAG 2220
PA            GGTGAGGCGCGCAAGGAGCAGGTGCAGGCGGCGGAGGAGCGCGCCTCCGGGAACCTCAAG 2220
PX            GGTGAGGCGCGCAAGGAGCAGGTGCAGGCGGCGGAGGAGCGCGCCTCCGGGAACCTCAAG 2220
              ************************************************************

NOQA          TGGGAGGTGTTCGCGAGGTACCTGGTCTCCGTGGACTCCTGGGCCATCGTGGCGCTCACG 2280
BTDELETED     TGGGAGGTGTTCGCGAGGTACCTGGTCTCCGTGGACTCCTGGGCCATCGTGGCGCTCACG 2279
BT            TGGGAGGTGTTCGCGAGGTACCTGGTCTCCGTGGACTCCTGGGCCATCGTGGCGCTCACG 2280
SBT           TGGGAGGTGTTCGCGAGGTACCTGGTCTCCGTGGACTCCTGGGCCATCGTGGCGCTCACG 2280
PA            TGGGAGGTGTTCGCGAGGTACCTGGTCTCCGTGGACTCCTGGGCCATCGTGGCGCTCACG 2280
PX            TGGGAGGTGTTCGCGAGGTACCTGGTCTCCGTGGACTCCTGGGCCATCGTGGCGCTCACG 2280
              ************************************************************

NOQA          CTCACCGCGATGCTCATCACGCAGGGGGCGGCGTCCTCCACCGACTACTGGCTCAGCTTC 2340
BTDELETED     CTCACCGCGATGCTCATCACGCAGGGGGCGGCGTCCTCCACCGACTACTGGCTCAGCTTC 2339
BT            CTCACCGCGATGCTCATCACGCAGGGGGCGGCGTCCTCCACCGACTACTGGCTCAGCTTC 2340
SBT           CTCACCGCGATGCTCATCACGCAGGGGGCGGCGTCCTCCACCGACTACTGGCTCAGCTTC 2340
PA            CTCACCGCGATGCTCATCACGCAGGGGGCGGCGTCCTCCACCGACTACTGGCTCAGCTTC 2340
PX            CTCACCGCGATGCTCATCACGCAGGGGGCGGCGTCCTCCACCGACTACTGGCTCAGCTTC 2340
              ************************************************************
```

```
NOQA          TGGACAAATCAAGTTGATGGATACATACAAGACCTGCCAGATGGGGAGGAACCAGATCCA 2400
BTDELETED     TGGACAAATCAAGTTGATGGATACATACAAGACCTGCCAGATGGGGAGGAACCAGATCCA 2399
BT            TGGACAAATCAAGTTGATGGATACATACAAGACCTGCCAGATGGGGAGGAACCAGATCCA 2400
SBT           TGGACAAATCAAGTTGATGGATACATACAAGACCTGCCAGATGGGGAGGAACCAGATCCA 2400
PA            TGGACAAATCAAGTTGATGGATACATACAAGACCTGCCAGATGGGGAGGAACCAGATCCA 2400
PX            TGGACAAATCAAGTTGATGGATACATACAAGACCTGCCAGATGGGGAGGAACCAGATCCA 2400
              ************************************************************

NOQA          AGTCTCGACACGCAAACGGGCATCCTGCAGACGGGCCAGTACGTGTACATCTACGGCGCG 2460
BTDELETED     AGTCTCGACACGCAAACGGGCATCCTGCAGACGGGCCAGTACGTGTACATCTACGGCGCG 2459
BT            AGTCTCGACACGCAAACGGGCATCCTGCAGACGGGCCAGTACGTGTACATCTACGGCGCG 2460
SBT           AGTCTCGACACGCAAACGGGCATCCTGCAGACGGGCCAGTACGTGTACATCTACGGCGCG 2460
PA            AGTCTCGACACGCAAACGGGCATCCTGCAGACGGGCCAGTACGTGTACATCTACGGCGCG 2460
PX            AGTCTCGACACGCAAACGGGCATCCTGCAGACGGGCCAGTACGTGTACATCTACGGCGCG 2460
              ************************************************************

NOQA          CTGGTGCTCACCATCATCGTGATGTCCTTCATGCGTCTGTTCGGCTTCGTGACCATGACC 2520
BTDELETED     CTGGTGCTCACCATCATCGTGATGTCCTTCATGCGTCTGTTCGGCTTCGTGACCATGACC 2519
BT            CTGGTGCTCACCATCATCGTGATGTCCTTCATGCGTCTGTTCGGCTTCGTGACCATGACC 2520
SBT           CTGGTGCTCACCATCATCGTGATGTCCTTCATGCGTCTGTTCGGCTTCGTGACCATGACC 2520
PA            CTGGTGCTCACCATCATCGTGATGTCCTTCATGCGTCTGTTCGGCTTCGTGACCATGACC 2520
PX            CTGGTGCTCACCATCATCGTGATGTCCTTCATGCGTCTGTTCGGCTTCGTGACCATGACC 2520
              ************************************************************

NOQA          ATGCGCGCCGCCGCCAACATACACGACCTCATGTTCCGCAACCTCATCCGCGCCACCATG 2580
BTDELETED     ATGCGCGCCGCCGCCAACATACACGACCTCATGTTCCGCAACCTCATCCGCGCCACCATG 2579
BT            ATGCGCGCCGCCGCCAACATACACGACCTCATGTTCCGCAACCTCATCCGCGCCACCATG 2580
SBT           ATGCGCGCCGCCGCCAACATACACGACCTCATGTTCCGCAACCTCATCCGCGCCACCATG 2580
PA            ATGCGCGCCGCCGCCAACATACACGACCTCATGTTCCGCAACCTCATCCGCGCCACCATG 2580
PX            ATGCGCGCCGCCGCCAACATACACGACCTCATGTTCCGCAACCTCATCCGCGCCACCATG 2580
              ************************************************************

NOQA          CGCTTCTTTGATACTAATCCTTCAGGTCGCGTGCTTAACCGGTTCTCCAAAGACATGGGC 2640
BTDELETED     CGCTTCTTTGATACTAATCCTTCAGGTCGCGTGCTTAACCGGTTCTCCAAAGACATGGGC 2639
BT            CGCTTCTTTGATACTAATCCTTCAGGTCGCGTGCTTAACCGGTTCTCCAAAGACATGGGC 2640
SBT           CGCTTCTTTGATACTAATCCTTCAGGTCGCGTGCTTAACCGGTTCTCCAAAGACATGGGC 2640
PA            CGCTTCTTTGATACTAATCCTTCAGGTCGCGTGCTTAACCGGTTCTCCAAAGACATGGGC 2640
PX            CGCTTCTTTGATACTAATCCTTCAGGTCGCGTGCTTAACCGGTTCTCCAAAGACATGGGC 2640
              ************************************************************

NOQA          GGTATGGACGAGCTGCTGCCCAGGTCCATCCTGCAGGCCTTCCAGATGTACCTGTCCATG 2700
BTDELETED     GGTATGGACGAGCTGCTGCCCAGGTCCATCCTGCAGGCCTTCCAGATGTACCTGTCCATG 2699
BT            GGTATGGACGAGCTGCTGCCCAGGTCCATCCTGCAGGCCTTCCAGATGTACCTGTCCATG 2700
SBT           GGTATGGACGAGCTGCTGCCCAGGTCCATCCTGCAGGCCTTCCAGATGTACCTGTCCATG 2700
PA            GGTATGGACGAGCTGCTGCCCAGGTCCATCCTGCAGGCCTTCCAGATGTACCTGTCCATG 2700
PX            GGTATGGACGAGCTGCTGCCCAGGTCCATCCTGCAGGCCTTCCAGATGTACCTGTCCATG 2700
              ************************************************************

NOQA          GCCAGCGTGCTCACGCTGAACGCCGTCTCTCTGCCCTGGACCCTCATACCCACGGTGCTG 2760
BTDELETED     GCCAGCGTGCTCACGCTGAACGCCGTCTCTCTGCCCTGGACCCTCATACCCACGGTGCTG 2759
BT            GCCAGCGTGCTCACGCTGAACGCCGTCTCTCTGCCCTGGACCCTCATACCCACGGTGCTG 2760
SBT           GCCAGCGTGCTCACGCTGAACGCCGTCTCTCTGCCCTGGACCCTCATACCCACGGTGCTG 2760
PA            GCCAGCGTGCTCACGCTGAACGCCGTCTCTCTGCCCTGGACCCTCATACCCACGGTGCTG 2760
PX            GCCAGCGTGCTCACGCTGAACGCCGTCTCTCTGCCCTGGACCCTCATACCCACGGTGCTG 2760
              ************************************************************
```

```
NOQA            CTGCTGGGGCTCTTCATCAGGTACCTCAAGTGGTACCTGAACGCTGCGCAGTCTGTGAAG 2820
BTDELETED       CTGCTGGGGCTCTTCATCAGGTACCTCAAGTGGTACCTGAACGCTGCGCAGTCTGTGAAG 2819
BT              CTGCTGGGGCTCTTCATCAGGTACCTCAAGTGGTACCTGAACGCTGCGCAGTCTGTGAAG 2820
SBT             CTGCTGGGGCTCTTCATCAGGTACCTCAAGTGGTACCTGAACGCTGCGCAGTCTGTGAAG 2820
PA              CTGCTGGGGCTCTTCATCAGGTACCTCAAGTGGTACCTGAACGCTGCGCAGTCTGTGAAG 2820
PX              CTGCTGGGGCTCTTCATCAGGTACCTCAAGTGGTACCTGAACGCTGCGCAGTCTGTGAAG 2820
                ************************************************************

NOQA            AGACTGGAGGGTACAACCAAGAGTCCGGTGTTTGGAATGATCGGCTCTACTCTATCGGGA 2880
BTDELETED       AGACTGGAGGGTACAACCAAGAGTCCGGTGTTTGGAATGATCGGCTCTACTCTATCGGGA 2879
BT              AGACTGGAGGGTACAACCAAGAGTCCGGTGTTTGGAATGATCGGCTCTACTCTATCGGGA 2880
SBT             AGACTGGAGGGTACAACCAAGAGTCCGGTGTTTGGAATGATCGGCTCTACTCTATCGGGA 2880
PA              AGACTGGAGGGTACAACCAAGAGTCCGGTGTTTGGAATGATCGGCTCTACTCTATCGGGA 2880
PX              AGACTGGAGGGTACAACCAAGAGTCCGGTGTTTGGAATGATCGGCTCTACTCTATCGGGA 2880
                ************************************************************

NOQA            ATGTCGACAATCAGAAGTTCAGACTCACAGGACAGACTAATCAAAACCTTCGACGACTGC 2940
BTDELETED       ATGTCGACAATCAGAAGTTCAGACTCACAGGACAGACTAATCAAAACCTTCGACGACTGC 2939
BT              ATGTCGACAATCAGAAGTTCAGACTCACAGGACAGACTAATCAAAACCTTCGACGACTGC 2940
SBT             ATGTCGACAATCAGAAGTTCAGACTCACAGGACAGACTAATCAAAACCTTCGACGACTGC 2940
PA              ATGTCGACAATCAGAAGTTCAGACTCACAGGACAGACTAATCAAAACCTTCGACGACTGC 2940
PX              ATGTCGACAATCAGAAGTTCAGACTCACAGGACAGACTAATCAAAACCTTCGACGACTGC 2940
                ************************************************************

NOQA            CAGAATCTCCATACTTCTGCCTTCCACACTTACATCGGGGGAGCCACGGCCTTCGGATTC 3000
BTDELETED       CAGAATCTCCATACTTCTGCCTTCCACACTTACATCGGGGGAGCCACGGCCTTCGGATTC 2999
BT              CAGAATCTCCATACTTCTGCCTTCCACACTTACATCGGGGGAGCCACGGCCTTCGGATTC 3000
SBT             CAGAATCTCCATACTTCTGCCTTCCACACTTACATCGGGGGAGCCACGGCCTTCGGATTC 3000
PA              CAGAATCTCCATACTTCTGCCTTCCACACTTACATCGGGGGAGCCACGGCCTTCGGATTC 3000
PX              CAGAATCTCCATACTTCTGCCTTCCACACTTACATCGGGGGAGCCACGGCCTTCGGATTC 3000
                ************************************************************

NOQA            TATTTGGATATGATATGCCTCGTCTACCTCGCCTCTATATTATCAATTTTCATCCTTATC 3060
BTDELETED       TATTTGGATATGATATGCCTCGTCTACCTCGCCTCTATATTATCAATTTTCATCCTTATC 3059
BT              TATTTGGATATGATATGCCTCGTCTACCTCGCCTCTATATTATCAATTTTCATCCTTATC 3060
SBT             TATTTGGATATGATATGCCTCGTCTACCTCGCCTCTATATTATCAATTTTCATCCTTATC 3060
PA              TATTTGGATATGATATGCCTCGTCTACCTCGCCTCTATATTATCAATTTTCATCCTTATC 3060
PX              TATTTGGATATGATATGCCTCGTCTACCTCGCCTCTATATTATCAATTTTCATCCTTATC 3060
                ************************************************************

NOQA            GACTTCGCGGACGTGATCCCAGTGGGCAGCGTGGGGCTGGCGGTGAGCCAGTCCATGGTG 3120
BTDELETED       GACTTCGCGGACGTGATCCCAGTGGGCAGCGTGGGGCTGGCGGTGAGCCAGTCCATGGTG 3119
BT              GACTTCGCGGACGTGATCCCAGTGGGCAGCGTGGGGCTGGCGGTGAGCCAGTCCATGGTG 3120
SBT             GACTTCGCGGACGTGATCCCAGTGGGCAGCGTGGGGCTGGCGGTGAGCCAGTCCATGGTG 3120
PA              GACTTCGCGGACGTGATCCCAGTGGGCAGCGTGGGGCTGGCGGTGAGCCAGTCCATGGTG 3120
PX              GACTTCGCGGACGTGATCCCAGTGGGCAGCGTGGGGCTGGCGGTGAGCCAGTCCATGGTG 3120
                ************************************************************

NOQA            CTGACGGTGCTGCTGCAGCTGGCCGCGCGGTTCACCAGCGACTTCCTGGCGCAGATGACC 3180
BTDELETED       CTGACGGTGCTGCTGCAGCTGGCCGCGCGGTTCACCAGCGACTTCCTGGCGCAGATGACC 3179
BT              CTGACGGTGCTGCTGCAGCTGGCCGCGCGGTTCACCAGCGACTTCCTGGCGCAGATGACC 3180
SBT             CTGACGGTGCTGCTGCAGCTGGCCGCGCGGTTCACCAGCGACTTCCTGGCGCAGATGACC 3180
PA              CTGACGGTGCTGCTGCAGCTGGCCGCGCGGTTCACCAGCGACTTCCTGGCGCAGATGACC 3180
PX              CTGACGGTGCTGCTGCAGCTGGCCGCGCGGTTCACCAGCGACTTCCTGGCGCAGATGACC 3180
                ************************************************************
```

```
NOQA            GCCGTGGAGCGCGTGCTCGAGTACACTAAACTGCCGCACGAGGAGAATATTAATGATGGA  3240
BTDELETED       GCCGTGGAGCGCGTGCTCGAGTACACTAAACTGCCGCACGAGGAGAATATTAATGATGGA  3239
BT              GCCGTGGAGCGCGTGCTCGAGTACACTAAACTGCCGCACGAGGAGAATATTAATGATGGA  3240
SBT             GCCGTGGAGCGCGTGCTCGAGTACACTAAACTGCCGCACGAGGAGAATATTAATGATGGA  3240
PA              GCCGTGGAGCGCGTGCTCGAGTACACTAAACTGCCGCACGAGGAGAATATTAATGATGGA  3240
PX              GCCGTGGAGCGCGTGCTCGAGTACACTAAACTGCCGCACGAGGAGAATATTAATGATGGA  3240
                ************************************************************

NOQA            CCTACGCAGCCGCCCAAGACATGGCCTGCTGAAGGGAACATCAAATTTGAAAACGTGTTC  3300
BTDELETED       CCTACGCAGCCGCCCAAGACATGGCCTGCTGAAGGGAACATCAAATTTGAAAACGTGTTC  3299
BT              CCTACGCAGCCGCCCAAGACATGGCCTGCTGAAGGGAACATCAAATTTGAAAACGTGTTC  3300
SBT             CCTACGCAGCCGCCCAAGACATGGCCTGCTGAAGGGAACATCAAATTTGAAAACGTGTTC  3300
PA              CCTACGCAGCCGCCCAAGACATGGCCTGCTGAAGGGAACATCAAATTTGAAAACGTGTTC  3300
PX              CCTACGCAGCCGCCCAAGACATGGCCTGCTGAAGGGAACATCAAATTTGAAAACGTGTTC  3300
                ************************************************************

NOQA            CTGACTTACTCGTTGGAGGACCCACCCGTGCTCAAAAATATCAACTTCGAGATCCAAAGC  3360
BTDELETED       CTGACTTACTCGTTGGAGGACCCACCCGTGCTCAAAAATATCAACTTCGAGATCCAAAGC  3359
BT              CTGACTTACTCGTTGGAGGACCCACCCGTGCTCAAAAATATCAACTTCGAGATCCAAAGC  3360
SBT             CTGACTTACTCGTTGGAGGACCCACCCGTGCTCAAAAATATCAACTTCGAGATCCAAAGC  3360
PA              CTGACTTACTCGTTGGAGGACCCACCCGTGCTCAAAAATATCAACTTCGAGATCCAAAGC  3360
PX              CTGACTTACTCGTTGGAGGACCCACCCGTGCTCAAAAATATCAACTTCGAGATCCAAAGC  3360
                ************************************************************

