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Identification et caractérisation de protéines ayant des propriétés entomotoxiques contre deux principaux ravageurs du maïs

Rania Jabeur

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Biologie des Interactions – Mécanismes des interactions Parasitaires Symbiotiques et Pathogènes

École doctorale GAIA

Unité mixte de recherche UM-INRAE 1333 DGIMI
En partenariat avec Limagrain Field Seeds

Identification et caractérisation de protéines ayant des propriétés entomotoxiques contre deux principaux ravageurs du maïs

Présentée par Rania JABEUR
Le 28 janvier 2022

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UNIVERSITÉ
DE MONTPELLIER

Dedication

I dedicate my dissertation work to my loving parents, Monia and Ahmed, whose words of encouragement and push for tenacity ring in my ears every day!! Thank you for your sacrifices, endless support and encouragement! I hope seeing you soon!

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Abstract: Due to their great adaptability to management strategies, insect pests are a constant threat to crops. Maize is the third most important staple crop in the world after wheat and rice. The western corn rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte, 1868) and the fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith, 1797) are among the most serious pests of maize and are responsible for massive yield losses every year. Bt maize expressing insecticidal proteins from *Bacillus thuringiensis* has long been a good alternative to chemicals. However, their overuse has led to the development of resistant populations. Hence, the need to find new compounds and strategies for sustainable control of these insects. In this PhD project, we aimed to develop biotech and biocontrol solutions for both WCR and FAW. In the first part, I was particularly interested in the study of binary insecticidal proteins named GDI0005A/GDI0006A identified by screening bacterial genomes and test against WCR larvae. These proteins were identified in *Chryseobacterium arthrosphaerae* genome. Heterologous expression of the two components was performed in *E. coli* system. One of the two components (GDI0006A) was a membrane lipoprotein and resulted in low amounts successfully purified. Nonetheless, the combination of the two proteins was active against WCR including WCR resistant to the currently marketed Cry3Bb1 and Gpp34Ab1/Tpp35Ab1 proteins. These results suggest that the new binary toxin may have different binding sites and thus a distinct mode of action compared to commercial toxins. The binary proteins were then expressed successfully in plant expression systems. However, the expression at low level of GDI0006A lead to no significant activity in planta. Finally, we tested the bacteria *C. arthrosphaerae* and show that it had activity in vitro against WCR, making of it a potential BCA against WCR.

The second part was devoted to studying the possible synergy between *Junonia coenia* densovirus (JcDV) and a Bt insecticidal protein Cry2Ab2 for biocontrol applications. Both are known to infect FAW and disturb the midgut permeability. However, when tested together either with *ex vivo* or *in vivo* approaches, we have noticed an antagonism suggesting a competition for the same midgut receptors. We also checked the feasibility of using VP4, the major capsid protein of JcDV in a biotech approach to enhance the activity of other gut binding insecticidal proteins. The preliminary results have shown a good expression of VP4 in *Nicotiana benthamiana* leaves without any sign of phytotoxicity. We demonstrated the ability of VP4 to self-assemble into virus-like particles (VLPs) in plant. When tested *ex vivo*, VLP-VP4 were able to penetrate the FAW intestinal cells. This proof of concept will be useful to explore the possible synergies with gut-binding insecticidal proteins to create new generations of transgenic maize resistant to FAW.

Keywords: *Diabrotica virgifera virgifera*, *Spodoptera frugiperda*, maize, insecticidal protein, *Junonia coenia* densovirus, insect midgut, synergy, biocontrol, resistance, transgenesis, virus-like particles

Résumé : Du fait de leur grande adaptabilité aux méthodes de lutte, les insectes demeurent une menace constante pour les plantes cultivées. Le maïs (*Zea mays* L.) est la troisième culture la plus importante dans le monde après le riz et le blé. La chrysomèle des racines du maïs (WCR) *Diabrotica virgifera virgifera* (LeConte, 1868) et la chenille légionnaire d'automne (FAW) *Spodoptera frugiperda* (J.E. Smith, 1797) sont parmi les ravageurs les plus importants du maïs et sont responsables de fortes pertes de rendement chaque année. Le maïs Bt exprimant des protéines insecticides provenant de *Bacillus thuringiensis* a été pendant longtemps une bonne alternative aux pesticides. Cependant, leur utilisation massive a conduit à l'apparition de populations de ravageurs résistantes, d'où la nécessité de trouver de nouvelles molécules et stratégies biotechnologiques ou de contrôle biologique pour gérer durablement ces insectes. Mes travaux de thèse s'inscrivent dans cet objectif et visent à trouver de nouvelles solutions contre WCR et FAW. Dans la première partie, je me suis particulièrement intéressée à une protéine binaire appelée GDI0005A/GDI0006A identifiée dans un criblage contre des larves de WCR. Ces protéines ont été isolées de la bactérie *Chryseobacterium arthrosphaerae* et ont montré une activité insecticide, lorsqu'elles sont exprimées sous forme de protéines recombinantes, contre des populations de WCR sensibles et résistantes aux protéines commerciales Cry3Bb1 et Gpp34Ab1/Tpp35Ab1. Ces résultats suggèrent que ces nouvelles protéines insecticides pourraient avoir des modes d'action différents de ceux des protéines Bt actuellement déployées et donc un nouveau mode d'action. Le deuxième membre de la protéine binaire (GDI0006A) est une lipoprotéine membranaire que j'ai purifiée partiellement. Cette propriété a été un facteur limitant pour évaluer le niveau d'activité de la protéine GDI0005A/GDI0006A. Nous avons essayé d'exprimer la protéine binaire en plante en utilisant deux systèmes d'expression : transitoire et stable. Les deux ont montré une faible accumulation de GDI0006A entraînant une perte de l'activité par rapport aux essais au labo avec des lysats bactériens. Nous avons également démontré que des cultures de la bactérie d'origine *C. arthrosphaerae* avait une activité contre WCR ce qui en fait un agent de biocontrôle potentiel contre les chrysomèles.

La deuxième partie a été consacrée à l'étude de l'éventuelle synergie entre *Junonia coenia* densovirus (JcDV) et une protéine insecticide Bt Cry2Ab2 dans une perspective de les combiner pour des applications de biocontrôle. Les deux sont connus pour infecter FAW et perturber la perméabilité intestinale de ce ravageur. Cependant, lorsqu'ils ont été testés ensemble avec des approches *ex vivo* et *in vivo*, nous avons plutôt remarqué un antagonisme suggérant une compétition avec les mêmes récepteurs intestinaux. Nous avons aussi vérifié la faisabilité de l'utilisation de VP4, la protéine majoritaire de la capsid de JcDV, dans une approche biotechnologique pour améliorer l'activité d'autres protéines insecticides qui ciblent l'intestin. Les résultats préliminaires ont montré une expression importante de VP4 dans les feuilles de

Nicotiana benthamiana sans aucun signe de phytotoxicité. Nous avons aussi mis en évidence la capacité de VP4 à s'auto-assembler en pseudo-particules virales (VLP) dans la plante. Ces VLP-VP4 ont été capables de pénétrer dans les cellules intestinales de FAW lorsqu'elles ont été testées *ex vivo*. Cette preuve de concept sera utile pour explorer les synergies possibles avec des protéines insecticides qui affectent l'intestin dans le but de créer de nouvelles variétés de maïs transgéniques résistants à FAW.