NOQA            GGTTGGAAGGTGGGTGTGGTCGGCAGGACGGGGGCTGGGAAGTCATCTCTCATATCTGCC  3420
BTDELETED       GGTTGGAAGGTGGGTGTGGTCGGCAGGACGGGGGCTGGGAAGTCATCTCTCATATCTGCC  3419
BT              GGTTGGAAGGTGGGTGTGGTCGGCAGGACGGGGGCTGGGAAGTCATCTCTCATATCTGCC  3420
SBT             GGTTGGAAGGTGGGTGTGGTCGGCAGGACGGGGGCTGGGAAGTCATCTCTCATATCTGCC  3420
PA              GGTTGGAAGGTGGGTGTGGTCGGCAGGACGGGGGCTGGGAAGTCATCTCTCATATCTGCC  3420
PX              GGTTGGAAGGTGGGTGTGGTCGGCAGGACGGGGGCTGGGAAGTCATCTCTCATATCTGCC  3420
                ************************************************************

NOQA            CTTTTCCGATTGACCAATTTAGACGGAAGCATTAAAATTGATGGGATCGATACAATAGGA  3480
BTDELETED       CTTTTCCGATTGACCAATTTAGACGGAAGCATTAAAATTGATGGGATCGATACAATAGGA  3479
BT              CTTTTCCGATTGACCAATTTAGACGGAAGCATTAAAATTGATGGGATCGATACAATAGGA  3480
SBT             CTTTTCCGATTGACCAATTTAGACGGAAGCATTAAAATTGATGGGATCGATACAATAGGA  3480
PA              CTTTTCCGATTGACCAATTTAGACGGAAGCATTAAAATTGATGGGATCGATACAATAGGA  3480
PX              CTTTTCCGATTGACCAATTTAGACGGAAGCATTAAAATTGATGGGATCGATACAATAGGA  3480
                ************************************************************

NOQA            ATAGCTAAGCAGGAGCTCAGGGCGAAGATTTCAATTATTCCGCAAGAGCCTGTTCTGTTC  3540
BTDELETED       ATAGCTAAGCAGGAGCTCAGGGCGAAGATTTCAATTATTCCGCAAGAGCCTGTTCTGTTC  3539
BT              ATAGCTAAGCAGGAGCTCAGGGCGAAGATTTCAATTATTCCGCAAGAGCCTGTTCTGTTC  3540
SBT             ATAGCTAAGCAGGAGCTCAGGGCGAAGATTTCAATTATTCCGCAAGAGCCTGTTCTGTTC  3540
PA              ATAGCTAAGCAGGAGCTCAGGGCGAAGATTTCAATTATTCCGCAAGAGCCTGTTCTGTTC  3540
PX              ATAGCTAAGCAGGAGCTCAGGGCGAAGATTTCAATTATTCCGCAAGAGCCTGTTCTGTTC  3540
                ************************************************************

NOQA            TCGGCCACGCTGCGCTACAACCTGGACCCCTTCGACTTGTACAGTGATGACGACATTTGG  3600
BTDELETED       TCGGCCACGCTGCGCTACAACCTGGACCCCTTCGACTTGTACAGTGATGACGACATTTGG  3599
BT              TCGGCCACGCTGCGCTACAACCTGGACCCCTTCGACTTGTACAGTGATGACGACATTTGG  3600
SBT             TCGGCCACGCTGCGCTACAACCTGGACCCCTTCGACTTGTACAGTGATGACGACATTTGG  3600
PA              TCGGCCACGCTGCGCTACAACCTGGACCCCTTCGACTTGTACAGTGATGACGACATTTGG  3600
PX              TCGGCCACGCTGCGCTACAACCTGGACCCCTTCGACTTGTACAGTGATGACGACATTTGG  3600
                ************************************************************
```

```
NOQA            AGAGCTTTGGAACAGGTGGAGTTGAAAGACGTGGTTCCGGCGCTCGACTACAAAGTGTCG  3660
BTDELETED       AGAGCTTTGGAGCAGGTGGAGTTGAAAGACGTGGTTCCGGCGCTCGACTACAAAGTGTCG  3659
BT              AGAGCTTTGGAGCAGGTGGAGTTGAAAGACGTGGTTCCGGCGCTCGACTACAAAGTGTCG  3660
SBT             AGAGCTTTGGAGCAGGTGGAGTTGAAAGACGTGGTTCCGGCGCTCGACTACAAAGTGTCG  3660
PA              AGAGCTTTGGAGCAGGTGGAGTTGAAAGACGTGGTTCCGGCGCTCGACTACAAAGTGTCG  3660
PX              AGAGCTTTGGAGCAGGTGGAGTTGAAAGACGTGGTTCCGGCGCTCGACTACAAAGTGTCG  3660
                *********** **************************************************

NOQA            GAAGGAGGTAGCAATTTCTCCGTGGGACAGCGGCAGCTGCTGTGCCTGGCGCGCGCCGTG  3720
BTDELETED       GAAGGAGGTAGCAATTTCTCCGTGGGACAGCGGCAGCTGCTGTGCCTGGCGCGCGCCGTG  3719
BT              GAAGGAGGTAGCAATTTCTCCGTGGGACAGCGGCAGCTGCTGTGCCTGGCGCGCGCCGTG  3720
SBT             GAAGGAGGTAGCAATTTCTCCGTGGGACAGCGGCAGCTGCTGTGCCTGGCGCGCGCCGTG  3720
PA              GAAGGAGGTAGCAATTTCTCCGTGGGACAGCGGCAGCTGCTGTGCCTGGCGCGCGCCGTG  3720
PX              GAAGGAGGTAGCAATTTCTCCGTGGGACAGCGGCAGCTGCTGTGCCTGGCGCGCGCCGTG  3720
                ************************************************************

NOQA            CTGCGCTCCAACAAGATACTAGTCATGGACGAGGCCACCGCCAATGTCGACCCGCAGACG  3780
BTDELETED       CTGCGCTCCAACAAGATACTAGTCATGGACGAGGCCACCGCCAATGTCGACCCGCAGACG  3779
BT              CTGCGCTCCAACAAGATACTAGTCATGGACGAGGCCACCGCCAATGTCGACCCGCAGACG  3780
SBT             CTGCGCTCCAACAAGATACTAGTCATGGACGAGGCCACCGCCAATGTCGACCCGCAGACG  3780
PA              CTGCGCTCCAACAAGATACTAGTCATGGACGAGGCCACCGCCAATGTCGACCCGCAGACG  3780
PX              CTGCGCTCCAACAAGATACTAGTCATGGACGAGGCCACCGCCAATGTCGACCCGCAGACG  3780
                ************************************************************

NOQA            GACGCGCTGATCCAGTCGACGATCCGGCGGCAGTTCGCGGCCTGCACCGTGCTCACCATC  3840
BTDELETED       GACGCGCTGATCCAGTCGACGATCCGGCGGCAGTTCGCGGCCTGCACCGTGCTCACCATC  3839
BT              GACGCGCTGATCCAGTCGACGATCCGGCGGCAGTTCGCGGCCTGCACCGTGCTCACCATC  3840
SBT             GACGCGCTGATCCAGTCGACGATCCGGCGGCAGTTCGCGGCCTGCACCGTGCTCACCATC  3840
PA              GACGCGCTGATCCAGTCGACGATCCGGCGGCAGTTCGCGGCCTGCACCGTGCTCACCATC  3840
PX              GACGCGCTGATCCAGTCGACGATCCGGCGGCAGTTCGCGGCCTGCACCGTGCTCACCATC  3840
                ************************************************************

NOQA            GCACATCGTCTTAACACCGTCATGGACTCCGACCGAGTGCTCGTCATGGACAAGGGTGAA  3900
BTDELETED       GCACATCGTCTTAACACCGTCATGGACTCCGACCGAGTGCTCGTCATGGACAAGGGTGAA  3899
BT              GCACATCGTCTTAACACCGTCATGGACTCCGACCGAGTGCTCGTCATGGACAAGGGTGAA  3900
SBT             GCACATCGTCTTAACACCGTCATGGACTCCGACCGAGTGCTCGTCATGGACAAGGGTGAA  3900
PA              GCACATCGTCTTAACACCGTCATGGACTCCGACCGAGTGCTCGTCATGGACAAGGGTGAA  3900
PX              GCACATCGTCTTAACACCGTCATGGACTCCGACCGAGTGCTCGTCATGGACAAGGGTGAA  3900
                ************************************************************

NOQA            GTGGTGGAGTTCGACCACCCCTACACGCTGCTGTCGGCGGCCGGCAGCCACATCAACTTC  3960
BTDELETED       GTGGTGGAGTTCGACCACCCCTACACGCTGCTGTCGGCGGCCGGCAGCCACCTCAACTTC  3959
BT              GTGGTGGAGTTCGACCACCCCTACACGCTGCTGTCGGCGGCCGGCAGCCACCTCAACTTC  3960
SBT             GTGGTGGAGTTCGACCACCCCTACACGCTGCTGTCGGCGGCCGGCAGCCACCTCAACTTC  3960
PA              GTGGTGGAGTTCGACCACCCCTACACGCTGCTGTCGGCGGCCGGCAGCCACCTCAACTTC  3960
PX              GTGGTGGAGTTCGACCACCCCTACACGCTGCTGTCGGCGGCCGGCAGCCACCTCAACTTC  3960
                ************************************************** ********

NOQA            ATGGTGGAGGAGACCGGAGACAACATGAGCAAGGCCCTTTAT------------------  4002
BTDELETED       ATGGTGGAGGAGACCGGAGACAACATGAGCAAGGCCCTCTATGACATGGCCAAGAAGAAA  4019
BT              ATGGTGGAGGAGACCGGAGACAACATGAGCAAGGCCCTCTATGACATGGCCAAGAAGAAA  4020
SBT             ATGGTGGAGGAGACCGGAGACAACATGAGCAAGGCCCTCTATGACATGGCCAAGAAGAAA  4020
PA              ATGGTGGAGGAGACCGGAGACAACATGAGCAAGGCCCTCTATGACATGGCCAAGA-----  4015
PX              ATGGTGGAGGAGACCGGAGACAACATGAGCAAGGCCCTCTATGACATGCCA---------  4011
                ************************************ ***
```

```
NOQA            ------------------------    4002
BTDELETED       TACTTCGACGATCATCCTCAATGA    4043
BT              TACTTCGACGATCATCCTCAATGA    4044
SBT             TACTTCGACGATCATCCTCAATGA    4044
PA              ------------------------    4015
PX              ------------------------    4011
```

After we exclude the overlapping parts and aligned the gene sequences, we translated the DNA sequences in to protein sequences to see the changes on the reading frame. This 1 base pair deletion of nucleotides showed that the protein sequence stops sooner and changes the reading frame. This type of mutation is called indel and inevitably changes the frameshift of the protein. We also compared our populations sequences to others *P. xylostella* ABCC2 gene present in the GenBank database. The alignment is shown below.

Multiple protein sequence alignment:

```
Btdeletion          MENGSGARKESEEKKEVKKGKPNVLYRLFMCWVCPVLVGGNRRDVEERDLIPPPSAKYKS  60
NOQA_WAITE          -------RKESEEKKEVKKGKPNVLSRLFMCWVCPVLVGGNRRDVEERDLIPPPSAKYKS  53
Bt                  MENGSGARKESEEKKEVKKGKPNVLYRLFMCWVCPVLVGGNRRDVEERDLIPPPSAKYKS  60
Pa                  MENGSGARKESEEKKEVKKGKPNVLYRLFMCWVCPVLVGGNRRDVEERDLIPPPSAKYKS  60
SBt                 MENGSGARKESEEKKEVKKGKPNVLYRLFMCWVCPVLVGGNRRDVEERDLIPPPSAKYKS  60
Px                  MENGSGARKESEEKKEVKKGKPNVLYRLFMCWVCPVLVGGNRRDVEERDLIPPPSAKYKS  60
NOQA                MENGSGARKESEEKKEVKKGKPNVLSRLFMCWVCPVLVGGNRRDVEERDLIPPPSAKYKS  60
NP_001296082        MENGSGARKESEEKKEVKKGKPNVLSRLFMCWVCPVLVGGNRRDVEERDLIPPPSAKYKS  60
DBM1Ac-S            MENGSGARKESEEKKEVKKGKPNVLSRLFMCWVCPVLVGGNRRDVEERDLIPPPSAKYKS  60
G88                 MENGSGARKESEEKKEVKKGKPNVLSRLFMCWVCPVLVGGNRRDVEERDLIPPPSAKYKS  60
                           ****************** *********************************

Btdeletion          ESLGDKFERYWLEELGLATQRGVAPSLWRALRRAFWLSYMPGALLLLGNAIPRTIQPLLF  120
NOQA_WAITE          ESLGDKFERYWLEELGLATQRGVAPSLWRALRRAFWLSYMPGALLLLGNAIPRTIQPLLF  113
Bt                  ESLGDKFERYWLEELGLATQRGVAPSLWRALRRAFWLSYMPGALLLLGNAIPRTIQPLLF  120
Pa                  ESLGDKFERYWLEELGLATQRGVAPSLWRALRRAFWLSYMPGALLLLGNAIPRTIQPLLF  120
SBt                 ESLGDKFERYWLEELGLATQRGVAPSLWRALRRAFWLSYMPGALLLLGNAIPRTIQPLLF  120
Px                  ESLGDKFERYWLEELGLATQRGVAPSLWRALRRAFWLSYMPGALLLLGNAIPRTIQPLLF  120
NOQA                ESLGDKFERYWLEELGLATQRGVAPSLWRALRRAFWLSYMPGALLLLGNAIPRTIQPLLF  120
NP_001296082        ESLGDKFERYWLEELGLATQRGVAPSLWRALRRAFWLSYMPGALLLLGNAIPRTIQPLLF  120
DBM1Ac-S            ESLGDKFERYWLEELGLATQRGVAPSLWRALRRAFWLSYMPGALLLLGNAIPRTIQPLLF  120
G88                 ESLGDKFERYWLEELGLATQRGVAPSLWRALRRAFWLSYMPGALLLLGNAIPRTIQPLLF  120
                    ************************************************************
```

```
Btdeletion        TRLLSYWSADSTMTRLEAGYWAMGMLLCNFLAMVCHHHNTLFVGRFGMKVRIACCSLLYR  180
NOQA_WAITE        TRLLSYWSADSTMTRLEAGYWAMGMLLCNFLAMVCHHHNTLFVGRFGMKVRIACCSLLYR  173
Bt                TRLLSYWSADSTMTRLEAGYWAMGMLLCNFLAMVCHHHNTLFVGRFGMKVRIACCSLLYR  180
Pa                TRLLSYWSADSTMTRLEAGYWAMGMLLCNFLAMVCHHHNTLFVGRFGMKVRIACCSLLYR  180
SBt               TRLLSYWSADSTMTRLEAGYWAMGMLLCNFLAMVCHHHNTLFVGRFGMKVRIACCSLLYR  180
Px                TRLLSYWSADSTMTRLEAGYWAMGMLLCNFLAMVCHHHNTLFVGRFGMKVRIACCSLLYR  180
NOQA              TRLLSYWSADSTMTRLEAGYWAMGMLLCNFLAMVCHHHNTLFVGRFGMKVRIACCSLLYR  180
NP_001296082      TRLLSYWSADSTMTRLEAGYWAMGMLLCNFLAMVCHHHNTLFVGRFGMKVRIACCSLLYR  180
DBM1Ac-S          TRLLSYWSADSTMTRLEAGYWAMGMLLCNFLAMVCHHHNTLFVGRFGMKVRIACCSLLYR  180
G88               TRLLSYWSADSTMTRLEAGYWAMGMLLCNFLAMVCHHHNTLFVGRFGMKVRIACCSLLYR  180
                  ************************************************************

Btdeletion        KLLRLNQRSLQSTAAGKLVNLMSNDVARFDYAFMFLHYFWMIPLQSAAVLYFMFRAAGWA  240
NOQA_WAITE        KLLRLNQRSLQSTAAGKLVNLMSNDVARFDYAFMFLHYFWMIPLQSAAVLYFMFRAAGWA  233
Bt                KLLRLNQRSLQSTAAGKLVNLMSNDVARFDYAFMFLHYFWMIPLQSAAVLYFMFRAAGWA  240
Pa                KLLRLNQRSLQSTAAGKLVNLMSNDVARFDYAFMFLHYFWMIPLQSAAVLYFMFRAAGWA  240
SBt               KLLRLNQRSLQSTAAGKLVNLMSNDVARFDYAFMFLHYFWMIPLQSAAVLYFMFRAAGWA  240
Px                KLLRLNQRSLQSTAAGKLVNLMSNDVARFDYAFMFLHYFWMIPLQSAAVLYFMFRAAGWA  240
NOQA              KLLRLNQRSLQSTAAGKLVNLMSNDVARFDYAFMFLHYFWMIPLQSAAVLYFMFRAAGWA  240
NP_001296082      KLLRLNQRSLQSTAAGKLVNLMSNDVARFDYAFMFLHYFWMIPLQSAAVLYFMFRAAGWA  240
DBM1Ac-S          KLLRLNQRSLQSTAAGKLVNLMSNDVARFDYAFMFLHYFWMIPLQSAAVLYFMFRAAGWA  240
G88               KLLRLNQRSLQSTAAGKLVNLMSNDVARFDYAFMFLHYFWMIPLQSAAVLYFMFRAAGWA  240
                  ************************************************************