Mots clés : *Diabrotica virgifera virgifera*, *Spodoptera frugiperda*, maïs, protéine insecticide, *Junonia coenia* densovirus, intestin de l'insecte, synergie, biocontrôle, résistance, transgénèse, pseudo-particule virale

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List of abbreviations

ABC:	ATP Binding Cassette
ALP:	Alkaline Phosphatase
APN:	Aminopeptidase
BBU:	BugBuster Protein Extraction Reagent
Bt:	<i>Bacillus thuringiensis</i>
CADR:	Cadherin like proteins
CHAPS:	3-[(3-cholamidopropyl)dimethylammonio]-1-propa-nesulfonate
Cry:	Crystal toxin
CsVMV:	Cassava Vein Mosaic Virus
Cyclic-AMP:	Cyclic Adenosine Monophosphate
Cys:	Cystein
Cyt:	Cytolytic toxin
DDM:	n-Dodecyl β -D-maltoside
DIDI:	Dynamics of Interactions between Densoviruses and Insects
dsRNA	double-stranded RNA
<i>E. coli:</i>	<i>Escherichia coli</i>
eGFP:	enhanced Green Fluorescent Protein
EPA:	Environmental Protection Agency (EPA)
ESCRT:	Endosomal Sorting Complex Required for Transport
FAO:	Food and Agriculture Organization of the United Nations
FAW	Fall Armyworm (<i>Spodoptera frugiperda</i>)
GMO:	Genetically Modified Organisms
GPI:	GlycosylPhosphatidylInositol
Ha:	<i>Helicoverpa armigera</i>
IgG:	Immunoglobulin G
INRAe:	French National Research Institute for Agriculture, Food and the Environment
IPM:	Integrated Pest Management
JcDV:	Junonia coenia densovirus
LD50:	Lethal median dose
LMN:	Lethal Maize Necrosis disease
MCMV:	Maize Chlorotic Mottle Virus
MNPV:	Multiple Nucleopolyhedrovirus
mRNA:	messenger RNA
NB:	<i>Nicotiana benthamiana</i>
NPV:	Nucleopolyhedrovirus
NS:	Non-Structural proteins
ORF:	Open Reading Frame
PBS:	Phosphate-Buffered Saline
PCBs:	polyhedrin crystalline bodies
PCR:	Polymerase Chain Reaction
PLA:	Phospholipase

PM:	Peritrophic Matrix
PVDF:	Polyvinylidene difluoride
qPCR:	“real-time” or quantitative Polymerase Chain Reaction
RNA	Ribo Nucleic Acid
RNAi:	RNA interference
SEMAE:	French Interprofessional Organisation for Seeds and Plants
Sip:	Secreted insecticidal protein
SNF:	Sucrose-Non-Fermenting genes
Spase:	Signal peptidase
Tris:	Tris-buffered saline
Vip:	Vegetative insecticidal proteins
VLP:	Virus-like Particles
VP:	Capsid Protein
WCR:	Western Corn Rootworm (<i>Diabrotica virgifera virgifera</i>)

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GENERAL INTRODUCTION

“Natural selection does not give any preference at all to anything that, in the long run, could be advantageous for the species but blindly rewards everything that, momentarily, affords greater procreative success.”

Konrad Lorenz (1903- 1989)

Austrian zoologist, ethologist, and ornithologist

Nobel Prize in Physiology / Medicine (1973)

Plant-insect relationships are complex and include a wide range of potential interactions. An evolutionary race between plant defenses and the methods deployed by phytophagous insects to continue feeding has helped shape the diversity of plants and insects. Since the emergence of agriculture, humans have intervened significantly in these relationships, on the one hand, through conventional breeding, which has improved plant resistance to insects, and on the other hand, with insecticides to reduce the impact of insects on the crop. Today, due to the global population explosion and climate change, the control of insect pests on crops remains a major concern in order to ensure food security for human beings.

Maize (*Zea mays* L.) (Cyperales: Poaceae) importance stems from its ability to be converted into a wide range of products, including biofuel and bioplastics, as well as its use as an essential source of food for humans and livestock (Aithani and Mohanty, 2006; FAOSTAT, 2018; Mosier and Ileleji, 2020). Together with wheat and rice, it is a staple diet for over 4 billion people, accounting for up to 60% of global food energy consumption according to the Food and Agriculture Organization of the United Nations (FAO) (<http://www.fao.org/3/u8480e/u8480e07.htm>). However, corn production is mostly threatened by insects throughout its vegetative cycle. Lepidopteran caterpillars which are stalk borers, ear or leaf feeders, and the coleopteran root-eating larvae are the two most injurious corn insect orders. For instance, the fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) and the western corn rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte, 1868) (Coleoptera: Chrysomelidae) are responsible of massive yield losses annually in maize fields (Wechsler and Smith, 2018; Chimweta et al., 2020). For a long time, control of these two insects relied on different classes of chemical insecticides such as pyrethroids and organophosphates (Levine and Oloumi-Sadeghi, 1991; Sikirou et al., 2020; Souza et al., 2019). However, in addition to the impacts on humans, the environment and pollinators (Hashimi et al., 2020), the excessive use of these chemicals has led to the appearance of resistance (Pereira et al., 2015; E. Pereira et al., 2017; Gutiérrez-Moreno et al., 2019). The use of these synthetic products has therefore been widely questioned, hence raising the need for alternative pests control solutions.