Btdeletion        PIVGLFSVMLLILPIQAGLTKLTAVYRRETAQRTDKRIKLMSEIINGIQVIKMYAWEVPF  300
NOQA_WAITE        PIVGLFSVMLLILPIQAGLTKLTAVYRRETAQRTDKRIKLMSEIINGIQVIKMYAWEVPF  293
Bt                PIVGLFSVMLLILPIQAGLTKLTAVYRRETAQRTDKRIKLMSEIINGIQVIKMYAWEVPF  300
Pa                PIVGLFSVMLLILPIQAGLTKLTAVYRRETAQRTDKRIKLMSEIINGIQVIKMYAWEVPF  300
SBt               PIVGLFSVMLLILPIQAGLTKLTAVYRRETAQRTDKRIKLMSEIINGIQVIKMYAWEVPF  300
Px                PIVGLFSVMLLILPIQAGLTKLTAVYRRETAQRTDKRIKLMSEIINGIQVIKMYAWEVPF  300
NOQA              PIVGLFSVMLLILPIQAGLTKLTAVYRRETAQRTDKRIKLMSEIINGIQVIKMYAWEVPF  300
NP_001296082      PIVGLFSVMLLILPIQAGLTKLTAVYRRETAQRTDKRIKLMSEIINGIQVIKMYAWEVPF  300
DBM1Ac-S          PIVGLFSVMLLILPIQAGLTKLTAVYRRETAQRTDKRIKLMSEIINGIQVIKMYAWEIPF  300
G88               PIVGLFSVMLLILPIQAGLTKLTAVYRRETAQRTDKRIKLMSEIINGIQVIKMYAWEIPF  300
                  ************************************************************:**

Btdeletion        QKVVGSSRAHEVEALKRASFVQGTFGGFMLFTERTSLFLTVMTLVLTGSMATATTVYPIQ  360
NOQA_WAITE        QKVVGSSRAHEVEALKRASFVQGTFGGFMLFTERTSLFLTVMTLVLTGSMATATTVYPIQ  353
Bt                QKVVGSSRAHEVEALKRASFVQGTFGGFMLFTERTSLFLTVMTLVLTGSMATATTVYPIQ  360
Pa                QKVVGSSRAHEVEALKRASFVQGTFGGFMLFTERTSLFLTVMTLVLTGSMATATTVYPIQ  360
SBt               QKVVGSSRAHEVEALKRASFVQGTFGGFMLFTERTSLFLTVMTLVLTGSMATATTVYPIQ  360
Px                QKVVGSSRAHEVEALKRASFVQGTFGGFMLFTERTSLFLTVMTLVLTGSMATATTVYPIQ  360
NOQA              QKVVGSSRAHEVEALKRASFVQGTFGGFMLFTERTSLFLTVMTLVLTGSMATATTVYPIQ  360
NP_001296082      QKVVGSSRAHEVEALKRASFVQGTFGGFMLFTERTSLFLTVMTLVLTGSMATATTVYPIQ  360
DBM1Ac-S          QKVVGSSRAHEVEALKRASFVQGTFGGFMLFTERTSLFLTVMTLVLTGSMATATTVYPIQ  360
G88               QKVVGSSRAHEVEALKRASFVQGTFGGFMLFTERTSLFLTVMTLVLTGSMATATTVYPIQ  360
                  ************************************************************

Btdeletion        QYFSIIQSNLALILPIAIAQLTEMLVSLERLQEFLMLDEREDLSVMPGGQADSAPVAFKY  420
NOQA_WAITE        QYFSIIQSNLALILPIAIAQLTEMLVSLERLQEFLMLDEREDLSVMPGGQADSAPVAFKY  413
Bt                QYFSIIQSNLALILPIAIAQLTEMLVSLERLQEFLMLDEREDLSVMPGGQADSAPVAFKY  420
Pa                QYFSIIQSNLALILPIAIAQLTEMLVSLERLQEFLMLDEREDLSVMPGGQADSAPVAFKY  420
SBt               QYFSIIQSNLALILPIAIAQLTEMLVSLERLQEFLMLDEREDLSVMPGGQADSAPVAFKY  420
Px                QYFSIIQSNLALILPIAIAQLTEMLVSLERLQEFLMLDEREDLSVMPGGQADSAPVAFKY  420
NOQA              QYFSIIQSNLALILPIAIAQLTEMLVSLERLQEFLMLDEREDLSVMPGGQADSAPVAFKY  420
NP_001296082      QYFSIIQSNLALILPIAIAQLTEMLVSLERLQEFLMLDEREDLSVMPGGQADSAPVAFKY  420
DBM1Ac-S          QYFSIIQSNLALILPIAIAQLTEMLVSLERLQEFLMLDEREDLSVMPGGQADTAPVAFKY  420
G88               QYFSIIQSNLALILPIAIAQLTEMLVSLERLQEFLMLDEREDLSVMPGGQTDSAPVAFKY  420
                  ************************************************:*:*******
```

```
Btdeletion        TKETTAPAYIVSKRYSKKEDDTGLAAELVERKSTSEFAVELNDVSASWGGEGDKDQHTLR  480
NOQA_WAITE        TKETTAPAYIVSKRYSKKEDDTGLAAELVERKATSEFAVELNDVSASWGGEGDKDQHTLR  473
Bt                TKETTAPAYIVSKRYSKKEDDTGLAAELVERKSTSEFAVELNDVSASWGGEGDKDQHTLR  480
Pa                TKETTAPAYIVSKRYSKKEDDTGLAAELVERKSTSEFAVELNDVSASWGGEGDKDQHTLR  480
SBt               TKETTAPAYIVSKRYSKKEDDTGLAAELVERKSTSEFAVELNDVSASWGGEGDKDQHTLR  480
Px                TKETTAPAYIVSKRYSKKEDDTGLAAELVERKSTSEFAVELNDVSASWGGEGDKDQHTLR  480
NOQA              TKETTAPAYIVSKRYSKKEDDTGLAAELVERKSTSEFAVELNDVSASWGGEGDKDQHTLR  480
NP_001296082      TKETTAPAYIVSKRYSKKEDDTGLAAELVERKSTSEFAVELNDVSASWGGEGDKDQHTLR  480
DBM1Ac-S          TKETTAPAYIVSKRYSKKEDDTGLAAELVERKSTSEFAVELNDVSASWGGEGDKDQHTLR  480
G88               TKETTAPAYIVSKRYSKKEDDTGLAAELVERKSTSEFAVELNDVSASWGGEGDKDQHTLR  480
                  ********************************:************************

Btdeletion        GVSMRVRRGKLAAIMDPWDPGSRRCSKCC------------------------------  509
NOQA_WAITE        GVSMRVRRGKLAAIIGPVGSGKSSLLQVLLKELPVSSGTVGVHGQISYACQESWLFSATV  533
Bt                GVSMRVRRGKLAAIIGPVGSGKSSLLQVLLKELPVSSGTVGVHGQISYACQESWLFSATV  540
Pa                GVSMRVRRGKLAAIIGPVGSGKSSLLQVLLKELPVSSGTVGVHGQISYACQESWLFSATV  540
SBt               GVSMRVRRGKLAAIIGPVGSGKSSLLQVLLKELPVSSGTVGVHGQISYACQESWLFSATV  540
Px                GVSMRVRRGKLAAIIGPVGSGKSSLLQVLLKELPVSSGTVGVHGQISYACQESWLFSATV  540
NOQA              GVSMRVRRGKLAAIIGPVGSGKSSLLQVLLKELPVSSGTVGVHGQISYACQESWLFSATV  540
NP_001296082      GVTLRVRRGKLAAIIGPVGSGKSSLLQVLLKEFPVSSGTVGVHGQISYACQESWLFSATV  540
DBM1Ac-S          GVSLRVRRGKLAAIIGPVGSGKSSLLQVLLKELPVSSGTVGVHGQISYACQESWLFSATV  540
G88               GVSLRVRRGKLAAIIGPVGSGKSSLLQVLLKELPVSSGTVGVHGQISYACQESWLFSATV  540
                  **::***********:.* . *.    :

Btdeletion        ------------------------------------------------------------  509
NOQA_WAITE        RDNILFGLPYDSKKYKKVCDACCLQPDFKQFPYGDLSLVGERGVSLSGGQRARINLARAV  593
Bt                RDNILFGLPYDSKKYKKVCDACCLQPDFKQFPYGDLSLVGERGVSLSGGQRARINLARAV  600
Pa                RDNILFGLPYDSKKYKKVCDACCLQPDFKQFPYGDLSLVGERGVSLSGGQRARINLARAV  600
SBt               RDNILFGLPYDSKKYKKVCDACCLQPDFKQFPYGDLSLVGERGVSLSGGQRARINLARAV  600
Px                RDNILFGLPYDSKKYKKVCDACCLQPDFKQFPYGDLSLVGERGVSLSGGQRARINLARAV  600
NOQA              RDNILFGLPYDSKKYKKVCDACCLQPDFKQFPYGDLSLVGERGVSLSGGQRARINLARAV  600
NP_001296082      RDNILFGLPYDSKKYKKVCDACCLQPDFKQFPYGDLSLVGERGVSLSGGQRARINLARAV  600
DBM1Ac-S          RDNILFGLPYDSKKYKKVCDACCLQPDFKQFPYGDLSLVGERGVSLSGGQRARINLARAV  600
G88               RDNILFGLPYDSKKYKKVCDACCLQPDFKQFPYGDLSLVGERGVSLSGGQRARINLARAV  600

Btdeletion        ------------------------------------------------------------  509
NOQA_WAITE        YRDADIYIFDDPLSAVDANVGRQLFEGCINGYLRGRTRVLVTHQIHFLKAADYIVILNEG  653
Bt                YRDADIYIFDDPLSAVDANVGRQLFEGCINGYLRGRTRVLVTHQIHFLKAADYIVILNEG  660
Pa                YRDADIYIFDDPLSAVDANVGRQLFEGCINGYLRGRTRVLVTHQIHFLKAADYIVILNEG  660
SBt               YRDADIYIFDDPLSAVDANVGRQLFEGCINGYLRGRTRVLVTHQIHFLKAADYIVILNEG  660
Px                YRDADIYIFDDPLSAVDANVGRQLFEGCINGYLRGRTRVLVTHQIHFLKAADYIVILNEG  660
NOQA              YRDADIYIFDDPLSAVDANVGRQLFEGCINGYLRGRTRVLVTHQIHFLKAADYIVILNEG  660
NP_001296082      YRDADIYIFDDPLSAVDANVGRQLFEGCINGYLRGRTRVLVTHQIHFLKAADYIVILNEG  660
DBM1Ac-S          YRDADIYIFDDPLSAVDANVGRQLFEGCINGYLRGRTRVLVTHQIHFLKAADYIVILNEG  660
G88               YRDADIYIFDDPLSAVDANVGRQLFEGCINGYLRGRTRVLVTHQIHFLKAADYIVILNEG  660

Btdeletion        ------------------------------------------------------------  509
NOQA_WAITE        AIENMGTYDDLTKLENSLLLPKQQEGSGDDSKDELAIPNAAKKPNMERGISVISVKSEDN  713
Bt                AIENMGTYDDLTKLENSLLLPKQQEGSGDDSKDELAIPNAAKKPIMERGISVISVKSEDN  720
Pa                AIENMGTYDDLTKLENSLLLPKQQEGSGDDSKDELAIPNAAKKPIMERGISVISVKSEDN  720
SBt               AIENMGTYDDLTKLENSLLLPKQQEGSGDDSKDELAIPNAAKKPIMERGISVISVKSEDN  720
Px                AIENMGTYDDLTKLENSLLLPKQQEGSGDDSKDELAIPNAAKKPIMERGISVISVKSEDN  720
NOQA              AIENMGTYDDLTKLENSLLLPKQQEGSGDDSKDELAIPNAAKKPIMERGISVISVKSEDN  720
NP_001296082      AIENMGTYDDLTKLENSLLLPKQQEGSGDDSKDELAIPNAAKKPIMERGVSVISVKSEDN  720
DBM1Ac-S          AIENMGTYDDLTKLENSLLLPKQQEGSGDDSKDELAIPNAANKPIMERGVSVISVKSEDN  720
G88               AIENMGTYDDLTKLENSLLLPKQQEGSGDDSKDELAVPNAANKPIMERGVSVVSVKSEDN  720
```

```
Btdeletion      ------------------------------------------------------------  509
NOQA_WAITE      GEARKEQVQAAEERASGNLKWEVFARYLVSVDSWAIVALTLTAMLITQGAASSTDYWLSF  773
Bt              GEARKEQVQAAEERASGNLKWEVFARYLVSVDSWAIVALTLTAMLITQGAASSTDYWLSF  780
Pa              GEARKEQVQAAEERASGNLKWEVFARYLVSVDSWAIVALTLTAMLITQGAASSTDYWLSF  780
SBt             GEARKEQVQAAEERASGNLKWEVFARYLVSVDSWAIVALTLTAMLITQGAASSTDYWLSF  780
Px              GEARKEQVQAAEERASGNLKWEVFARYLVSVDSWAIVALTLTAMLITQGAASSTDYWLSF  780
NOQA            GEARKEQVQAAEERASGNLKWEVFARYLVSVDSWAIVALTLTAMLITQGAASSTDYWLSF  780
NP_001296082    GEARKEQVQAAEERASGNLKWEVFARYLVSVDSWAIVALTLTAMLITQGAASSTDYWLSF  780
DBM1Ac-S        GEARKEQVQAAEERASGNLKWEVFARYLVSVDSWAIVALTLTAMLITQGAASSTDYWLSF  780
G88             GEARKEQVQAAEERASGNLKWEVFARYLVSVDSWAIVALTLTAMLITQGAASSTDYWLSF  780

Btdeletion      ------------------------------------------------------------  509
NOQA_WAITE      WTNQVDGYIQDLPDGEEPDPSLGTQTGILQTGQYVYIYGALVLTIIVMSFMRLFGFVTMT  833
Bt              WTNQVDGYIQDLPDGEEPDPSLDTQTGILQTGQYVYIYGALVLTIIVMSFMRLFGFVTMT  840
Pa              WTNQVDGYIQDLPDGEEPDPSLDTQTGILQTGQYVYIYGALVLTIIVMSFMRLFGFVTMT  840
SBt             WTNQVDGYIQDLPDGEEPDPSLDTQTGILQTGQYVYIYGALVLTIIVMSFMRLFGFVTMT  840
Px              WTNQVDGYIQDLPDGEEPDPSLDTQTGILQTGQYVYIYGALVLTIIVMSFMRLFGFVTMT  840
NOQA            WTNQVDGYIQDLPDGEEPDPSLDTQTGILQTGQYVYIYGALVLTIIVMSFMRLFGFVTMT  840
NP_001296082    WTNQVDGYIQDLPDGEEPDPSLGTQTGILLTGQYVYIYGALVLTIIVMSFMRLFGFVTMT  840
DBM1Ac-S        WTNQVDGYIQDLPDGEEPDPSLGTQTGILLTGQYVYIYGALVLTIIVMSFMRLFGFVTMT  840
G88             WTNQVDGYIQDLPDGEEPDPSLGTQTGILLTGQYVYIYGALVLTIIVMSFMRLFGFVTMT  840

Btdeletion      ------------------------------------------------------------  509
NOQA_WAITE      MRAAANIHDLMFRNLIRATMRFFDTNPSGRVLNRFSKDMGGMDELLPRSILQAFQMYLSM  893
Bt              MRAAANIHDLMFRNLIRATMRFFDTNPSGRVLNRFSKDMGGMDELLPRSILQAFQMYLSM  900
Pa              MRAAANIHDLMFRNLIRATMRFFDTNPSGRVLNRFSKDMGGMDELLPRSILQAFQMYLSM  900
SBt             MRAAANIHDLMFRNLIRATMRFFDTNPSGRVLNRFSKDMGGMDELLPRSILQAFQMYLSM  900
Px              MRAAANIHDLMFRNLIRATMRFFDTNPSGRVLNRFSKDMGGMDELLPRSILQAFQMYLSM  900
NOQA            MRAAANIHDLMFRNLIRATMRFFDTNPSGRVLNRFSKDMGGMDELLPRSILQAFQMYLSM  900
NP_001296082    MRAAANIHDLMFRNLIRATMRFFDTNPSGRVLNRFSKDMGGMDELLPRSILQAFQMYLSM  900
DBM1Ac-S        MRAAANIHDLMFRNLIRATMRFFDTNPSGRVLNRFSKDMGGMDELLPRSILQAFQMYLSM  900
G88             MRAAANIHDLMFRNLIRATMRFFDTNPSGRVLNRFSKDMGGMDELLPRSILQAFQMYLSM  900

Btdeletion      ------------------------------------------------------------  509
NOQA_WAITE      ASVLTLNAVSLPWTLIPTVLLLGLFIRYLKWYLNAAQSVKRLEGTTKSPVFGMIGSTLSG  953
Bt              ASVLTLNAVSLPWTLIPTVLLLGLFIRYLKWYLNAAQSVKRLEGTTKSPVFGMIGSTLSG  960
Pa              ASVLTLNAVSLPWTLIPTVLLLGLFIRYLKWYLNAAQSVKRLEGTTKSPVFGMIGSTLSG  960
SBt             ASVLTLNAVSLPWTLIPTVLLLGLFIRYLKWYLNAAQSVKRLEGTTKSPVFGMIGSTLSG  960
Px              ASVLTLNAVSLPWTLIPTVLLLGLFIRYLKWYLNAAQSVKRLEGTTKSPVFGMIGSTLSG  960
NOQA            ASVLTLNAVSLPWTLIPTVLLLGLFIRYLKWYLNAAQSVKRLEGTTKSPVFGMIGSTLSG  960
NP_001296082    ASVLTLNAVSLPWTLIPTVLLLGLFIRYLKWYLNAAQSVKRLEGTTKSPVFGMIGSTLSG  960
DBM1Ac-S        ASVLTLNAVSLPWTLIPTVLLLGLFIRYLKWYLNAAQSVKRLEGTTKSPVFGMIGSTLSG  960
G88             ASVLTLNAVSLPWTLIPTVLLLGLFIRYLKWYLNAAQSVKRLEGTTKSPVFGMIGSTLSG  960