Since its first commercialization in 1996, transgenic maize expressing insecticidal proteins from the soil bacteria *Bacillus thuringiensis* (Bt maize/corn) has marked the dawn of a new era in crop protection against both lepidopteran and coleopteran insects (Ostlie et al., 1997; Sanahuja et al., 2011). The principle is that when ingested by the target insect, these proteins bind to specific receptors in the brush border midgut cells leading to a pore formation resulting in cell damages and insect death (Bravo et al., 2007; Palma et al., 2014). The use of

Bt corn has been beneficial for farmers and the environment. It has been proved that Bt maize has better grain yield and nitrogen uptake (Haegele and Below, 2013). For instance, the average yield increase in Brazil from 2008 to 2016 has been estimated to be over 11.8 percent (Brookes and Barfoot, 2018). The quality of corn kernels has also improved by decreasing fungal infection rate caused by insect damage (Dowd, 2000). Moreover, the use of Bt corn has resulted in less damage to the environment and human health (farmers and consumers as well) by reducing insecticide use. According to (Brookes and Barfoot, 2020), between 1996 and 2018, the use of insecticide active ingredients for corn was reduced by 112 million kg due to the adoption of transgenic Bt corn. Because of its success and extensive use, insects resistant to Bt corn appeared rapidly. As soon as resistance emerged, numerous studies were conducted to identify new strategies to delay the development of resistant insect strains. The refuge strategy appears to be one of the solutions. It consists in sowing a proportion of non-Bt corn in a field of Bt corn. The refuge size is mandated by the Environmental Protection Agency (EPA) and it is highly dependent on the Bt protein and the targeted insect (Tabashnik and Gould, 2012). This approach genetically dilutes insect resistance by allowing susceptible insects in the non-Bt corn to mate with resistant insects in the Bt corn (Tabashnik and Carrière, 2017, 2019). The refuge strategy has been successful especially when using high dose Bt maize expressing two or more distinct insecticidal proteins either in pyramid combinations or in seeds mixtures (Bravo and Soberón, 2008; Tabashnik et al., 2013). Unfortunately, the efficacy failed when resistance to Bt proteins was dominant or when insects developed cross-resistance to the same family of Bt proteins used in combinations (Gassmann et al., 2014; Farias et al., 2016; Jakka et al., 2016b). The ability of insects to adapt and overcome resistance is inevitable, hence the need to keep fueling research for new insecticidal molecules and pest-management strategies.

Many seeds companies have investigated an RNA interference (RNAi) approach targeting the insect pest in their control strategies. The RNAi technique allows to silence essential genes and could be a good option in combination with other tools such as microbial insecticidal proteins (Christiaens et al., 2020). However, resistance to the RNAi approach has already been shown in the laboratory of Khajuria et al., (2018) for a WCR gene crucial for cell cycle (transmembrane protein sorting). It is also important to note that the success of this technique is limited to a certain order of insect. Indeed, this approach has proven to have poor efficacy against lepidopteran pests compared to coleopterans. For example, dsRNA was shown to be less stable in the gut of the European corn borer *Ostrinia nubilalis* (ECB) than in the gut of rootworms. Therefore, a greater amount of dsRNA is required to induce gene silencing in the corn borer (Cooper et al., 2020). A rapid degradation of dsRNA was observed in the gut and

hemolymph of other lepidopteran species. It could be due to nucleases or to the physiological pH (Arimatsu et al., 2007; Baum and Roberts, 2014; Guan et al., 2018; Cooper et al., 2020). Further development is needed to stabilize or protect the dsRNA once ingested, as well as to identify more genes whose silencing could control insect populations (Nitnavare et al., 2021).

The integration of agents from various origins into pest control has also been the subject of much research using a variety of strategies, ranging from classical microbial control to the development of genetically modified products. For maize pests, as mentioned above, Bt has been an extremely useful tool. Today, more and more research is being carried out to identify new molecules and or bio-control solutions that are not derived from *Bacillus*. For instance, research on fungi revealed the activity of the Aegerolysin/pleurotolysin B complex proteins from the fungal genus *Pleurotus* against the WCR. Interestingly, these fungal proteins have different binding sites compared with the commercial Bt aegerolysin Gpp34Ab1 protein (previously known as Cry34Ab1) opening the door to a new generation of bioinsecticides and transgenic corns (Panevska et al., 2019).

Another type of pathogens thoroughly investigated are entomopathogenic viruses. Among them, baculoviruses of the nucleopolyhedrovirus (NPV) genera are known to be highly infectious to economically important insect pests such as *Helicoverpa spp.* and *Spodoptera spp.* For this reason, many NPV-based commercial bioinsecticides are currently available, such as *Spodoptera exigua* nuclear polyhedrosis virus Spod-X (Certis) and *Helicoverpa zea* nuclear polyhedrosis virus GemStar (Certis) against these 2 insects. One of the limiting factors of this approach is the production and storage of NPVs (Sree and Varma, 2015). Besides, resistance to baculoviruses have been also reported for some lepidopteran at the lab and in the field as well (Siegwart et al., 2015).

Densoviruses from the *Parvoviridae* family were also explored for insecticidal activity. *Junonia coenia* densovirus (JcDV), for example, has shown to be specifically effective against few lepidopterans such as FAW larvae (Rivers and Longworth, 1972; Bergoin and Tijssen, 2008; Mutuel et al., 2010).

PHD THESIS CONTEXT AND GOALS

This PhD thesis is a Public-Private Partnership between the seed company Limagrain (based in Chappes-France) and the French National Research Institute for Agriculture, Food and the Environment (INRAe) DGIMI of Montpellier. It is co-financed by the French Ministry of Higher Education and Research and Limagrain through a CIFRE fellowship.

Limagrain has developed a research program in biotechnology on insect control, in partnership with the German company KWS, by creating the joint venture Genective. The main objective of the company is to develop new generations of transgenic corn with a strong efficacy against insects. It is targeting the American markets and especially the North American market for which the two companies have a joint seed company (AgReliant). The thesis project is linked to a project called DIETETIC (2016-2020). The objective of the DIETETIC project was to identify insecticidal proteins against the western corn rootworm (WCR), a pest that has appeared in France in recent years. The DIETETIC project is a collaboration with another private partner (PROTEUS), two public laboratories of the CEA (Genoscope and LEMIRE) and a laboratory of an international organisation, CABI, located in Hungary. This project has received financial support from SEMAE (Interprofessional Organisation for Seeds and Plants, formerly GNIS).

The PhD project includes an additional target, the fall armyworm. This part is a collaboration between Limagrain Europe and the DIDI team (Dynamics of Interactions between Densovirus and Insects) that is part of the mix research unit DGIMI between INRAe and the University of Montpellier (<https://www6.montpellier.inrae.fr/dgimi/Nous-contacter/Nos-coordonnees>) in which we studied the potential interaction between a FAW virus and bacterial insecticidal proteins.

Thus, the principal goal of this thesis is to explore the microbial world to identify novel bacteria / compounds active against maize pests, particularly WCR, and distinct from those currently used either in biocontrol applications or in transgenic maize. As discussed, the finding of new active molecules is not enough to control insects regarding their high physiological adaptation. Hence, we first aimed to find candidate insecticidal proteins derived from bacteria other than *Bacillus* spp. and having a novel mode of action compared to what is commercially available. In this part, we focused on the characterization of a couple of pesticidal proteins that have shown activity against WCR susceptible and resistant populations to the two-existing mode of action (Cry3 and Gpp34Ab1/Tpp35Ab1). In a second step, we conducted preliminary assays using commercial Bt proteins to assess whether the combination between microbial

proteins and densoviruses could enhance pathogenicity of the first and therefore, in the future, enhance the efficacy of our candidate proteins.