Btdeletion      ------------------------------------------------------------  509
NOQA_WAITE      MSTIRSSDSQDRLIKNFDGCQNLHTSAFHTYIGGATAFGFYLDMICLVYLASILSIFILI  1013
Bt              MSTIRSSDSQDRLIKTFDDCQNLHTSAFHTYIGGATAFGFYLDMICLVYLASILSIFILI  1020
Pa              MSTIRSSDSQDRLIKTFDDCQNLHTSAFHTYIGGATAFGFYLDMICLVYLASILSIFILI  1020
SBt             MSTIRSSDSQDRLIKTFDDCQNLHTSAFHTYIGGATAFGFYLDMICLVYLASILSIFILI  1020
Px              MSTIRSSDSQDRLIKTFDDCQNLHTSAFHTYIGGATAFGFYLDMICLVYLASILSIFILI  1020
NOQA            MSTIRSSDSQDRLIKTFDDCQNLHTSAFHTYIGGATAFGFYLDMICLVYLASILSIFILI  1020
NP_001296082    MSTIRSSDSQDRLIKNFDDCQNLHTSAFHTYIGGATAFGFYLDMICLVYLASILSIFILI  1020
DBM1Ac-S        MSTIRSSDSQDRLIKNFDDCQNLHTSAFHTYIGGATAFGFYLDMICLVYLASILSIFILI  1020
G88             MSTIRSSDSQDRLIKNFDDCQNLHTSAFHTYIGGATAFGFYLDMICLVYLASILSIFILI  1020
```

```
Btdeletion      ------------------------------------------------------------  509
NOQA_WAITE      DFADVIPVGSV----------TVLLQLAARFTSDFLAQMTAVERVLEYTKLPHEENINDG  1063
Bt              DFADVIPVGSVGLAVSQSMVLTVLLQLAARFTSDFLAQMTAVERVLEYTKLPHEENINDG  1080
Pa              DFADVIPVGSVGLAVSQSMVLTVLLQLAARFTSDFLAQMTAVERVLEYTKLPHEENINDG  1080
SBt             DFADVIPVGSVGLAVSQSMVLTVLLQLAARFTSDFLAQMTAVERVLEYTKLPHEENINDG  1080
Px              DFADVIPVGSVGLAVSQSMVLTVLLQLAARFTSDFLAQMTAVERVLEYTKLPHEENINDG  1080
NOQA            DFADVIPVGSVGLAVSQSMVLTVLLQLAARFTSDFLAQMTAVERVLEYTKLPHEENINDG  1080
NP_001296082    DFADVIPVGSVGLAVSQSMVLTVLLQLAARFTSDFLAQMTAVERVLEYTKLPHEENINDG  1080
DBM1Ac-S        DFADVIPVGSVGLAVSQSMVLTVLLQLAARFTSDFLAQMTAVERVLEYTKLPHEENINDG  1080
G88             DFADVIPVGSVGLAVSQSMVLTVLLQLAARFTSDFLAQMTAVERVLEYTKLPHEENINDG  1080

Btdeletion      ------------------------------------------------------------  509
NOQA_WAITE      PTQPPKTWPAEGNIKFENVFLTYSLEDPPVLKNINFEIQSGWKVGVVGRTGAGKSSLISA  1123
Bt              PTQPPKTWPAEGNIKFENVFLTYSLEDPPVLKNINFEIQSGWKVGVVGRTGAGKSSLISA  1140
Pa              PTQPPKTWPAEGNIKFENVFLTYSLEDPPVLKNINFEIQSGWKVGVVGRTGAGKSSLISA  1140
SBt             PTQPPKTWPAEGNIKFENVFLTYSLEDPPVLKNINFEIQSGWKVGVVGRTGAGKSSLISA  1140
Px              PTQPPKTWPAEGNIKFENVFLTYSLEDPPVLKNINFEIQSGWKVGVVGRTGAGKSSLISA  1140
NOQA            PTQPPKTWPAEGNIKFENVFLTYSLEDPPVLKNINFEIQSGWKVGVVGRTGAGKSSLISA  1140
NP_001296082    PTQPPKTWPAEGNIKFENVFLTYSLEDPPVLKNINFEIQSGWKVGVVGRTGAGKSSLISA  1140
DBM1Ac-S        PTQPPKTWPAEGNIKFENVFLTYSLEDPPVLKNINFEIQSGWKVGVVGRTGAGKSSLISA  1140
G88             PTQPPKTWPSQGNIKFENVFLTYSLEDPPVLKNINFEIQSGWKVGVVGRTGAGKSSLISA  1140

Btdeletion      ------------------------------------------------------------  509
NOQA_WAITE      LFRLTNLDGSIKIDGIDTIGIAKQELRAKISIIPQEPVLFSATLRYNLDPFDLYSDDDIW  1183
Bt              LFRLTNLDGSIKIDGIDTIGIAKQELRAKISIIPQEPVLFSATLRYNLDPFDLYSDDDIW  1200
Pa              LFRLTNLDGSIKIDGIDTIGIAKQELRAKISIIPQEPVLFSATLRYNLDPFDLYSDDDIW  1200
SBt             LFRLTNLDGSIKIDGIDTIGIAKQELRAKISIIPQEPVLFSATLRYNLDPFDLYSDDDIW  1200
Px              LFRLTNLDGSIKIDGIDTIGIAKQELRAKISIIPQEPVLFSATLRYNLDPFDLYSDDDIW  1200
NOQA            LFRLTNLDGSIKIDGIDTIGIAKQELRAKISIIPQEPVLFSATLRYNLDPFDLYSDDDIW  1200
NP_001296082    LFRLTNLDGSIKIDGIDTIGIAKQELRAKISIIPQEPVLFSATLRYNLDPFDLYSDDDIW  1200
DBM1Ac-S        LFRLTNLDGSIKIDGIDTIGIAKQELRAKISIIPQEPVLFSATLRYNLDPFDLYSDDDIW  1200
G88             LFRLTNLDGSIKIDGIDTIGIAKQELRAKISIIPQEPVLFSATLRYNLDPFDLYSDDDIW  1200

Btdeletion      ------------------------------------------------------------  509
NOQA_WAITE      RALEQVELKDVVPALDYKVSEGGSNFSVGQRQLLCLARAVLRSNKILVMDEATANVDPQT  1243
Bt              RALEQVELKDVVPALDYKVSEGGSNFSVGQRQLLCLARAVLRSNKILVMDEATANVDPQT  1260
Pa              RALEQVELKDVVPALDYKVSEGGSNFSVGQRQLLCLARAVLRSNKILVMDEATANVDPQT  1260
SBt             RALEQVELKDVVPALDYKVSEGGSNFSVGQRQLLCLARAVLRSNKILVMDEATANVDPQT  1260
Px              RALEQVELKDVVPALDYKVSEGGSNFSVGQRQLLCLARAVLRSNKILVMDEATANVDPQT  1260
NOQA            RALEQVELKDVVPALDYKVSEGGSNFSVGQRQLLCLARAVLRSNKILVMDEATANVDPQT  1260
NP_001296082    RALEQVELKDVVPALDYKVSEGGSNFSVGQRQLLCLARAVLRSNKILVMDEATAHVDPQT  1260
DBM1Ac-S        RALEQVELKDVVPALDYKVSEGGSNFSVGQRQLLCLARAVLRSNKILVMDEATANVDPQT  1260
G88             RALEQVELKDVVPALDYKVSEGGSNFSVGQRQLLCLARAVLRSNKILVMDEATANVDPQT  1260

Btdeletion      ------------------------------------------------------------  509
NOQA_WAITE      DALIQSTIRRQFAACTVLTIAHRLNTVMDSDRVLVMDKGEVVEFDHPYTLLSAPGSHLNF  1303
Bt              DALIQSTIRRQFAACTVLTIAHRLNTVMDSDRVLVMDKGEVVEFDHPYTLLSAAGSHLNF  1320
Pa              DALIQSTIRRQFAACTVLTIAHRLNTVMDSDRVLVMDKGEVVEFDHPYTLLSAAGSHLNF  1320
SBt             DALIQSTIRRQFAACTVLTIAHRLNTVMDSDRVLVMDKGEVVEFDHPYTLLSAAGSHLNF  1320
Px              DALIQSTIRRQFAACTVLTIAHRLNTVMDSDRVLVMDKGEVVEFDHPYTLLSAAGSHLNF  1320
NOQA            DALIQSTIRRQFAACTVLTIAHRLNTVMDSDRVLVMDKGEVVEFDHPYTLLSAAGSHINF  1320
NP_001296082    DALIQSTIRRQFAACTVLTIAHRLNTVMDSDRVLVMDKGEVVEFDHPYTLLSAAGSHLNF  1320
DBM1Ac-S        DALIQSTIRRQFAACTVLTIAHRLNTVMDSDRVLVMDKGEVVEFDHPYTLLSAPGSHLNF  1320
G88             DALIQSTIRRQFAACTVLTIAHRLNTVMDSDRVLVMDKGEVVEFDHPYTLLSAPGSHLNF  1320
```