MANUSCRIPT OUTLINE

As illustrated in Figure 1, this manuscript is composed of two parts. The first section is committed to the-state-of-the-art review in which I present the two insects models this work focuses on: the western corn rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte, 1868) and the fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith, 1797). Their biology, geographical distribution, the damages they cause on host plants and the different existing management tools to control them will be described. Then, I discuss the frequently used Bt proteins in the corn industry, their mode of action, the possible resistance scenarios, and the potential synergy with viruses. The second section will be devoted to my contributions. In the first chapter, I explain the strategy that we used for identification and characterization of a new family of binary pesticidal proteins active against WCR larvae. Assays toward evaluating the potentialities of these proteins in pest control are also presented. We will describe the possible production of transgenic maize expressing these proteins as well as the potential use of the originating bacteria as a biocontrol agent. The second chapter is dedicated to the evaluation of the possible interactions between the densovirus JcDV / its major capsid protein and Bt insecticidal proteins by using FAW as an insect model. Then, in a perspective of using the combination of the two in a GM approach, I evaluated the expression of the capsid protein in plant using a transient expression in *Nicotiana benthamiana* leaves and its ability to pass through the midgut as the virus does.

The manuscript concludes with a general discussion that summarizes the contributions and viewpoints of this thesis.

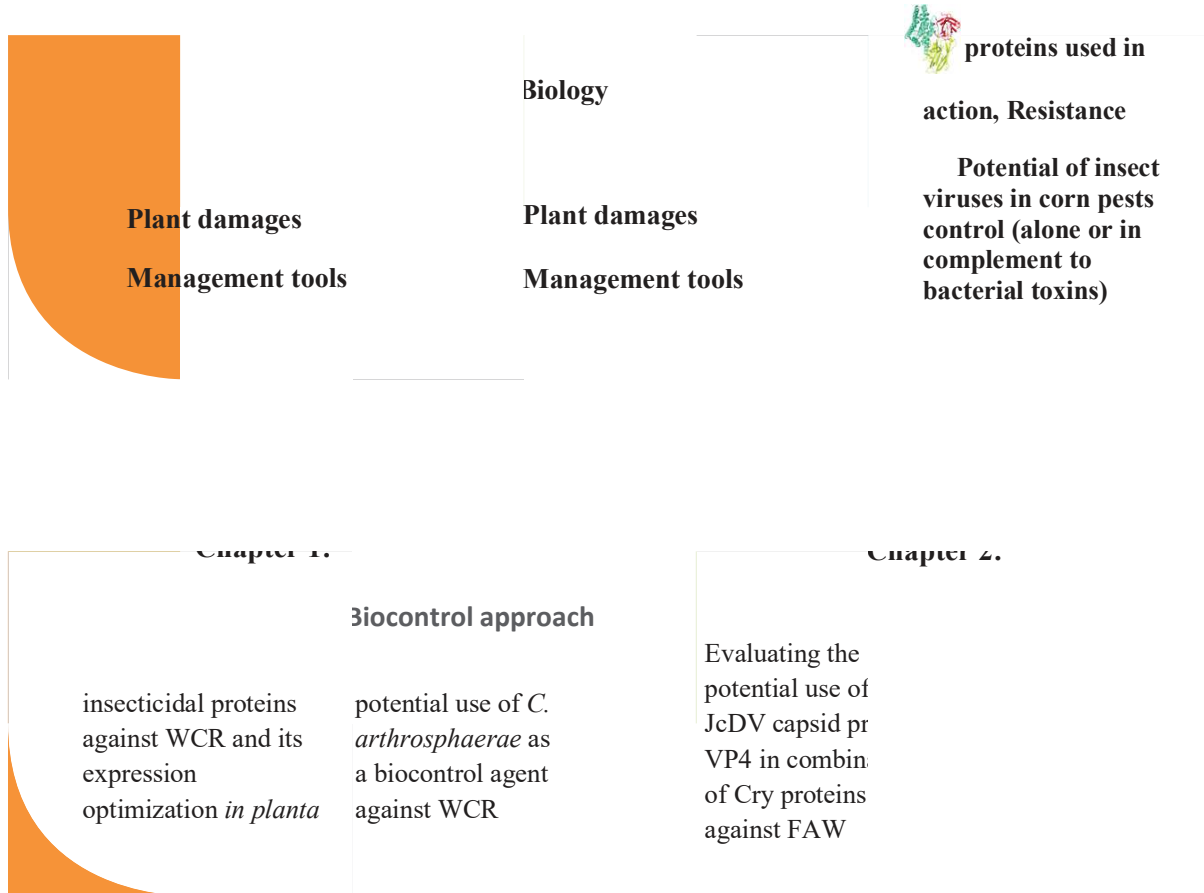


Figure 1: Manuscript outline

Part 1: State of the art

1 Two invasive insect pests threatening corn crops

1.1 The Western Corn Rootworm (WCR) *Diabrotica virgifera virgifera*

The western corn rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte, 1868) (Coleoptera: Chrysomelidae) is believed to be native in Central America (Krysan and Smith, 1987). It became the most serious pest across the US corn belt (Gray et al., 2009). It was accidentally introduced into Europe and was spotted for the first time in 1992 near Belgrade, Serbia. Since then, this insect has spread throughout 32 European countries, making its eradication no longer possible (CABI, 2021).

1.1.1 Description

The adult is approximately 5 mm long. A sexual dimorphism has been reported. Females are longer (between 4.4 and 6.8 mm) than males (between 4.2 and 6.6 mm). The beetle's body is consistently bright yellow, except for the head and elytra, which are characterized by longitudinal black lines. In the male, these bands are fused between the wings while in the female a band is located outside each wing and a third in the middle of both. The antennae, implanted on the forehead, are longer in the male than in the female (Krysan and Smith, 1987). The eggs are oval, beige, and less than 1 mm in size (CABI, 2020). Larvae are white to pale yellow in color, with a yellowish-brown head capsule and a brownish plate on the last abdominal segment. Their size varies between 2 mm and 13 mm at the third instar (Chiang, 1973). Pupae are usually found naked in clay cells around plant roots. They are white but change into brownish before adult emergence (CABI, 2020).