```
Btdeletion          --------------------------  509
NOQA_WAITE          MVEETGDNM-----------------  1312
Bt                  MVEETGDNMSKALYDMAKKKYFDDHPQ  1347
Pa                  MVEETGDNMSKALYDMAK---------  1338
SBt                 MVEETGDNMSKALYDMAKKKYFDDHPQ  1347
Px                  MVEETGDNMSKALYDMP----------  1337
NOQA                MVEETGDNMSKALY-------------  1334
NP_001296082        MVEETGDNMSKALYDMAKKKYFDDHPQ  1347
DBM1Ac-S            MVEETGDNMSKALYDMAKKKYFDDHPQ  1347
G88                 MVEETGDNMSKALYDMAKKKYFDDHPQ  1347
```

There are some differences between the populations tested on this research and others from the database and besides the deletion of 1 base par on the Bt population, the other interesting thing that we found is that the NO-QA population that is supposed to have a deletion (BAXTER, 2011), does not have any mutations.

The ABCC2 protein has two distinct sections, which mirror each other and is comprised of a transmembrane domain (TMD) containing multiple transmembrane regions and a nucleotide-binding domain (NBD). The TMDs and NBDs were arranged in the N- to C-terminus order of TMD-NBD-TMD-NBD, which is the classical domain architecture of a full transporter. By coupling and hydrolyzing ATP, NBDs provide energy and work together with TMDs to remove excessive substrates. Generally, two molecules of ATP are consumed during a single transportation cycle (WILKENS, 2015). To better show the extent of the deletion has on the protein we did a schematic of the ABCC2 demonstrating the deleted region (Figure 21).

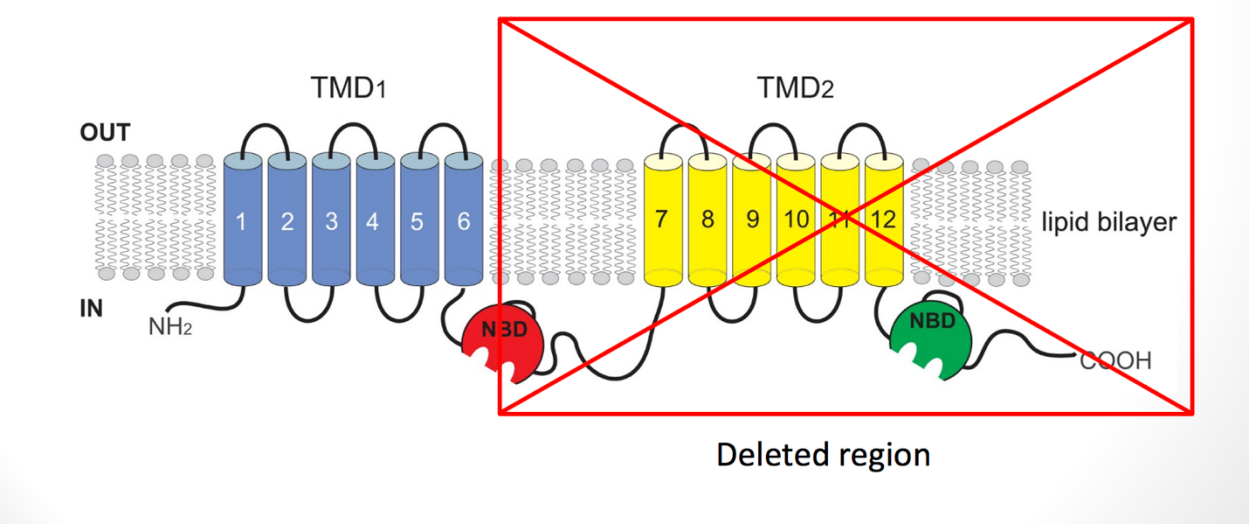

Figure 21: Schematic of ABCC2 displaying 12 transmembrane domains predicted using Phobius (http://phobius.sbc.su.se/) and two nucleotide-binding domains (NBD), showing the deleted region, in the Bt-resistant individuals, of the protein.

From the 1 base par deletion, the reading will be changed and, the frameshift mutation will result in the synthesis of a nonfunctional protein.

4.10. Backcrossing the resistant population with the lab susceptible population

We have sequenced the ABCC2 DNA from five larvae from the 4th generation back cross. Two possible outcomes are as follows, (DNA positions in the ABCC2 gene are shown):

a) Px sequence without T deletion:

1459 ATC A**T**C GGA CCC GT 1472

b) Px sequence with T deletion:

1459 ATC ACG GAC CCG TG 1473

The results below showed that there was no deletion of T1463. The highlighted region shows the sequence used to search for the region of interest. In bold and underlined is the T that was found deleted in the Bt population and the bold region is normally found deleted in the ABCC2 gene of the NO-QA population. The 4GA1 sequencing results show that this T is still present.

>4GA1

AGCTGACGACGTGAGTGCGTCGTGGGGGGGTGAGGGGGACAAGGACCAGCACAC
GCTGCGCGGGGTGTCGATGCGCGTGCGCCGCGGCAAGCTGGCCGCCATCA**T**CGG
ACCCGTGGGATCCGGGAAGACCTTTAT

As the deletion was not found, the reamplification method was used on 4 additional fourth generation alive larvae: 4GA3, 4GA4, 4GA5, 4GA6 to obtain PCR product to be sequenced. Following reamplification, all 4 samples 4GA3-4GA6 now contained a band at 168bp as expected. All four of these would now be sequenced just like 4GA1. The sequencing results of 4GA4, 4GA5 and 4GA6 all show that there was also no deletion of T1463 in the ABCC2 gene. 4GA3 had problems with the sequencing so the sequence was not obtained. The sequencing results of 4GA4, 4GA5 and 4GA6 are shown below, underlined and in bold shows that T1463 is still present:

>4GA4

GGTCGAGCTGAACGACGTGAGTGCGTCGTGGGGGGGGTGTAAGGGGGACAAGGA
CCAGCACACGCTGCGCGGGGTGTCGATGCGCGTGCGCCGCGGCAAGCTGGCCG
CCATCA**T**CGGACCCGTGGGATCCGGGAAGAAGAACTTT

>4GA5
GCGTCGTGGGGGGGGTGAAGGGGGACAAGGACCAGCACACGCTGCGCGGGGTG
TCGATGCGCGTGCGCCGCGGCAAGCTGGCCGCCATCATCGGACCCGTGGGATCC
GGGAAGATGAGAAT

>4GA6
CGTCGTGGGGGGGGTGAAGGGGGACAAGGACCAGCACACGCTGCGCGGGGTGT
CGATGCGCGTGCGCCGCGGCAAGCTGGCCGCCATCATCGGACCCGTGGGATCCG
GGAAGACAGTCTTT

4.11. Complementation tests between Bt and NO-QAGE

The LC_{50} in the first generations, both for Cry1Ac and HD1, showed that the Bt and NO-QA populations are still resistant after the crossing (Tables 8 to 11). Therefore, this complementation test indicated that the resistance Brazilian population shares a resistance allele with NO-QA, where resistance has been linked to a deletion in ABCC2.

Table 8. Probit analysis concentration-mortality response with Cry1Ac protein of *Bacillus thuringiensis* against second-instar *Plutella xylostella* larvae of crossing between NO-QA resistant male population X Bt Brazilian resistant female population.

Generations	n[a]	LC_{50} (95% C.I.) (µg/mL)	Slope ± SE	χ^2	DF	P-value
First	60	2.08 (1.75-2.44)	2.02 ± 0.23	4.57	4	0.3345
Second	60	0.37 (0.13-0.63)	1.07 ± 0.20	6.78	6	0.3417
Sixth			Not available			
Seventh	60	2.12 (1.68-2.63)	1.47 ± 0.20	7.63	4	0.1301
Eighth	60	11.5 (7.15-45.92)	0.98 ± 0.27	5.19	3	0.1582

[a] n = number of insects per concentration.

Table 9. Probit analysis concentration-mortality response with Cry1Ac protein of *Bacillus thuringiensis* against second-instar *Plutella xylostella* larvae of crossing between NO-QA resistant female population X Bt Brazilian resistant male population.

Generations	n[a]	LC_{50} (95% C.I.) (µg/mL)	Slope ± SE	χ^2	DF	P-value
First	60	1.73 (1.18-2.29)	2.24 ± 0.21	2.43	3	0.4883
Second	60	0.25 (0.12-0.39)	1.50 ± 0.25	5.53	4	0.2370
Sixth	60	2.05 (1.60-2.81)	1.37 ± 0.23	2.74	3	0.4333
Seventh	60	6.66 (4.49-12.29)	0.95 ± 0.60	1.08	4	0.3662
Eighth	60	7.78 (2.67-20.51)	0.52 ± 0.16	4.68	4	0.3224

[a] n = number of insects per concentration.

Table 10. Probit analysis concentration-mortality response with HD-1 strain of *Bacillus thuringiensis* against second-instar *Plutella xylostella* larvae of crossing between NO-QA resistant male population X Bt Brazilian resistant female population.

Generations	n[a]	LC_{50} (95% C.I.) (spores/mL)	Slope ± SE	χ^2	DF	P-value
First	60	2.6×10^6 (1.4×10^6-5.7×10^6)	0.55 ± 0.09	5.79	4	0.2156
Second	60	7.1×10^4 (2.07×10^5-4.5×10^5)	0.79 ± 0.23	6.75	3	0.0803
Sixth	60	1.1×10^6 (7.9×10^5-1.6×10^6)	0.75 ± 0.08	5.10	6	0.5308
Seventh	60	1.9×10^6 (1.2×10^6-3.1×10^6)	1.19 ± 0.16	11.92	6	0.0636

[a] n = number of insects per concentration.

Table 11. Probit analysis concentration-mortality response with HD-1 strain of *Bacillus thuringiensis* against second-instar *Plutella xylostella* larvae of crossing between NO-QA resistant female population X Bt Brazilian resistant male population.

Generations	n[a]	LC_{50} (95% C.I.) (spores/mL)	Slope ± SE	χ^2	DF	P-value
First	60	$2.3x10^6$ ($1.3x10^6$-$4.4x10^6$)	1.00 ± 0.16	9.67	5	0.0850
Second	60	Not available				
Sixth	60	$1.6x10^6$ ($1.1x10^6$-$2.3x10^6$)	1.07 ± 0.13	4.78	3	0.1882
Seventh	60	$1.2x10^7$ ($1.2x10^7$-$8.2x10^9$)	0.46 ± 0.15	1.88	3	0.1307

[a] n = number of insects per concentration.

There are no genetic reason why the LC_{50} drop in the 2nd generation. However our abcc2 sequencing did suggest that the Bt population was not fully resistant – i.e. we saw a mixture of mutated and non-mutated sequences. If there is a fitness cost associated with resistance in the Bt population then there might be an enrichment for non-resistant insects. Yet, after the sixth generation the LC_{50} increases, indicating that resistance in Bt population is also in ABCC2.

5. DISCUSSION

Resistance of insects to Bt toxins has been mainly observed in the laboratory, including species such as *P. interpunctella*, *C. virescens*, *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae), *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera: Crambidae), and *Culex quinquefasciatus* Say, 1823 (Diptera: Culicidae). Until recently only *P. xylostella* has shown field resistance and resistant insects have been selected from fields in Hawaii, Florida, Pennsylvania, Indonesia, Malaysia, Central America, several states of USA, and Philippines (SCHNEPF et al., 1998).

Due to many studies performed with Bt, there are a number of models to explain the mode of action of the toxins produced by this microorganism. As a result, the resistance development seems to involve various mechanisms, depending on the type of insect, the toxin, and the Bt strain. Reduced solubilization of the protoxin, alteration in the proteolytic processing, elimination of active toxin by precipitation and/or degradation, reduction of binding of the toxin to the membrane receptors in the midgut of insects, ABBC2 mutations in this gene, have been reported as mechanisms of resistance. Combination of these mechanisms might be involved in acquisition of resistance; however, it is possible that other mechanisms may be involved as well.

High levels of resistance to Cry1 toxins, due to reduced toxin binding, have been genetically linked to mutations or expression alterations of receptor genes. Many of these genes are potentially involved in protecting the insect against Bt toxins, since they respond to the pathogen once the larvae ingest the toxin. The diversity of genes that are differently expressed in the midgut of resistant insects, implying in a variety of cell processes that are related to the resistance preservation (AYRA-PARDO et al., 2015). Some of these genes were tested in this study.

According to the literature (GUO et al., 2015)., resistant populations tested, would show down-regulation ALP, APN, ABCC2 genes and up-regulation of CDKAL1 and MAPK genes; the genes tested in this research demonstrate that there are no patterns in their expression in the resistant and/or the susceptible populations tested. Even

though previous reports supported that differential expressions of these genes are linked to loss toxin binding to the receptor and consequently resistance. Therefore we cannot conclude that the down-regulation of ALP, APN and ABCC2 genes and/or the up-regulation of CDKAL1 and MAPK are linked to resistant strain tested in this work.

Since it was proposed that the role of ABCC2 is a receptor protein involved in facilitating insertion of Cry1A oligomers into the membrane (HECKEL, 2012). The toxin has been proposed to bind to the open state of the ABCC2 transporter; closure of the channel then forces the toxin into the cell membrane where it forms a pore. Mutations in these genes were liked to create resistant colonies from different insect species such as *P. xyostella*, *T. ni* (BAXTER et al., 2011) and *B. mori* (ATSUMI et al., 2012).

Alternatively ABCC2 might not be acting as a receptor but mutations in its gene could indirectly affect susceptibility to Bt. One possibility is that an altered function of ABCC2 could mimic a pathogen attack and result in physiological changes that protect the cell from toxin. Such a change might be the down-regulation of a receptor gene.

Our results didn't demonstrate any link between Bt resistance and an increase of basal expression of CDKAL1 and MAPK genes or/and a decrease of ALP, APN, ABCC2 genes.

Since no pattern was found on expression of genes linked to Bt resistance we decided to sequence the whole ABCC2 gene; as the ABCC2 gene is correlated with resistance to Cry1Ac in *C. virescens* (BAXTER et al., 2010), and mutations on this gene have been shown to cause Bt resistance in several other insect species, and in particular a deletion was identified in a resistant population of *P. xylostella* (BAXTER, 2011), it was decided to check whether or not there were such deletions or other mutations in this region in the populations under test. When we sequenced the Bt population it showed two different sequences, one of which has a 1 base pair deletion. Clearly this deletion is responsible for the resistant phenotype. We considered three explanations as to why there was a mixture of sequences:

1) That there had been differential splicing – if this region lay across a splice site then perhaps some exons were not joining properly. We checked this though and the region was in the middle of exon 9.

2) That the resistance allele is not recessive but dominant or partially dominant. In this case individuals that are heterozygous for the deletion would also be resistant. Possible, but unlikely given what we know about *P. xylostella* resistance.

3) That the population isn't fully resistant and that we extracted mRNA from a mixture of homozygotes and heterozygotes. This seems the most likely explanation. The best way to test that would be to extract mRNA only from individuals that had survived a reasonable dose of toxin.

The backcrossing assays was supposed to confirm that the resistance was due to the 1bp deletion, unfortunately that wasn't the case. The results showed that there was no deletion of T1463. There are two immediate explanations:

1) The deletion is not causing the resistance phenotype. This would be consistent with our "NO-QA" population that is resistant but doesn't have the 30bp deletion. It rather brings us back to stage one in terms of what is causing the resistance. If it's not a mutation, and not loss of expression, then what is it?

2) The second possibility is that the dose of toxin is not high enough to kill all of the homozygous/heterozygous susceptible larvae. By classic genetics 25% of the larvae in the F2 self-cross should be resistant and therefore the dose of toxin used should be killing 75% of the larvae.

On the other hand, the complementation assays indicated that the resistant Brazilian population shares a resistance allele with NO-QA, where resistance has been linked to a deletion in ABCC2. This indication was due to the LC_{50} that was still high after the crossing, both for Cry1Ac and HD1.

Our resistant population became resistant with HD1 selection that consists of 4 toxins, such as Cry1Ac, Cry1Ab, Cry1Aa, Cry2Aa (LIU et al., 1996). Does this population has cross resistance to others toxins that are not present in the HD1 strain? This question could be answered by testing other toxins and/or products that vanquish this resistance. Pathogenicity and virulence assays should be carried out to discover novel toxins that are active against this insect strain (STEPHEN; ELKINGTON, 2004). If there are other alternative toxins and/or products that kills the resistance phenotype, this

could be applied in Genetically Modified Plants (YI et al., 2011). The toxins that are active against this mutated *P. xylostella* could be incorporated in cruciferous plants.

Also, genetic manipulation of Bt toxins for improvement on toxicity can be carried out to overcome this resistance. Domain switch is a possibility to produce toxins that are more toxic and by this killing *P. xylostella* that are resistant (DEIST et al., 2014). So, research on other alternative methods should be carried out to overcome this resistance, improving tactics for *P. xylostella* Integrated Pest Management.

5. CONCLUSIONS

- NO-QA and Bt populations are proven to be resistant;
- No link between Bt resistance and an increase of basal expression of CDKAL1 and MAPK genes or/and a decrease of ALP, APN, ABCC2 genes were found;
- A 1 base pair deletion was found in the ABCC2 gene of the resistant Brazilian *Plutella xylostella* population;
- Clearly this deletion is responsible for the resistant phenotype;
- Backcrossing assays didn't confirm that the resistance was due to the 1bp deletion;
- Complementation assays indicated that the resistant Brazilian population shares a resistance allele with NO-QA;
- The mechanism of Bt resistance in *P. xylostella* is via mutations in the same locus that results in mutation of ABCC2.

7. REFERENCES

AGAISSE, H.; LERECLUS, D. How does *Bacillus thuringiensis* produce so much insecticidal crystal protein. **Journal of Bacteriology.** Washington DC, v. 177, n. 21, p. 6027-6032, 1995.

ALVES, S. B. Patologia e controle microbiano: vantagens e desvantagens. In: ALVES, S. B. (Ed.). **Controle microbiano de insetos**. Piracicaba: FEALQ, p. 21-37, 1998.

AMANCHI, N. R.; HUSSAIN, M. M. Cytotoxic effects of delfin insecticide (*Bacillus thuringiensis*) on cell behavior, phagocytosis, contractile vacuole activity and macronucleus in a protozoan ciliate *Paramecium caudatum*. **African Journal of Biotechnology**, Pretoria, v. 7, n. 15, p. 2637-2643, 2008.

ANGUS, T. A. The use of *Bacillus thuringiensis* as a microbial insecticide. **World Review of Pest Control**, Cambridge, v. 7, p. 1-26, 1968.

ANKERSMIT, G. W. DDT resistance in *Plutella maculipennis* (Curt.) Lepidoptera in Java. **Bulletin of Entomological Research**, Cambridge, v. 44, p. 421-425, 1953.

ARONSON, A. I.; HAN, E.; MCGAUGHEY, W.; JOHNSON, D. The solubility of the inclusion proteins from *Bacillus thuringiensis* is dependent upon protoxin composition and is a factoring toxicity to insects. **Applied and Environmental Microbiology**, Washington, v. 57, n. 12, p. 981-086, 1991.

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. 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 of The United States of America - PNAS Plus**, Washington, v. 109, p. 9674–9675, 2012.

AYRA-PARDO, C.; RAYMOND, B.; GULZAR, A.; RODRIGUEZ-CABRERA, L.; MORAN-BERTOT, I.; CRICKMORE, N.; WRIGHT, D. J. Novel genetic factors involved in resistance to *Bacillus thuringiensis* in *Plutella xylostella*. **Insect Molecular Biology**, Hoboken, v. 24, n. 6, p. 589-600, 2015.

BADENAS-PEREZ, F. R.; SHELTON, A. M.; NAULT, B. A. Evaluating trait troops for diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae). **Journal of Economic Entomology**, Lanham, v. 97, p. 1365-1372, 2004.

BALLESTER, V.; GRANERO, F.; TABASHNIK, B. E.; MALVAR, T.; FERRÉ, J. Integrative model for binding of *Bacillus thuringiensis* toxins in susceptible and resistant larvae of the diamondback moth (*Plutella xylostella*). **Applied and Environmental Microbiology**, Washington, v. 65, p. 1413-1419, 1999.

BARROS, R.; VENDRAMIN, J.D. Efeito de cultivares de repolho, utilizados para criação de *Plutella xylostella* (L.) (Lepidoptera: Plutelidae), no desenvolvimento de *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae). **Anais da Sociedade Entomológica do Brasil**, Londrina, v. 28, p. 469-476, 1999.

BAXTER, S. W.; CHEN, M.; DAWSON, A.; ZHAO, J. Z.; VOGEL, H.; SHELTON, A. M. HECKEL, D. G.; JIGGINS, C. D. Mis-spliced transcripts of nicotinic acetylcholine receptor alfa6 are associated with field evolved spinosad resistance in *Plutella xylostella* (L.). **PLoS Genetics,** San Francisco, v. 6, n. 1, p. 1-10, 2010.

BAXTER, S. W.; BADENES-PÉREZ, F. R.; MORRISON, A.; HEIKO VOGEL, H.; CRICKMORE, N.; KAIN, W.; PING WANG, HECKEL, D. G.; JIGGINGS, C. D. Parallel Evolution of *Bacillus thuringiensis* toxin resistance in Lepidoptera. **Genetics**, v. 189, n. 2, p. 675–679, 2011.

BEEGLE, C. C.; YAMAMOTO, T. Invitation paper (C.P. Alexander Fund): history of *Bacillus thuringiensis* Berliner research and development. **Canadian Entomologist**, Ottawa, v. 124, n. 4, p. 587-616, 1992.

BERLINER, E. Eber die schlaffsucht der Mehlmottenraupe. (*Ephesis kuehniella* Zell.) undihren Erreger *Bacillus thuringiensis*, n. sp. **Zeitschrift fuer Angewandte Entomologie**, Berlin, v. 2, p. 29–56, 1911.

BERNHARD, K.; JARRET, P.; MEADOWS, M. Natural isolates of *Bacillus thuringiensis*: worldwide distribution, characterization, and activity against insects pests. **Journal of Invertebrate Pathology**, San Diego, v. 70, n. 1, p. 59–68, 1997.

BIZZARRI, M. F.; BISHOP, A. H. Recovery of *Bacillus thuringiensis* in vegetative form from phylloplane of clover (*Trifolium hybridum*) during a growing season. **Journal of Invertebrate Pathology**, San Diego, v. 94, n. 1, p. 38–47, 2007.

BRAVO, A., JANSENS, S.; PEFEROEN, M. Immunocytochemical localization of *Bacillus thuringiensis* insecticidal crystal proteins in intoxicated insects. **Journal of Invertebrate Pathology**, San Diego, v. 60, p. 237-246, 1992.

BRAVO, A.; SARABIA, S.; LOPEZ, L.; ONTIVEROS, H.; ABARCA, C.; ORTIZ, A.; ORTIZ, M.; LINA, L.; VILLALOBOS, F. J.; GUADALUPE PENHA, G.; VALDEZ, M. E.; SOBERON, M.; QUINTERO, R. Characterization of cry genes in Mexican *Bacillus thuringiensis* strain collection. **Applied and Environmental Microbiology**, Washington DC, v. 64, n. 2, p. 4965–4972, 1998.

BRAVO, A.; GILL, S. S.; SOBERÓN, M. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. **Toxicon**, Elmsford, v. 49, n. 4, p. 423–435, 2007.

BRAVO, A.; LIKITVIVATANAVONG, S.; GILL, S. S.; SOBERÓN, M. *Bacillus thuringiensis*: a story of a successful bioinsecticide. **Insect Biochemistry and Molecular Biology**, Kidlington, v. 41, n. 7, p. 1–9, 2011.

BRODERICK, N. A.; RAFFA, K. F.; HANDELSMAN, J. Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. **Proceedings of the National Academy Sciences USA**, Washington, v. 103, p. 15196–15199, 2006.

BRODERICK, N. A.; ROBINSON, C. J.; MCMAHON, M. D.; HOLT, J.; HANDELSMAN, J.; RAFFA K. F. Contributions of gut bacteria to *Bacillus thuringiensis* induced mortality vary across a range of Lepidoptera. **BMC Biology**, London, v. 7, p. 11, 2009.

BUNTIN, G. D. Canola insect issues in the Southeast. In: PROCEEDINGS OF CANOLA CROP PROTECTION SYMPOSIUM. University of Tennessee, Memphis, 1990. p. 28–33.

CAB International Centre. The 2010 Worldwide Biopesticides Market Summary. Wallingford: CAB International Centre, 2010. 40 p.

CANCINO-RODEZNO, A.; ALEXANDER, C.; VILLASEÑOR, R.; PACHECO, S.; PORTA, H.; PAUCHET, Y.; SOBERÓN, M.; GILL, S. S.; BRAVO, A. The mitogen-activated protein kinase p38 is involved in insect defense against Cry toxins from *Bacillus thuringiensis*. **Insect Biochemistry and Molecular Biology**, Kidlington, v. 40, p. 58-63, 2010.

CANCINO-RODEZNO, A.; LOZANO, L.; OPPERT, C.; CASTRO, J. I.; LANZ-MENDOZA, H.; ENCARNACIÓN, S., EVANS, A. E.; GILL, S. S.; SOBERÓN, M.; JURAT-FUENTES, J. L.; BRAVO, A. Comparative proteomic analysis of *Aedes aegypti* larval midgut after intoxication with Cry11Aa toxin from *Bacillus thuringiensis*. **PloS One**, San Francisco, v. 7, n. 5, 2012.

CANDAS, M.; LOSEVA, O.; OPPERT, B.; KOSARAJU, P.; BULLA JUNIOR, L. A. Insect resistance to *Bacillus thuringiensis*: alterations in the indianmeal moth larval gut proteome. **Molecular Cellular Proteomics**, Rockville, v. 2, n. 1, p. 19-28, 2003.

CARBALLO, V. M. Investigación sobre manejo integrado de *Plutella xylostella* L. en el cultivo de repolho en Costa Rica. **News Cerba**, Paris, v. 33, p. 617-622, 1992.

CASTELO BRANCO, M.; FRANCA, F. H.; VILLAS BOAS, G. L. Traça-das-crucíferas (*Plutella xylostella*). Brasília: EMBRAPA, 1997. 4 p. (Comunicado Técnico - hortaliças).

CASTELO BRANCO, M.; FRANÇA, F. H.; MEDEIROS, M. A.; LEAL, J. G. T. Uso de inseticidas para o controle da traça-do-tomateiro e traça-das-crucíferas: um estudo de caso. **Horticultura Brasileira**, Brasília, v. 19, n. 1, p. 60-63, 2001.

CASTELO BRANCO, M.; MELO, C. A Resistência a abamectin e cartap em populações de traça-das-crucíferas. **Horticultura Brasileira**, Brasília, v. 20, n. 4, p. 541-543, 2002.

CASTELO BRANCO, M.; FRANÇA, F. H.; PONTES, L. A.; AMARAL, P. S. T. Avaliação da suscetibilidade a inseticidas em populações da traça-das-crucíferas de algumas áreas do Brasil. **Horticultura Brasileira**, Brasília, v. 21, n. 3, p. 549-552, 2003.

CHAPMAN, J. W.; REYNOLDS, D. R.; SMITH, A. D.; RILEY, J. R.; PEDGLEY, D. E.; WOIWOD, I. P. High altitude migration of the diamondback moth *Plutella xylostella* to the UK: a study using radar, aerial netting, and ground trapping. **Ecological Entomology**, West Sussex, v. 27, p. 641-650, 2002.

CHARLESTON, D. S.; KFIR, R. The possibility of using Indian mustard, *Brassica juncea*, as a trap crop for the diamondback moth, *Plutella xylostella*, in South Africa. **Crop Protection**, Guildford, v. 19, p. 455-460, 2000.

CONTRERAS, E.; RAUSELL, C.; REAL, M. D. Proteome response of *Tribolium castaneum* larvae to *Bacillus thuringiensis* toxin producing strains. **PloS One**, San Francisco, v. 8, n. 1, 2013.

COPPING, L. G.; MENN, J. J. Review biopesticides: a review of their action, applications and efficacy. **Pest Management Science**, Sussex, v. 56, n. 5, p. 651–676, 2000.

COULSON, S. J.; HODKINSON, I. D.; WEBB, N. R.; MIKKOLA, K.; HARRISON, J. A.; PEDGLEY, D. E. Aerial colonization of high Arctic islands by invertebrates: the diamondback moth *Plutella xylostella* (Lepidoptera: Yponomeutidae) as a potential indicator specie. **Diversity and Distributions**, Oxford, v. 8, p. 327-334, 2002.

CRICKMORE, N. Beyond the spore – past and future developments of *Bacillus thuringiensis* as a biopesticide. **Journal of Applied Microbiology**, Chichester, v. 101, n. 3, p. 616–619, 2006.

CRICKMORE, N.; ZEIGLER, D. R.; FEITELSON, J.; SCHNEPF, E.; VAN RIE, J.; LERECLUS, D.; BAUM, J.; DEAN, D. H. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. **Microbiology and Molecular Biology Reviews**, Washington DC, v. 62, n. 3, p. 807–813, 1998.

CRICKMORE, N. *Bacillus thuringiensis* toxin gene nomenclature. 2013. Disponível em: http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/#Research. Acesso em: 27 nov. 2013.

CRICKMORE, N.; BAUM, J.; BRAVO, A.; LERECLUS, D.; NARVA, K.; SAMPSON, K.; SCHNEPF, E.; SUN, M.; ZEIGLER, D.R. *Bacillus thuringiensis* toxin nomenclature http://www.btnomenclature.info/. 2016.

CZEPAK, C; FERNANDES, P. M.; SANTANA, H. G. TAKATSUKA, F. S.; ROCHA, C. L. Eficiência de inseticidas para o controle de *Plutella xylostella* (Lepidoptera: Plutellidae) na cultura do repolho (*Brassica oleracea* var. *capitata*). **Pesquisa Agropecuária Tropical**, Goiânia, v. 35, n. 2, p. 129-131, 2005.

DE BORTOLI, S. A.; VACARI, A. M.; MAGALHAES, G. O.; DIBELLI, W.; DE BORTOLI, C. P.; ALVES, M. P. Subdosagens de *Bacillus thuringiensis* em *Plutella xylostella* (Lepidoptera: Plutellidae) e *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae). **Revista Caatinga**, Mossoró, v. 25, n. 2, p. 50–57, 2012.

DE BORTOLI, S. A.; VACARI, A. M.; GOULART, R. M.; FERRAUDO. A. S.; VOLPE, H. X. L. Classification of cruciter cultivars based on the life history of diamondback moth (*Plutella xylostella*). **International Journal of Pest Management,** Abingdon, v. 59, p. 73-78, 2013.

DEIST, B. R.; RAUSCH, M. A.; FERNANDEZ-LUNA, M. T.; ADANG, M.J.; BONNING, B. C. Bt toxin modification for enhanced efficacy. **Toxins**, Basel, v. 6, n. 10, p. 3005-3027, 2014.

DE MAAGD, R. A.; BRAVO, A.; CRICKMORE, N. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. **Trends in Genetics**, Cambridge, v. 17, n. 4, p. 193–199, 2001.

DIAS, D. G. S.; SOARES, C. M. S.; MONNERAT, R. G. Avaliação de larvicidas de origem microbiana no controle da traça-das-crucíferas em couve-flor. **Horticultura Brasileira**, Brasília, v. 22, n. 3, p. 553-556, 2004.

DÍAZ-GOMEZ, O.; RODRÍGUEZ, J. C.; SHELTON, A. M.; LAGUNES, A.; BUJANOS, R. Susceptibility of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) populations in Mexico to comercial formulations of *Bacillus thuringiensis*. **Journal of Economic Entomology**, Lanham, v. 93, n. 3, p. 963–970, 2000.

DORSCH, J. A.; CANDAS, M.; GRIKO, N. B.; MAATY, W. S.; MIDBOE, E. G.; VADLAMUDI, R. K.; BULLA JUNIOR, L. A. Cry1A toxins of *Bacillus thuringiensis* bind specifically to a region adjacent to the membrane-proximal extracellular domain of BT-R(1) in *Manduca sexta*: involvement of a cadherin in the entomopathogenicity of *Bacillus thuringiensis*. **Insect Biochemistry and Molecular Biology**, Kidlington, v. 32, p. 1025-1036, 2002.

DOSDALL, L. M.; MASON, P. G.; OLFERT, O.; KAMINSKI, L.; KEDDIE, B. A. The origins of infestations of diamondback moth, *Plutella xylostella* (L.), in canola in western Canada. In: ENDERSBY, N. M.; RIDLAND, P. M. (Eds.). **The Management of Diamondback Moth and Other Crucifer Pests**. PROCEEDINGS OF THE FOURTH INTERNATIONAL WORKSHOP, 26–29 November. Department of Natural Resources and Environment, Melbourne, Australia, 2001, p. 95–100.

EDWARDS, D. L.; PAYNE, J.; SOARES, G. G. Novel isolates of *Bacillus thuringiensis* having activity against nematodes. **European Patent Application EP** O 303 426 A2, 1988.

EL-SADAWY, H. A.; EL-HAG, H. A.; GEORGY, J. M.; EL HOSSARY, S. S.; KASSEM, H. A. In vitro activity of *Bacillus thuringiensis* (H14) 43 kDa crystal protein against *Leishmania major*. **American-Eurasian Journal of Agriculture & Environmental Science**, Dubai, v. 3, n. 4, p. 583–589, 2008.

FEITELSON, J. S. Novel pesticidal delta-endotoxins from *Bacillus thuringiensis*. In: PROCEEDINGS OF THE ANNUAL MEETING OF THE SOCIETY FOR INVERTEBRATE PATHOLOGY, 27, Montpellier, p. 184, 1994.

FERRÉ, J.; REAL, M. D.; VAN RIE, J.; JANSENS, S.; PEFEROEN, M. Resistance to the *Bacillus thuringiensis* bioinsecticide in the field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. **Proceedings of the National Academy of Science**, Washington DC, v. 88, n. 12, p. 5119–5123, 1991.

FERRÉ, J.; VAN RIE, J. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis.* **Annual Review of Entomology**, Palo Alto, v. 47, p. 501-533, 2002.

FINNEY, D. J. **Probit analysis**. 3ª ed. Cambridge: Cambridge University Press, 1971. 205 p.

FIUZA, L. M.; POLANCZYK, R. A.; CRICKMORE, N. ***Bacillus thuringiensis* and *Lysinibacillus sphaericus* – Characterization and the use in the field of biocontrol**. Gewerbestrasse: Springer, 2017. 288 p.

FLANNAGAN, R. D.; YU, C. G.; MATHIS, J. P.; MEYER, T. E.; SHI, X.; SIQUEIRA, H. A. A.; SIGFRIED, B. D. Identification, cloning and expression of a Cry1Ab cadherin receptor from European corn borer, *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae). **Insect Biochemistry and Molecular Biology**, Kidlington, v. 35, p. 33-40, 2005.

FORSYTH, G.; LOGAN, N. A. Isolation of *Bacillus thuringiensis* Northern Victoria Land, Antarctica. **Letters in Applied Microbiology**, Medford, v. 30, n. 2, p. 263–266, 2000.

FREITAS LUZ, F. J.; SABOYA, R. C. C.; SILVA-PEREIRA, P. R. V. **O cultivo do repolho em Roraima**. Boa Vista: EMBRAPA, 2002. 17 p. (Circular Técnica).

GEORGHIOU, G. P.; LAGUNES-TEJADA, A. **The occurrence of resistance to pesticides in arthropods**: an index of cases reported through 1989. Roma: FAO, 1991. 318 p.

GLARE, T. R.; O'CALLAGHAN, M. *Bacillus thuringiensis*: biology, ecology and safety. Chichester: John Wiley, 2000. 350 p.

GOMEZ, I.; OLTEAN, D. I.; GILL, S. S.; BRAVO, A.; SOBERON, M. Mapping the epitope in cadherin-like receptors involved in *Bacillus thuringiensis* Cry1A toxin interaction using phage display. **The Journal of Biological Chemistry**, Bathesda, v. 276, p. 28906-28912, 2001.

GONZÁLEZ-CABRERA, J.; HERRERO, S.; SAYYED, A. H.; ESCRICHE, B.; LIU, Y. B.; MEYER, S. K.; WRIGHT, D. J.; TABASHNIK, B. E.; FERRÉ, J. Variation in susceptibility to *Bacillus thuringiensis* toxins among unselected strains of *Plutella xylostella*. **Applied and Environmental Microbiology**, Washington DC, v. 67, n. 10, p. 4610–4613, 2001.

GONG, Y.; WANG, C.; YANG, Y.; WU, S.; WU, Y. Characterization of resistance to *Bacillus thuringiensis* toxin Cry1Ac in *Plutella xylostella* from China. **Journal of Invertebrate Pathology**, San Diego, v. 104, n. 2, p. 90–96, 2010.

GRIFFITS, B. S.; CAUL, S.; THOMPSON, J.; BIRCH, A. N. E.; SCRIMGEOUR, C.; ANDERSEN, M. N.; CORTET, J.; MESSEAN, A.; SAUSSE, C.; LACROIX, B.; KROGH, P. H. A comparison of soil microbial community structure, protozoa and nematodes in field plots of conventional and genetically modified maize expressing the *Bacillus thuringiensis* Cry1Ab toxin. **Plant and Soil,** Dordrecht, v. 275, n. 1-2, p. 135-146, 2005.

GUAN-SOON, L. Overview of vegetable IPM in Asia. **Plant Protection Bulletin**, Roma, v. 38, p. 73-87, 1990.

GUO, Z.; KANG, S.; ZHU, X.; XIA, J.; WU, Q.; WANG, S.; XIE, W.; ZHANG, Y. 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**, Kidlington, v. 59, p. 30-40, 2015.