1.1.2 Life cycle and biology

The WCR is a univoltine species that overwinters as an egg in the soil. Larvae hatch in the spring with the rise in temperature. Neonates are attracted to carbon dioxide (CO₂) emitted by host plants (Arce et al., 2020). They go through three instars of growth in and on the host roots before reaching pupal stage in about a month (Strnad and Bergman, 1987). Adults emerge from June to September. Males appear before females and reach sexual maturity at the time females appear (Branson, 1987). The reproduction rate of rootworms is quite high. A single female can lay up to 1000 eggs in moist soil or in cracks and crevices at the base of host plants (Hill, 1975; Kirk, 1979, 1981). Adults die in the autumn, eggs go through diapause in the winter, and a new cycle begins in the next spring (Figure 3).

Because they are mostly pollinivorous, adults of the WCR can feed on a broad variety of plants. They are most commonly found on flowers, particularly those of the cucurbit family.

They can feed on floral organs, maize silks, and immature kernels as well (Moeser and Hibbard, 2005). Larvae, on the other hand, are more specialized and only develop on the roots of a few number of Poaceae species, making them particularly harmful, particularly to maize (Branson and Ortman, 1970; Clark and Hibbard, 2004; Moeser and Vidal, 2004).

1.1.3 Damage and symptoms

The WCR is particularly voracious in the larval stage. The larvae feed by mining the maize roots, altering root system structure and function which results in decreased water and nutrients uptake by the plants and significant yield losses (Wesseler and Fall, 2010; Schumann and Vidal, 2012). In case of a severe attack, the root system being weaker, the plants tend to take on, in adverse weather conditions, a characteristic "gooseneck" appearance (Figure 3). This may cause additional economical losses due to mechanical difficulties at harvest time (Manole et al., 2017; Sivcev et al., 2012).

Even though the primary damages are caused by larvae, WCR adults, which are highly mobile across corn fields, can also cause supplemental damage by feeding on silks, pollen and immature kernels. Cutting of silks by WCR adults can limit pollination and, as a result, reduce the number of grains. They can also feed on young corn leaves and by this transmit phytoviruses. For instance, WCR is a vector of the Maize Chlorotic Mottle Virus (MCMV) which, when combined with any potyvirus, can cause the Lethal Maize Necrosis disease (LMN) (Jensen, 1985; Gray et al., 2009; Awata et al., 2019), a disease that can drastically reduce corn yields (Chapwanya et al., 2021).

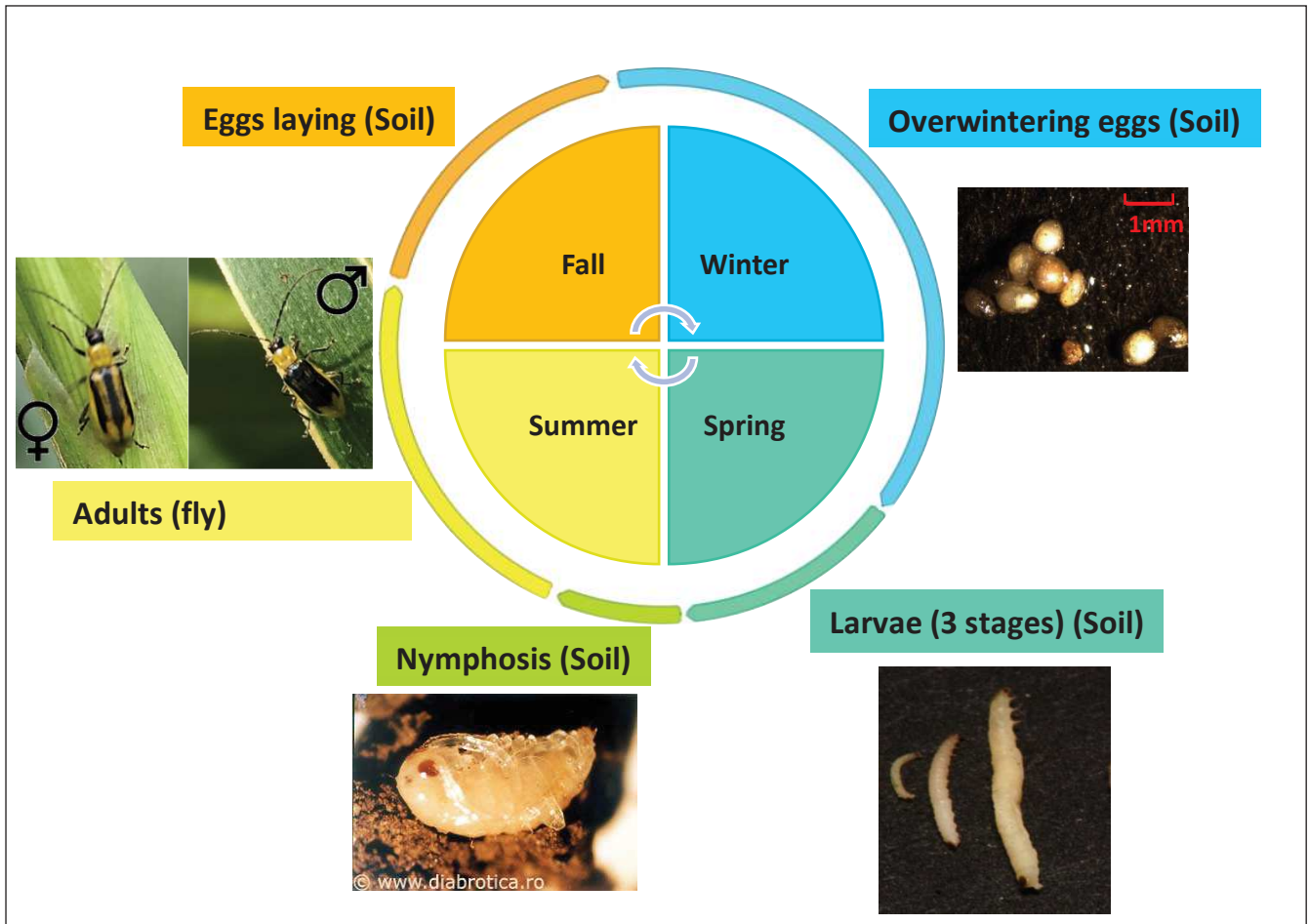


Figure 2: *Diabrotica virgifera virgifera* life cycle (adapted from: <https://arena-auximore.fr>)



Figure 3: *Diabrotica virgifera virgifera* damages on maize plants. A: adult feeding on silks and young grains. B: severe larvae attack on roots (Hungary, 2018)

Photos by Christophe Sallaud & Virginie Guyon

1.1.4 WCR management strategies

Chemical control

Insecticides targeting the larvae stage either by seeds and soil treatments or targeting adults through aerial applications have been widely used in both the USA and Europe to minimize WCR damages in corn fields. Pyrethroids, organophosphates and neonicotinoids were the most frequently used products against both adults and larvae (Levine and Oloumi-Sadeghi, 1991; Souza et al., 2019). Whereas they remain largely employed in the Americas, their use is now limited in Europe. Indeed, restrictions have been implemented in Europe particularly for neonicotinoids due to their undesired side effects on the agroecosystems and non-target organisms (Girolami et al., 2012). Their use in the long term is also compromised because of the appearance of resistances of WCR to many insecticide classes (Meinke et al., 1998; Wright et al., 2000a; Souza et al., 2019).

Cultural practices: crop rotation

Cultural practices are commonly used in insect management to disrupt the insect life cycle. For years, maize rotation with other non-host crops such as soybean has been a good solution to control WCR populations in both Europe and USA. However, annual rotation has led to the appearance of rotation-resistant populations. In the USA, WCR populations overcame the maize-soybean rotation strategy by losing their preference to maize for oviposition (O'Neal et al., 2002). As a result, eggs were laid on soybean plants, which after wintering hatched to larvae already settled in the field planted with corn the following year (Levine et al., 2002). According to (Onstad et al., 2003) rotation-resistance seems also inevitable in Europe, though at a slower rate. This is probably because maize fields account for only 9,32% of overall agricultural lands (Eurostat, 2020).

Use of transgenic plants

- Use of Bt-corn

Pesticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) have been successfully used to control rootworms when expressed in maize (Sanahuja et al., 2011). A detailed description of the mode of action of these proteins will be discussed below. The MON683 event, developed by Monsanto in 2003, has been the first transgenic maize against WCR larvae producing the Bt insecticidal protein Cry3Bb1 (Vaughn et al., 2005). Then several pesticidal proteins have been registered: the binary proteins Gpp34Ab1/Tpp35Ab1 (previously known as Cry34Ab1/Cry35Ab1), the modified mCry3A and the chimeric eCry3.1Ab (modified mCry3A and Cry1Ab). Except for eCry3.1Ab, all these proteins were initially expressed

separately. Then, to delay the establishment of resistance, pyramids combining Cry3Bb1, Gpp34Ab1/Tpp35Ab1, mCry3A, or eCry3.1Ab were commercialized (Environmental Protection Agency, 2009, 2011, 2012; Carrière et al., 2015)). Despite their effectiveness, these proteins were not highly dosed and led to the appearance of resistance. Thus, field-evolved resistance to Cry3Bb1 has been detected (Gassmann et al., 2011) before extending to mCry3A and eCry3.1Ab via cross resistance (Gassmann et al., 2014). Besides, root injuries caused by WCR on transgenic maize expressing Gpp34Ab1/Tpp35Ab1 have been also observed (Gassmann et al., 2016).

- **Refuge strategy**

After the first detection of WCRs resistant to Bt proteins, it was suggested to use the refuge strategy. The later consists in planting non-Bt plants in a “refuge area” (suitable for the pest) in close vicinity to Bt plants. Thus, susceptible insects are maintained in the population; they can mate with resistant WCR issued from Bt plants, what lowers the resistance frequency in the pest population. However, the success of this strategy requires a low initial resistance in the pest population, the recessive nature of the resistance and Bt plants producing a high concentration of Bt proteins (Tabashnik and Carrière, 2017, 2019). In the case of WCR, the frequency of resistance was high, and the Bt proteins concentrations were quite low in commercially available Bt-corns. Therefore, this strategy failed in controlling WCR populations (Andow et al., 2016; Gassmann et al., 2014). Another approach was tested, named “the refuge in the bag”, that consists in selling to farmers seeds comprising a fixed percentage of non-Bt seeds blended with Bt seeds (expressing singly or as pyramids Bt proteins against WCR) (Yang et al 2015). However, this approach wasn't a long-term success especially with the appearance of WCR cross-resistance to the Cry3 family proteins used in the combinations (Gassmann et al., 2014).

- **Use of RNAi**

RNA interference (RNAi) is one approach intensively studied in recent years as an alternative to Bt proteins against WCR. It consists of the post-transcriptional downregulation of a given gene by the introduction of a specific double stranded RNA (dsRNA) into the cells (Whangbo and Hunter, 2008). The effectiveness of this gene silencing method depends on the targeted insect and the manner in which the dsRNAs is introduced (injection or ingestion) (reviewed in Terenius et al., 2011; Vélez and Fishilevich, 2018). Promising results have been reported against WCR larvae using dsRNA that target a vital gene *DvSnf7* (*Diabrotica virgifera* sucrose-non-fermenting genes SNF7) encoding for a class E vacuolar sorting protein that belongs to the ESCRT (Endosomal Sorting Complex Required for Transport)-III complex and

required for transmembrane protein sorting. Significant mortality and stunting have been observed after WCR exposure to DvSnf7 dsRNA on both artificial diet and transgenic corn (Baum et al., 2007; Bolognesi et al., 2012). Thus, many seeds company have been interested in integrating this dsRNA gene with other microbial insecticidal proteins. For example, MON87411 is a cultivar commercialized by Monsanto company expressing Bt proteins (Cry3Bb1 and Gpp34Ab1/Tpp35Ab1) and dsRNA targeting DvSnf7. (Levine et al., 2015; EFSA Panel on Genetically Modified Organisms (GMO) et al., 2021). However, it has been demonstrated in Khajuria et al., (2018) the ability of rootworms to develop resistance to dsRNAs whatever the gene target. Further development is needed to stabilize the dsRNA once ingested, as well as to identify more genes whose silencing could control insect populations.

Biological control

The increased interest of citizens in organic products, as well as the restrictions in several countries affected by WCR on conventional pesticide use, have underlined the need of using natural enemies to fight this pest. The possible use of fungus such *Beauveria spp.* and *Metarhizium spp.* to reduce damage caused by WCR has been studied. Pilz et al., (2009), for example, have shown that treatment with *Metarhizium brunneum* induced a 31% reduction of WCR emergence in corn fields comparing to the negative control. Toxicity of *Beauveria bassiana*, and *Beauveria brongniartii* have been also evaluated on WCR larvae and adults. Both have been shown to have a significant effect on larvae survival and adults fecundity (Mulock and Chandler, 2001; Cagán et al., 2019). Moreover, some studies have focused on identifying the molecules responsible for mortality of WCR. Recently, a study has shown the possible use of aegerolysins from the fungal genus *Pleurotusnematodes* as a bioinsecticide against WCR (Panevska et al., 2020).

Entomopathogenic nematodes from the Steinernematidae and Heterorhabditidae families have also caught the interest of researchers for biological applications against WCR. Similar efficacies of *Heterorhabditis bacteriophora* Poinar to conventional insecticides on WCR larvae have been reported (Pilz et al., 2009; Modic et al., 2020).