GUNNING, R. V.; DANG, H. T.; KEMP, F. C.; NICHOLSON, I. C.; MOORES, G. D. New resistance mechanism in *Helicoverpa armigera* threatens transgenic crops expressing *Bacillus thuringiensis* Cry1Ac toxin. **Applied and Environmental Microbiology**, Washington DC, v. 71, n. 5, p. 2558-2563, 2005.

GUO, Y.; MA, L.; CRISTOFANILLI, M.; HART, R. P.; HAO, A.; SCHACHNER, M. Transcription factor Sox11b is involved in spinal cord regeneration in adult zebrafish. **Neuroscience**, San Juan de Alicante, v. 172, p. 329-341, 2011.

GUPTA, B. L.; DOW, J. A. T.; HALL, T. A.; HARVEY, W. R. Electron probe X-ray microanalysis of the effects of *Bacillus thuringiensis* var. *kurstaki* crystal protein insecticide on ions in an electrogenic K+-transporting epithelium of the larval midgut in the lepidopteran, *Manduca sexta*, in vitro. **Journal of Cell Science**, London, v. 74, p. 137-152, 1985.

GUTIÉRREZ, M. E. M.; GONÇÁLVES, E. F. Selección de cepas de *Bacillus thuringiensis* con efecto nematicida. **Manejo Integrado de Plagas y Agroecologia**, Turrialba, n. 78, p. 63-66, 2006.

HABIB, M. E. M.; ANDRADE, C. E. S. Bactérias entomopatogênicas. In: ALVES, S. B. (Ed.). **Controle microbiano de insetos**. Piracicaba: FEALQ, 1998. p. 383-446.

HAMA, H.; KONO, Y.; SATO, Y. Decreased sensitivity of central nerve to fenvalerate in the pyrethroid-resistant diamondback moth, *Plutella xylostella* Linn. (Lepidoptera: Yponomeutidae). **Applied Entomology and Zoology**, Tokyo, v. 22, p. 176–180, 1987.

HANNAY, C. L. Crystalline inclusions in aerobic spore forming bacteria. **Nature**, London, v. 172, p. 1004, 1953.

HAYAKAWA, T.; SHITOMI, Y.; MIYAMOTO, K.; HORI, H. GalNAc pretreatment inhibits trapping of *Bacillus thuringiensis* Cry1Ac on the peritrophic membrane of *Bombyx mori*. **FEBS Letters**, Amsterdam, v. 579, n. 3, p. 331-335, 2004.

HECKEL, D. G.; GAHAN, L. J.; LIU, Y. B.; TABASHNIK. B. E. Genetic mapping of resistance to *Bacillus thuringiensis* toxins in diamondback moth using biphasic linkage analysis. **Proceedings of the National Academy of Sciences of The United States of America - PNAS Plus**, Washington, v. 96, n. 15, p. 8373– 8377, 1999.

HECKEL, D. G.; GAHAN, L. J.; BAXTER, S. W.; ZHAO, J. Z.; SHELTON, A. M.; GOULD, F.; TABASHNIK, B. E. The diversity of Bt resistance genes in species of Lepidoptera. **Journal of Invertebrate Pathology,** San Diego, v. 95, n. 3, p. 192-197, 2007.

HECKEL, D. G. 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 Biochemestry and Physiology**, Maryland Heights, v. 104, n. 2, p. 103–110, 2012.

HERNÁNDEZ-MARTÍNEZ, P.; NAVARRO-CERRILLO, G.; CACCIA, S.; DE MAAGD, R. A.; MOAR, W. J.; FERRÉ, J.; ESCRICHE, B.; HERRERO, S. Constitutive activation of the midgut response to *Bacillus thuringiensis* in Bt-resistant *Spodoptera exigua*. **PLoS One**, San Francisco, v. 5, n. 9, p. 1–10, 2010.

HERNÁNDEZ-MARTÍNEZ, P.; HERNÁNDEZ-RODRÍGUEZ, C. S.; VIDISHA KRISHNAN, V.; CRICKMORE, N.; ESCRICHE, B.; FERRE, J. Lack of Cry1Fa binding to the midgut brush border membrane in a resistant colony of *Plutella xylostella* moths with a mutation in the ABCC2 Locus. **Applied and Environmental Microbiology**, Washington DC, v. 78, n. 18, p. 6759–6761, 2012.

HERNÁNDEZ-RODRÍGUEZ, C. S.; FERRÉ, J.; HERRERO, S. Genomic structure and promoter analysis of pathogen-induced repat genes from *Spodoptera exigua*. **Insect Molecular Biology**, Hoboken, v. 18, n. 1, p. 77–85, 2009.

HERRERO, S.; ANSEMS, M.; VAN OERS, M. M.; VLAK, J. M.; BAKKER, P. L.; DE MAAGD, R. A. REPAT, a new family of proteins induced by bacterial toxins and baculovirus infection in *Spodoptera exigua*. **Insect Biochemistry and Molecular Biology**, Kidlington, v. 37, n. 11, p. 1109–1118, 2007.

HOFTE, H.; WHITELEY, H. R. Insecticidal crystal proteins of *Bacillus thuringiensis*. **Microbiological Reviews**, Birmingham, v. 53, n. 3, p. 242–255, 1989.

HUA, G.; JURAT-FUENTES, J. L.; ADANG, M. J. Bt-R1a extracellular cadherin repeat 12 mediates *Bacillus thuringiensis* Cry1Ab binding and cytotoxicity. **The Journal of Biological Chemistry**, Bethesda, v. 279, p. 28051-28056, 2004.

HUNG, C. F.; SUN, C. N. Microsomal monooxygenases in diamondback moth larvae resistant to fenvalerate and piperonyl butoxide. **Pesticide Biochemistry and Physiology**, San Diego, v. 33, p. 168–175, 1989.

IMENES, S. D. L.; CAMPOS, T. B.; RODRIGUES NETTO, S. M.; BERGMANN, E. C. Avaliação da atratividade de feromônio sexual sintético da traça-das-crucíferas, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), em cultivo orgânico de repolho. **Arquivos do Instituto Biológico**, São Paulo, v. 69, n. 1, p. 81-84, 2002.

ISHIWATA, S. On a kind of severe flacherie (sotto disease). **Danihon Sanshi Kaiho**, Tóquio, v. 114, p. 1–5, 1901.

JAMES, C. Global status of commercialized biotec/GM crops: 2016. ISAAAA Briefs No 52. 2016.

JOHNSON, D. E.; MCGAUGHEY W. H. Natural mortality among Indian meal moth larvae with resistance to *Bacillus thuringiensis*. **Journal of Invertebrate Pathology**, San Diego, v. 68, p. 170–172, 1996.

JONHSTON, P. R.; CRICKMORE, N. Gut bacteria are not required for the insecticidal activity of *Bacillus thuringiensis* toward the tabacco hornworm, *Manduca sexta*. **Applied and Environmental Microbiology**, Washinghton DC, v. 75, p. 5094-5099, 2009.

JURAT-FUENTES, J. L.; ADANG, M. J. Cry toxin mode of action in susceptible and resistant *Heliothis virescens* larvae. **Journal of Invertebrate Pathology**, San Diego, v. 92, n. 3, p. 166-171, 2006.

JURAT-FUENTES, J. L; JACKSON, T. A. Bacterial entomopathogens. In: KAYA, H; VERA, F. (Eds.). **Insect Pathology**. 2nd ed. London: Academic Press, 2012. p. 268-349.

KAO, C. H.; HUNG, C. F.; SUN, C. N. Parathion and methyl parathion resistance in diamondback moth (Lepidoptera: Plutellidae) larvae. **Journal of Economic Entomology**, Lanham, v. 82, p. 1299–1304, 1989.

KAO, C. Y.; LOS, F. C. O.; HUFFMAN, D. L.; WACHI, S.; KLOFT, N.; HUSMANN, M.; KARABRAHIMI, V.; SCHWARTZ, J. L.; BELLIER, A.; HA, C.; SAGONG, Y.; FAN, H.; GOSH, P.; HSIEH, M.; HSU, C. S.; CHEN, L.; AROIAN, R. V. Global functional analyses of cellular responses to pore-forming toxins. **PLoS Pathogens**, San Francisco, v. 7, n. 3, 2011.

KIRSCH, K.; SCHMUTTERER, H. Low efficacy of a *Bacillus thuringiensis* (Berl.) formulation in controlling the diamondback moth, *Plutella xylostella* (L.), in the Philippines. **Journal of Applied Entomology**, Hoboken, v. 105, n. 1-5, p. 249-255, 1988.

KNOWLES, B. H. Mechanism of action of Bacillus thuringiensis insecticidal δ–endotoxins. **Advances in Insect Physiology**, London, v. 24, p. 275–308, 1994.

KONECKA, E.; KAZNOWSKI, A.; ZIEMNICKA, J. Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated during epizootics in *Cydia pomonella* L. **Journal of Invertebrate Pathology**, San Diego, v. 94, n. 1, p. 56–63, 2007.

LAMBERT, B.; PEFEROEN, M. Insecticidal promise of *Bacillus thuringiensis*. **BioScience**, Washington, v. 42, n. 2, p. 112-122, 1992.

LEI, Y.; ZHU, X.; XIE, W.; WU, Q.; WANG, S.; GUO, Z.; XU, B.; LI, X.; ZHOU, X.; ZHANG, Y. Midgut trascriptome response to Cry toxin in the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae). **Gene**, Amsterdam, v. 533, n. 1, p. 180-187, 2014.

LI, J.; CARROLL, J.; ELLAR , D. J. Crystal structure of insecticidal delta–endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. **Nature,** London, v. 353, p. 815–821, 1991.

LIU, M. Y.; TZENG, Y. J.; SUN, C. N. Insecticide resistance in the diamondback moth. **Journal of Economic Entomology**, Lanham, v. 75, p. 153–155, 1982.

LIU, Y. B.; TABASHNIK, B. E.; PUSZTAI-CAREY, M. Field-evolved resistance to *Bacillus thuringiensis* toxin Cry1C in diamondback moth (Lepidoptera: Plutellidae). **Journal of Economic Entomology**, Lanham, v. 89, p. 798-804, 1996.

MA, G.; ROBERTS, H.; SARJAN, M.; FEATHERSTONE, N.; LAHNSTEIN, J.; AKHURST, R.; SCHMIDT, O. Is the mature endotoxin Cry1Ac from *Bacillus thuringiensis* inactived by a coagulation reaction in the gut lumen of resistant *Helicoverpa armigera* larvae? **Insect Biochemistry and Molecular Biology,** Kidlington, v. 35, n. 7, p. 729-739, 2005.

MAA, C. J. W.; LIU, J. F.; TZAU, Y. Y.; CHENG, C. H. Involvement of acetylcholinesterase in malathion-resistance of the diamondback moth. In: SIVAPRAGASAM, A.; LOKE, W. H.; HUSSAN, A. K.; LIM, G. S. (Eds.). **The management of diamondback moth and other crucifer pests**. PROCEEDINGS OF THE THIRD INTERNATIONAL WORKSHOP, Kuala Lumpur, Malaysia, 1996. p. 201–205.

MAA, W. C. J.; LIAO, S. C. Culture dependent variation in esterase isozymes and malathion susceptibility of diamondback moth, *Plutella xylostella*. L. **Zoological Studies**, Taipei, v. 39, p. 375–386, 2000.

MAU, R. F. L.; KESSING, J. L. M. *Plutella xylostella* (Linnaeus): diamondback moth. Crop Knowledge Masters. April 2007. Disponível em: http://www.extento.hawaii.edu/Kbase/Crop/Type/plutella.htm. Acesso em: 14 set. 2013.

MEDEIROS, P. T.; DIAS, J. M. C. S.; MONNERAT, R. G.; SOUZA, N. R. **Instalação e manutenção de criação massal da traça-das-crucíferas (*Plutella xylostella*)**. Brasília: EMBRAPA, 2003. 4 p. (Circular Técnica).

MEDEIROS, P. T.; FERREIRA, M. N.; MARTINS, E. S.; GOMES, A. C. M. M.; FALCÃO, R.; DIAS, J. M. C. S.; MONNERAT, R. G. Seleção e caracterização de estirpes de *Bacillus thuringiensis* efetivas no controle da traça-das-crucíferas *Plutella xylostella*. **Pesquisa Agropecuária Brasileira**, Brasília, v. 40, n. 11, p. 1145-1148, 2005.

MELO, P. E.; CASTELO BRANCO, M.; MADEIRA, N. R. Avaliação de genótipos de repolho para a resistência à traça das crucíferas. **Horticultura Brasileira**, Brasília, v. 12, n. 1, p. 19-24, 1994.

MEUNIER, L.; PRÉFONTAINE, G.; VAN MUSTER, M.; BROUSSEAU, R.; MASSON, L. Transcriptional response of *Choristoneura fumiferana* to sublethal exposure of CryAb protoxin from *Bacillus thuringiensis*. **Insect Molecular Biology**, Hoboken, v. 15, n. 4, p. 475-483, 2006.

MICHEREFF, M. F. F.; VILELA, E. F.; MICHEREFF FILHO, M.; MAFRA NETO, A. Uso do feromônio sexual sintético para captura de machos da traça-das-crucíferas. **Pesquisa Agropecuária Brasileira**, Brasília, v. 35, n. 10, p. 1919-1926, 2000.

MONNERAT, R. G. **Interrelations entre la teigne des crucuferes, *Plutella xylostella*, son parasitoide *Diadegma* sp. et la bacteria entomopathogene *Bacillus***

***thuringiensis* Berliner**. 1995. 160 f. These (Doctoral en Science Agronomiques) - Ecole Nationale Superieure Agronominique de Montpellier. Montpellier, 1995.

MONNERAT, R. G.; MASSON, L.; BROUSSEAU, R.; PUSZTAICAREY, M.; BORDAT, D.; FRUTOS, R. Differential activity and activation of *Bacillus thuringiensis* insecticidal proteins in diamondback moth, *Plutella xylostella*. **Current Microbiology**, New York, v. 39, p. 159-162, 1999.

MONNERAT, R. G.; BRAVO, A. **Proteínas bioinseticidas produzidas pela bactéria *Bacillus thuringiensis*: modo de ação e resistência.** In: MELO, I. S.; AZEVEDO, J. L. (Eds.). **Controle Biológico**. Jaguariúna: Embrapa Meio Ambiente, 2000. p. 163-200.

MONNERAT, R. G.; QUEIROZ, P.; ORDUZ, S.; BENITENDE, G.; COZZI, J.; REAL, M. D.; IBARRA, J.; BRAVO, A. Genetic variability in *Spodoptera frugiperda* Smith populations in Latin America is associated to variations in susceptibility to *Bacillus thuringiensis* Cry toxins. **Applied and Environmental Microbiology**, Washington DC, v. 72, n. 11, p. 7029–7035, 2006.

MONNERAT, R. G.; BATISTA, A. C.; MEDEIROS, P. T. S.; MARTINS, E.; MELATTI, V.; PRAÇA, L.; DUMAS, V.; DEMO, C.; GOMES, A. C. M.; FALCAO, R.; BERRY, C. Screening of Brazilian *Bacillus thuringiensis* strains active against *Spodoptera frugiperda*, *Plutella xylostella* and *Anticarsia gemmatalis*. **Biological Control**, College Station v. 41, n. 3, p. 291–295, 2007.

MORAES, I. O.; CAPALBO, D. M. F. Produção de bactérias entomopatogênicas. In: ALVES, S. B. (Ed.). **Controle microbiano de insetos**. Piracicaba: Manole, 1986. p. 127-170.

NAGAMATSU, Y.; KOIKE, T.; SASAKI, K.; YOSHIMOTO, A.; FURUKAWA, Y. The cadherin-like protein is essential to specificity determination and cytotoxic action of the *Bacillus thuringiensis* insecticidal CryIAa toxin. **FEBS Letters**, Amsterdam, v. 460, p. 385-390, 1999.

NAVARRO-CERRILLO, G.; FERRÉ, J.; DE MAAGD, R. A.; HERRERO, S. Functional interactions between members of the REPAT family of insect pathogen-induced proteins. **Insect Molecular Biology**, Hoboken, v. 21, n. 3, p. 335–342, 2012.

NAVARRO-CERRILLO, G.; HERNÁNDEZ-MARTÍNEZ, P.; VOGEL, H.; FERRÉ, J.; HERRERO, S. A new gene superfamily of pathogen-response (repat) genes in Lepidoptera: classification and expression analysis. **Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology**, Kingston, v. 164, n. 1, p. 10–17, 2013.

NEVES, B. P.; NOGUEIRA, J. C. M. Cultivo e utilização do nim indiano (*Azadirachta indica* A. Juss.). Goiânia: EMBRAPA, 1996. 32 p. (Circular Técnica, 28).

NOPPUN, V.; SAITO, T.; MIATA, T. Cuticular penetration of S-fenvalerate in fenvalerate-resistant and susceptible strains of the diamondback moth, *Plutella xylostella* (L.). **Pesticide Biochemistry and Physiology**, San Diego, v. 33, p. 83–87, 1989.

OCELOTL, J.; SÁNCHES, J.; GÓMEZ, I.; TABASHNIK, B. E.; BRAVO, A.; SOBERÓN, M. ABCC2 is associated with *Bacillus thuringiensis* Cry1Ac toxin oligomerization and membrane insertion in diamondback moth. **Scientific Reports**, London, v. 7, n. 2386, p. 1-9, 2017.

OPPERT, B. Protease interactions with *Bacillus thuringiensis* insecticidal toxins. **Archives of Insect Biochemistry and Physiology,** Haboken, v. 42, n. 1, p. 1-12, 1999.

PARDO-LÓPEZ, L.; SOBERÓN, M.; BRAVO, A. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. **FEMS Microbiology Reviews**, Chichester, v. 37, p. 3-22, 2013.

PARK, Y.; GONZÁLEZ-MARTÍNEZ, R.; NAVARRO-CERRILLO, G.; CHAKROUN, M.; KIM, Y.; ZIARSOLO, P.; BLANCA, J.; CAÑIZARES, J.; FERRÉ, J.; HERRERO, S. ABCC transports mediate insect resistance to multiple Bt toxins revealed by bulk segregant analysis. **BMC Biology**, London, v. 12, n. 46, 2014.

PASCUAL, L.; JAKUBOWSKA, A. K.; BLANCA, J. M.; CAÑIZARES, J.; FERRÉ, J.; GLOECKNER, G.; VOGEL, H.; HERRERO, S. The transcriptome of *Spodoptera exigua* larvae exposed to different types of microbes. **Insect Biochemistry Molecular Biology**, Riverside, v. 42, n. 8, p. 557-570, 2012.

PEREZ, C. J.; SHELTON, A. M. Resistance of *Plutella xylostella* (Lepidoptera: Plutellidae) to *Bacillus thuringiensis* Berliner in Central America. **Journal of Economic Entomology**, Lanham, v. 90, n. 1, p. 87–93, 1997.

POLANCZYK, R. A.; ALVES, S. B. *Bacillus thuringiensis*: uma breve revisão. **Revista Brasileira de Agrociência**, Pelotas, v. 7, n. 2, p. 1-10, 2003.

POLANCZYK, R. A.; ALVES, S. B.; PADULLA, L. F. Screening of *Bacillus thuringiensis* against three Brazilian populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). **Biopesticides International**, Jalandhar, v. 1, n. 1/2, p. 114–124, 2005.

POLANCZYK, R. A.; DE BORTOLI, S. A.; DE BORTOLI, C. P. *Bacillus thuringiensis* - based biopesticides against agricultural pests in Latin America. In: LARRAMENDY, M. L.; SOLONESKI, S. (Orgs.). **Integrated Pest Management and Pest Control** - Current and Future Tactics. Rijeka: Intech, 2012. p. 445-462.

POLANCZYK, R. A.; VAN FRANKENHUYZEN, K.; PAULI, G. The American *Bacillus thuringiensis* based biopesticides market. In: FIUZA, L. M.; POLANCZYK, R. A.; CRICKMORE, N. (Eds.). ***Bacillus thuringiensis* and *Lysinibacillus sphaericus*** – Characterization and the use in the field of biocontrol. Gewerbestrasse: Springer, 2017. p. 173-184.

PRAÇA, L. B.; BATISTA, A. C.; MARTINS, E. S.; SIQUEIRA, C. B.; DIAS, D. G. S.; GOMES, A. C. M. M.; FALCÃO, R.; MONNERAT, R. G. Estirpes de *Bacillus thuringiensis* efetivas contra insetos das ordens Lepidoptera, Coleoptera e Diptera. **Pesquisa Agropecuária Brasileira**, Brasília, v. 39, p. 11-16, 2004.

PRAÇA, L. B.; RAMOS, F. R.; WAGNER, F. O.; SOARES, C. M. S.; SUJII, E.; MONNERAT, R. G. Suscetibilidade da traça-das-crucíferas a *Bacillus thuringiensis* em repolho no Distrito Federal. **Universitas**, Brasília, v. 8, p. 17-31, 2010.

RAHMAN, M. M.; ROBERTS, H. L. S.; SARJAN, M.; ASGARI, S.; SCHMIDT, O. Induction and transmission of *Bacillus thuringiensis* tolerance in the flour moth *Ephestia kuehniella*. **Proceeding of the National Academy of Sciences of the United States of America**, Washington, v. 101, n. 9, p. 2596-2699, 2004.

RAMACHANDRAN, S.; BUNTIN, G. V. D.; ALL, J. V. N. Response of canola to simulated diamondback moth (Lepidoptera: Plutellidae) defoliation at different growth stages. **Canadian Journal of Plant Science**, Ottawa, v. 80, p. 639–646, 2000.

RAMOS, F. R. **Avaliação a campo de uma estirpe de *Bacillus thuringiensis* tóxica à Lepidoptera e seu possível efeito adverso sobre espécies não-alvo**. 2008. 87 f. Dissertação (Mestrado em Ciências Agrárias) - Faculdade de Agronomia e Medicina Veterinária, Universidade de Brasília, Brasília, 2008.

RAYMOND, B.; JOHNSTON, P. R.; NIELSEN-LEROUX, C.; LERECLUS, D.; CRICKMORE, N. *Bacillus thuringiensis*: an impotent pathogen? **Trends in Microbiology**, Oxford, v. 18, p. 189-194, 2010.

RODRÍGUEZ-CABRERA, L.; TRUJILLO-BACALLAO, D.; BORRÁS-HIDALGO, O.; WRIGHT, D. J.; AYRA-PARDO, C. RNAi-mediated knockdown of a *Spodoptera frugiperda* trypsin-like serine-protease gene reduces susceptibility to a *Bacillus thurigiensis* Cry1Ca1 protoxin. **Environmental Microbiology**, Washington DC, v. 12, n. 11, p. 2894-2903, 2010.

ROUX, O.; GEVREY, M.; ARVANITAKIS, L.; GERS, C.; BORDAT, D.; LEGAL, L. ISSR-PCR: Tool for discrimination and genetic structure analysis of *Plutella xylostella* populations native to different geographical areas. **Molecular Phylogenetics and Evolution**, Urbana, v. 43, n. 1, p. 240–250, 2007.

SANCHIS, V. From microbial sprays to insect-resistant transgenic plants: history of the biospesticide *Bacillus thuringiensis*. A review. **Agronomy for Sustainable Development**, Reims, v. 31, n. 1, p. 217–231, 2011.

SAMPSON, C. Producción de repolho en Belice: programa de investigación para el control de la palomilla dorso de diamante. **News Cerba**, Paris, v. 33, p. 623-628, 1992.

SARFRAZ, M.; DOSDALL, L. M.; KEDDIE, B. A. Diamondback moth-host plant interactions: implications for pest management. **Crop Protection**, Guildford, v. 25, n. 7, p. 625–639, 2006.

SAS INSTITUTE 2002. SAS/STAT User`s Guide, version 9.00 TS level 2MO. SAS Institute Inc., Cary, NC: SAS Institute Inc., 2002.

SASTROSISWOJO, S.; SASTRODIHARDJO, S. Status of biological of Diamondback moth by introduction of parasitoid *Diadegma eucerophaga* in Indonesia. In: TALEKAR, N. S.; GRIGGS, T. D. (Eds). **Diamondback moth management**. PROCEEDINGS OF THE 1ST INTERNATIONAL WORKSHOP. Shanhua: Asian Vegetable Research and Development Centre, 1986. p. 185-194.

SAYYED, A. H.; GATSI, R.; PALACIOS, S. I.; ESCRICHE, B.; WRIGHT, D. J.; CRICKMORE, N. Common, but complex, mode of resistance of *Plutella xylostella* to *Bacillus thuringiensis* toxins Cry1Ab and Cry1Ac. **Applied of Environmental Microbiology**, Cardiff, v. 71, n. 11, p. 6863–6869, 2005.

SAYYED, A. H.; MOORES, G.; CRICKMORE, N.; WRIGHT, D. J. Cross-resistance between a *Bacillus thuringiensis* Cry toxin and non-Bt insecticides in the diamondback moth. **Pest Management Science**, Sussex, v. 64, n. 8, p. 813–819, 2008.

SCHNEPF, H. E.; CRICKMORE, N.; VAN RIE, J.; LERECLUS, D.; BAUM, J.; FEITELSON, J.; ZEIGLER, D. R.; DEAN, D. H. *Bacillus thuringiensis* and its pesticidal crystal proteins. **Microbiology and Molecular Biology Reviews**, Whashington DC, v. 62, p. 775–806, 1998.

SERAFINI, L. A.; BARROS, N. M.; AZEVEDO, J. A. **Biotecnologia: avanços na agricultura e na agroindústria**. Caxias do Sul: EDUCS, 2002. 433 p.

SHELTON, A. M.; ROBERTSON, J. L.; TANG, J. D. Resistance of diamondback moth (Lepidoptera: Plutellidae) to *Bacillus thuringiensis* subspecies in the field. **Journal of Economic Entomology**, Lanham, v. 86, p. 697–705, 1993.

SHELTON, A. M. Management of the diamondback moth: de´ ja` vu all over again? In: ENDERSBY, N. M.; RIDLAND, P. M. (Eds.). **The management of diamondback moth and other crucifer pests.** PROCEEDINGS OF THE FOURTH INTERNATIONAL WORKSHOP. Department of Natural Resources and Environment**,** Melbourne, 2004. p. 3–8.

SILVA, V. C. A.; BARROS, R.; MARQUES, E. J.; TORRES, J. B. Suscetibilidade de *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) aos fungos *Beauveria bassiana* (Bals.) Vuill. e *Metarhizium anisopliae* (Metsch.**)** Sorok**. Neotropical Entomology**, Londrina, v. 32, n. 4, p. 653-665, 2003.

SILVEIRA, L. F. V.; POLANCZYK, R. A.; PRATISSOLI, D.; FRANCO, C. R. Seleção de isolados de *Bacillus thuringiensis* para *Tetranychus urticae* Koch. **Arquivos do Instituto Biológico**, São Paulo, v. 78, n. 2, p. 273–278, 2007.

STEPHEN, R. T.; ELKINTON, J. S. Pathogenicity and virulence. **Journal of Invertebrate Pathology**, San Diego, n. 85, v. 3, p. 146-151, 2004.

SWIECICKA, I.; FIEDORUK, K.; BEDNARZ, G. The occurrence and properties of *Bacillus thuringiensis* isolated from free-living animals. **Letters in Applied Microbiology**, Medford, v. 34, n. 3, p. 194–198, 2002.

TABASHNIK, B. E.; FINSON, N.; SCHWARTZ, J. M.; CAPRIO, M. A.; JOHNSON, M. W. Diamondback moth resistance to *Bacillus thuringiensis* in Hawaii. In: PROCEEDINGS OF THE SECOND INTERNATIONAL WORKSHOP, Tainan, p. 175–183, 1990.

TABASHNIK, B. E.; FINSON, N.; JOHNSON, M. W. Managing resistance to *Bacillus thuringiensis*: lessons from the diamondback moth (Lepidoptera: Plutellidae). **Journal of Economic Entomology**, Lanham, v. 84, n. 1, p. 49-55, 1991.

TABASHNIK, B. E.; FINSON, N. ; GROETERS, F. R. ; MOAR, W. J. ; JOHNSON, M. W.; LUO, K.; ADANG, M. J. Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. **Proceedings of the National Academy of Sciences of the USA**, Washington, v. 91, p. 4120-4124, 1994.

TABASHNIK, B. E.; MALVAR, T.; LIU, Y. B.; FINDON, N.; BORTHAKUR, D.; SHIN, B. S.; PARK, S. H.; MASSON, L.; MAAGD, R. A.; BOSCH, D. Cross-resistance of the diamondback moth indicates altered interactions with domain II of *Bacillus thuringiensis* toxins. **Applied and Environmental Microbiology**, Washington, v. 62, n. 8, p. 2839-2844, 1996.

TABASHNIK, B. E.; LIU, Y. B.; FINSON, N.; MASSON, L.; HECKEL, D. G. One gene in diamondback moth confers resistance to four *Bacillus thuringiensis* toxins. **Proceeding of the National Academy of Sciences of USA,** Washington, v. 94, n. 5, p. 1640-1644, 1997a.

TABASHNIK, B. E.; LIU, Y. B.; MALVAR, T.; HECKEL, D. G.; MASSON, L.; BALLESTER, V.; GRANERO, F.; MÉNSUA, J. L.; FERRÉ, J. Global variation in the genetic and biochemical basis of diamondback moth resistance to *Bacillus thuringiensis.* **Proceeding of the National Academy of Sciences of the USA**, Washington, v. 94, n. 24, p. 12780-12785, 1997b.

TABASHNIK, B. E.; LIU, Y. B.; MALVAT, T.; HECKEL, D. G.; MASSON, L.; FERRÉ, J. Insect resistance to *Bacillus thuringiensis*: uniform or diverse? **Philosophical Transactions of the Royal Society B. Biological Sciences**, Colchester, v. 353, n. 1376, p. 1751-1756, 1998.

TABASHNIK, B. E.; VAN RENSBURG, J. B.; CARRIÈRE, Y. Field-evolved insect resistance to Bt crops: definition, theory, and data. **Journal of Economic Entomology**, Lanham, v. 102, v. 6, p. 2011-2025, 2009.

TABASHNIK, B. E.; CARRIÈRE, Y. Surge in insect resistance to transgenic crops and prospects for sustainability. **Nature Biotechnology**, London, v. 35, p. 926 – 935, 2017.

TANG, J. D.; SHELTON, A. M.; VAN RIE, J.; DE ROECK, S.; MOAR, W. J.; ROUSH, R. T.; PEFEROEN, M. Toxicity of *Bacillus thuringiensis* spore and crystal protein to resistant diamondback moth (*Plutella xylostella*). **Applied and Environmental Microbiology**, Washington DC, v. 62, n. 2, p. 564–569, 1996.

TAYLOR, R.; TIPPET, J.; GIBB, G. Identification and characterization of a novel *Bacillus thuringiensis* d-endotoxin entomocidal to coleopteran and lepidopteran larvae. **Molecular Microbiology**, Medford, v. 6, n. 9, p. 1211–1217, 1992.

THAMMASITTIRONG, A.; ATTATHOM, T. PCR-based method for the detection of cry genes in local isolates of *Bacillus thuringiensis* from Thailand. **Journal of Invertebrate Pathology**, San Diego, v. 98, n. 2, p. 121–126, 2008.

THIERY, I.; DELÉCLUSE, A.; TAMAYO, M. C.; ORDUZ, S. Identification of a gene for Cyt1A-like hemolysin from *Bacillus thuringiensis* subsp. medellin and expression in a crystal-negative *B. thuringiensis* strain. **Applied and Environmental Microbiology**, Washington, v. 63, n. 2, p. 468-473, 1997.

THOMAS, W. E.; ELLAR, D. J. *Bacillus thuringiensis* var. *israelensis* crystal–endotoxin: effects on insect and mammalians cells in vitro and in vivo. **Journal of Cell Science**, London, v. 60, p. 181–197, 1983.

THULER, A. M. G.; THULER, R. T.; CICERO, E. S.; DE BORTOLI, S. A.; LEMOS, M. V. F. Estudo da variabilidade gênica em isolados brasileiros de *Bacillus thuringiensis* para emprego no controle biológico de *Plutella xylostella*. **Boletin de Sanidad Vegetal Plagas**, Madrid, v. 33, p. 409-417, 2007.

THULER, R. T. Criação de *Plutella xylostella*. In: DE BORTOLI, S. A. (Ed.). **Criação de insetos: da base à biofábrica**. Jaboticabal: Edição própria, 2009. p. 58-68.

THULER, R. T. ***Plutella xylostella*** **(L.) (Lepidoptera: Plutellidae): táticas para o manejo integrado em brássicas**. 2006. 79 f. Tese (Doutorado em Entomologia Agrícola) – Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, 2006.

THULER, R. T.; DE BORTOLI, S. A.; HOFFMANN-CAMPO, C. B. Classificação de cultivares de brássicas com relação à resistência à traça-das-crucíferas e à presença de glucosinolatos. **Pesquisa Agropecuária Brasileira**, Brasília, v. 42, p. 467-474, 2007.

TIBA, L. M. **Efeito de alguns inseticidas sobre a mariposa *Plutella xylostella* (L., 1758) (Lepidoptera, Plutellidae) por meio de iscas esterilizantes.** 2008. 58 f. Dissertação (Mestrado Entomologia) – Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo, Piracicaba, 2008.

TIEWSIRI, K.; WONG, P. Differential alteration of two aminopeptidases N associated with resistance to *Bacillus thuringiensis* toxin Cry1Ac in cabbage looper. **Proceedings of the National Academy of Sciences of the USA**, Washinghton, v. 108, n. 34, p. 14037-1442, 2011.

TOJO, A.; AIZAWA, K. Dissolution and degradation of *Bacillus thuringiensis* delta–endotoxin by gut juice protease of the silkworm *Bombyx mori*. **Applied and Environmental Microbiology**, Washington, v. 45, p. 576–580, 1983.

TORRES, A. L.; BARROS, R.; OLIVEIRA, J. V. de. Efeito de extratos aquosos de plantas no desenvolvimento de *Plutella xylostella* (L., 1758) (Lepidoptera: Plutellidae). **Neotropical Entomology**, Londrina, v. 30, n. 1, p. 151-156, 2001.

VACARI, A. M.; VOLPE, H. X. L.; GOULART, R. M.; VIANA, C. L. T. P.; BENVENGA, S. R.; CARVALHO, J. S.; THULER, R. T.; DE BORTOLI, S. A. Integração de métodos de controle de pragas em hortaliças: experiência prévia para uma aplicação segura. In: ARAUJO, E. S.; VACARI, A. M.; CARVALHO, J. S.; GOULART, R. M.; CAMPOS, A. P.; VOLPE, H. X. L. (Eds.). **Tópicos em entomologia agrícola**. Ribeirão Preto: Maxicolor Gráfica e Editora, 2008. p. 84-99.

VACHON, V.; LAPRADE, R.; SCHWARTZ, J. L. Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: A critical review. **Journal of Invertebrate Pathology**, San Diego, v. 111, n. 1, p. 1–12, 2012.

VAN FRANKENHUYZEN, K. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. **Journal of Invertebrate Pathology**, San Diego, v. 101, n. 1, p. 1–16, 2009.

VAN FRANKENYUZEN, K. Cross-order and cross phylum activity of *Bacillus thuringiensis* pesticidal toxins. **Journal of Invertebrate Pathology,** San Diego, v. 114, p. 76-85, 2013.

VAN MUNSTER, M.; PRÉFONTAINE, G.; MEUNIER, L.; ELIAS, M.; MAZZA, A.; BROUSSEAU, R.; MASSON, L. Altered gene expression in *Choristoneura fumiferana* and *Manduca sexta* in response to sublethal intoxication by *Bacillus thuringiensis* Cry1Ab toxin. **Insect Molecular Biology**, Hoboken, v. 6, n. 1, p. 25-35, 2007.

XIE, R.; ZHUANG, M.; ROSS, L. S.; GOMEZ, I.; OLTEAN, D. I.; BRAVO, A. Single amino acid mutations in the cadherin receptor from *Heliothis virescens* affect its toxin binding ability to Cry1A toxins. **The Journal of Biological Chemistry**, Bathesda, v. 280, p. 8416-8425, 2005.

YAMAMOTO, T.; DEAN, D. H. Insecticidal proteins produced by bacteria pathogenic to agricultural pests. In: CHARLES, J. F.; DELÉCLUSE, A.; NIELSEN-LE ROUX, C. (Eds.). **Entomopathogenic bacteria**: from laboratory to field application. Netherlands: Kluwer Academic Publishers, 2000. p. 81–100.

YANG, Y.; ZHU, Y. C.; OTTEA, J.; HUSSENEDER, C.; LEONARD, B. R.; ABEL, C.; HUANG, F. Molecular characterization and RNA interference of three midgut aminopeptidase N isozimes from *Bacillus thuringiensis*-susceptible and –resistance strains of sugarcane borer, *Diatraea saccharalis*. **Insect Biochemistry and Molecular Biology**, Kidlington, v. 40, p. 592-603, 2010.

YAO, J.; BUSCHMAN, L. L.; LUN, N.; KHAJURIA, C.; ZHU, K. Y. Changes in gene expression in the larval gut of *Ostrinia nubilalis* in response to *Bacillus thuringiensis* Cry1Ab protoxin ingestion. **Toxins,** Basel, v. 6, n. 4, p. 1274-1294, 2014.

YAO, M. G.; HUNG, C. F.; SUN, C. N. Fenvalerate resistance and aldrin epoxidation in larvae of the diamondback moth. **Pesticide Biochemistry and Physiology,** San Diego, v. 30, p. 272–278, 1988.

YI, D.; CUI, L.; LIU, Y. U.; ZHUANG, M.; ZHANG, Y.; FANG, Z.; YANG, L. Transformation of cabbage (*Brassica oleracea* L. var. *capitata*) with Bt cry1Ba3 gene for control of diamondback moth. **Agricultural Sciences in China**, Beinjing, v. 11, n. 10, p. 1693-1700, 2011.

YU, S. J.; NGUYEN, S. N. Detection and biochemical characterization of insecticide resistance in the diamondback moth. Pesticide Biochemistry and Physiology, San Diego, v. 44, p. 74–81, 1999.

WAG, B. Achiasmy or heterochiasmy: Does meiotic recombination occur in female Lepidoptera?. **The Journal of Research on the Lepidoptera**, California, v. 44, p. 43-45, 2011.

WILKINGS, S. Structure and mechanism of ABC transporters**. F1000Prime Reports**, London, v. 7, n. 14, 2015.

WRIGHT, D. J.; IQBAL, M.; GRANERO, F.; FERRE, J. A change in a single midgut receptor in the diamondback moth (*Plutella xylostella*) is only part responsible for field resistance to *Bacillus thuringiensis* subsp. *kurstaki* and *Bacillus thuringiensis* subsp. *aizawai*. **Applied and Environmental Microbiology**, Washington DC, v. 63, n. 5, p. 1814–1819, 1997.

WU, Y.; LEI, C. F.; YI, D.; LIU, P. M.; GAO, M. Y. Novel *Bacillus thuringiensis* endotoxin active against *Locusta migratoria manilensis*. **Applied and Environmental Microbiology**, Washington DC, v. 77, n. 10, p. 3227–3233, 2011.

ZHANG, X.; CANDAS, M.; GRIKO, N. B.; ROSE-YOUNG, L.; BULLA JUNIOR, L. A. 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 & Differentiation**, London, v. 12, p. 1407–1416, 2005.

ZHANG, X.; CANDAS, M.; GRIKO, N. B.; TAUSSIG. R.; BULLA JUNIOR, L. A. 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 of the USA,** Washington, v. 103, p. 9897–9902, 2006.

ZHANG, X.; TIEWSIRI, K.; KAIN, W.; HUANG, L.; WANG, P. Resistance of *Trichoplusia ni* to *Bacillus thuringiensis* toxin Cry1Ac is independent of alteration of the cadherin-like receptor for Cry toxins. **PLoS One,** San Francisco, v. 7, n. 5, p. 1-9, 2012.

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. Development and characterization of diamondback moth resistance to transgenic broccoli expressing high levels to Cry1C. **Applied and Environmental Microbiology**, Washington DC, v. 66, n. 9, p. 3784–3789, 2000.