Unfortunately, some variability in the effectiveness of biological agents (nematodes and fungi as well) against rootworms have been noticed when applied under field conditions (Pilz et al., 2009). Thus, since they appeared not sufficient by themselves, researchers rather recommend the use of biological agents within integrated pest management (IPM) strategies (Rauch et al., 2017).

1.2 Fall armyworm: *Spodoptera frugiperda*

1.2.1 Geographical distribution

The fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith, 1797), is a noctuid moth belonging to the lepidopteran order originating from the tropical and subtropical regions of the Americas. This species has a strong dispersal ability and a high invasive potential (Early et al., 2018). To date, FAW has spread from the Americas to over 70 countries from different continents. It was first found in Central and Western Africa in early 2016 and has spread in nearly all of the Sub-Saharan Africa in less than two years (CABI, 2021; Sisay et al., 2018). Between 2018 and late 2019, FAW has been confirmed in several Asian nations such as India, China, and Japan. In February 2020, it reached Australia where it has largely expended. Recently, New Caledonia and Spain's Canary Islands have reported the presence of FAW (CABI, 2021) (Figure 5).

1.2.2 Description

Adults are nocturnal. A sexual dimorphism has also been reported for this insect. Females are longer (1.7 cm length with 3.8 cm of wingspan) than males (1.6 cm length with 3.7 cm of wingspan). Besides, females have uniform greyish brown forewings that may be finely mottled of grey and brown, whereas males have mottled forewings (light brown, grey, straw). The eggs are oval, pale yellow, and less than 0.5 mm in size. Larvae are pale green to dark brown with longitudinal stripes. Their size varies between 2 mm and 45 mm depending on the larval stage. A yellow inverted Y-shape on the head and four black spots grouped in a square on the last abdominal segment are the most distinguishing features of large larvae. Chrysalids are shiny brown with 15-20 mm of length (Rwomushana, 2020).

1.2.3 Life cycle

FAW is a non-diapausing insect with a life cycle highly dependent on temperature (Du Plessis et al., 2020). During their lifespan, females can lay up to 1000 eggs in tight clusters of 100-200 eggs on host plants leaves or young stems. Larvae hatch in 2-3 days in warm regions and go through 6 growth stages in general. The last instar larvae feed substantially more than the others to store lipids (fatty substance) and prepare for pupation. After 14-22 days, the fully grown caterpillar will fall to the ground where it pupates. Adults emerge within 7-13 days. They are known for their strong migratory behavior. They can fly over 500 km after mating and before oviposition (Rwomushana, 2020) (Figure 5).

The life cycle of the FAW takes about 30 days in the summer, 60 days in the early spring and autumn, and 80 to 90 days in the winter. As a result, the number of generations differs by geography and can reach up to eight in tropical environments (Capinera, 1969).

1.2.4 FAW biology and damage

FAW is a polyphagous insect pest that can feed on around 353 host plants according to (Montezano et al., 2018), but with a preference for some staple grasses such as maize, rice, sorghum and wheat (Silva et al., 2017). Two FAW host strains have been reported: a corn strain which feeds preferentially on maize, sorghum and cotton, and a rice strain that is mostly found in rice, Bermuda grass and sugar cane (Pashley, 1986; Juárez et al., 2012). Although they are morphologically indistinguishable, it has been demonstrated that the corn and the rice strains are genetically different suggesting they might be different species (Dumas et al., 2015).

FAW attacks maize at all developmental stages. Young larvae usually feed on the underside of leaves creating a characteristic windowing effect. Later larval stages are more voracious and make holes on the whorls. Thus, damaged plants present characteristic shot holes with ragged leaf symptoms. Even though they are often cannibalistic, one or 2 larvae left per whorl can defoliate and destroy a whole plant. Whorl damage and ear feeding are responsible for reduced grain quality and yield (Barlow and Kuhar, 2009; Lima et al., 2010) (Figure 6). Without any treatment, the FAW has been estimated to have the potential to cause annual yield losses in corn from 4.1 to 17.7 million tons depending on the country (estimates for 12 African countries), which is equivalent to US\$ 1,088 and US\$ 4,661 million (Rwomushana et al., 2018).

1.2.5 FAW management strategies

Chemical control

FAW control is challenging due to its strong dispersal capacities and adaptability to various control strategies. FAW management in the Americas relies mostly on insecticide sprays and genetically engineered maize expressing Bt proteins (Hruska, 2019). Insecticide applications mostly target the young larvae before they reach the whorl, which limits their efficacy. Besides, intensive use of insecticides has led to the appearance of resistance of FAW to many insecticide classes (Gutiérrez-Moreno et al., 2019).

Since FAW appearance in other continents, concerns about controlling this pest have increased. Lessons have been taken from the America's experience and it was advised by the FAO to consider integrated approaches for managing FAW (FAO, 2018). Thus, association of different options such as cultural, chemical, and biological practices have been deployed to help decreasing population diversity and adaptation potential as well as reducing insecticide

applications (Hutchison et al., 2010). In such IPM strategies, monitoring of FAW populations via pheromones traps is essential and allows to decide which methods to apply or integrate.

Cultural practices

Cultural practices also can be helpful in FAW management. They rely essentially on early planting to reduce larvae densities (that reach it higher rate in late season) and on deep plowing to kill pupae. Hand picking and eggs/larvae killing, as well as placing sand or wood-ash in whorls of maize plants could be efficient for smallholder farmers especially in Africa (Matova et al., 2020).

Use of transgenic crops

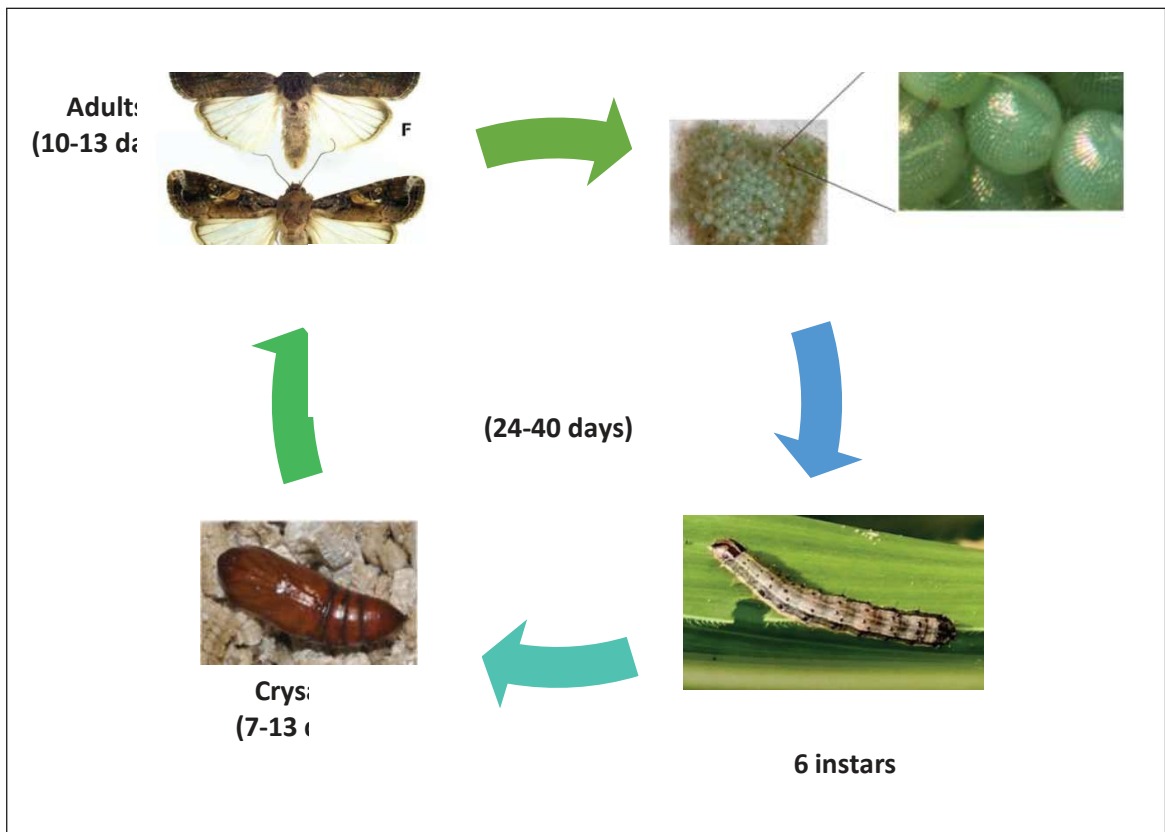
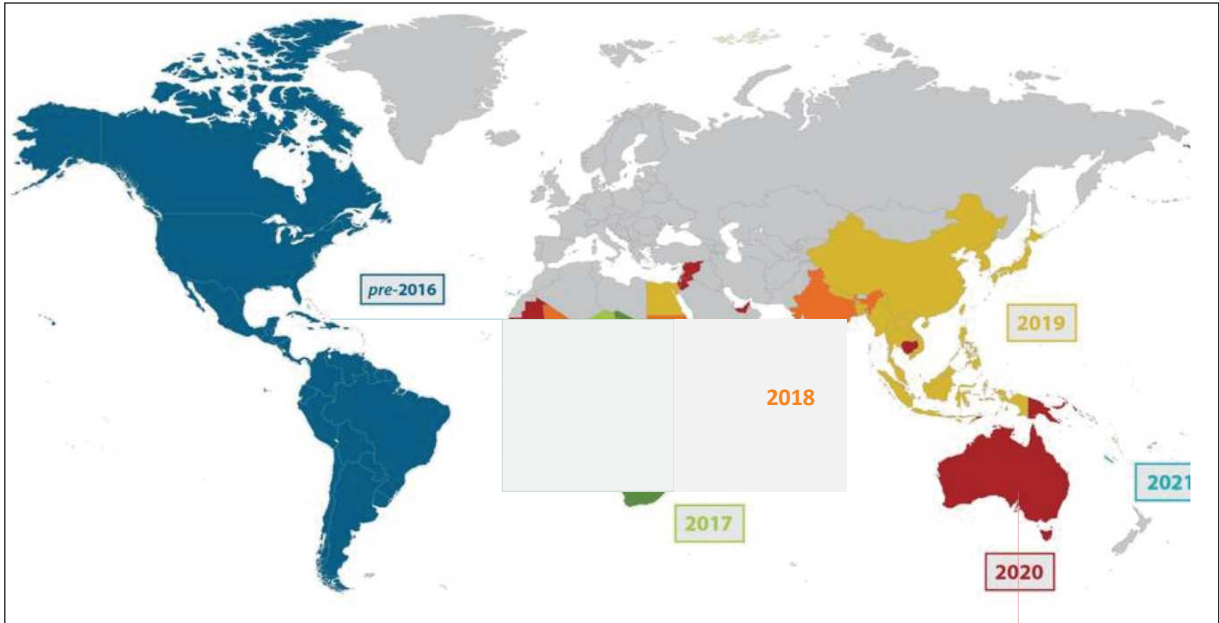
Transgenic maize hybrids expressing Bt toxins in the stationary growth phase (Cry1A, Cry2A and Cry1F types) or the vegetative growth phase (Vip3Aa20) have been used against FAW either singly or coexpressed together. Except for Vip3Aa20, their excessive use as well as their low-dose efficacy are responsible for the appearance of resistance in a short period (Farias et al., 2014; Huang et al., 2014; Storer et al., 2010). The emergence of FAW resistant to Cry1Ab, Cry2A, and Cry1F was reported in Brazil barely three years after their market introduction (Faretto et al., 2017).

Biological Control

Biological control using natural enemies has been also largely investigated in FAW control. For instance, following arrival of FAW in Africa, different native parasitoids wasps (*Cotesia icipe* Fernandez-Triana & Fiobe (Braconidae), *Chelonus curvimaculatus* Cameron (Braconidae), *Coccygidium luteum* (Brulle) (Braconidae), *Charops ater* Szepligeti (Ichneumonidae)) and the dipteran (*Palexorista zonata* (Curran) (Tachinidae)) have been evaluated for their potential as biological control agents against FAW in Ethiopia, Kenya and Tanzania. The results are encouraging but with some variability among the countries suggesting their use in an integrated management program (Sisay et al., 2018, 2019; Gebreziher, 2020).

Besides, baculoviruses from the genera of nuclear polyhedrosis virus (NPV) are among the mostly used entomopathogens and the most commercialized. Due to its specificity, biosafety (no detectable toxicity to mammalian) and virulence level, the *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) has shown an important potential for FAW management (Landwehr, 2021). The pest is infected by ingesting the baculovirus occlusion bodies (able to withstand environmental conditions). Once solubilized in the insect gut, virions are released and reach the epithelium cells nuclei where they replicate. Virus multiplication occurs in the target tissues such as muscles, tracheas and hemocytes. At the end of replication cycle, virions

are included in polyhedrin crystalline bodies (PCBs) to form the occlusion bodies that will be released upon death of the infected insect, allowing their dissemination (Airenne et al., 2013; Harrison et al., 2018; Ohkawa et al., 2010, 2005; Rohrmann, 2013; Williams et al., 2017). Baculovirus infection is commonly accompanied with severe deterioration of the host integument (Vieira et al., 2012). Infected insects usually have blemishes, yellow skin, and a decrease in feeding behavior (Rwomushana, 2020).



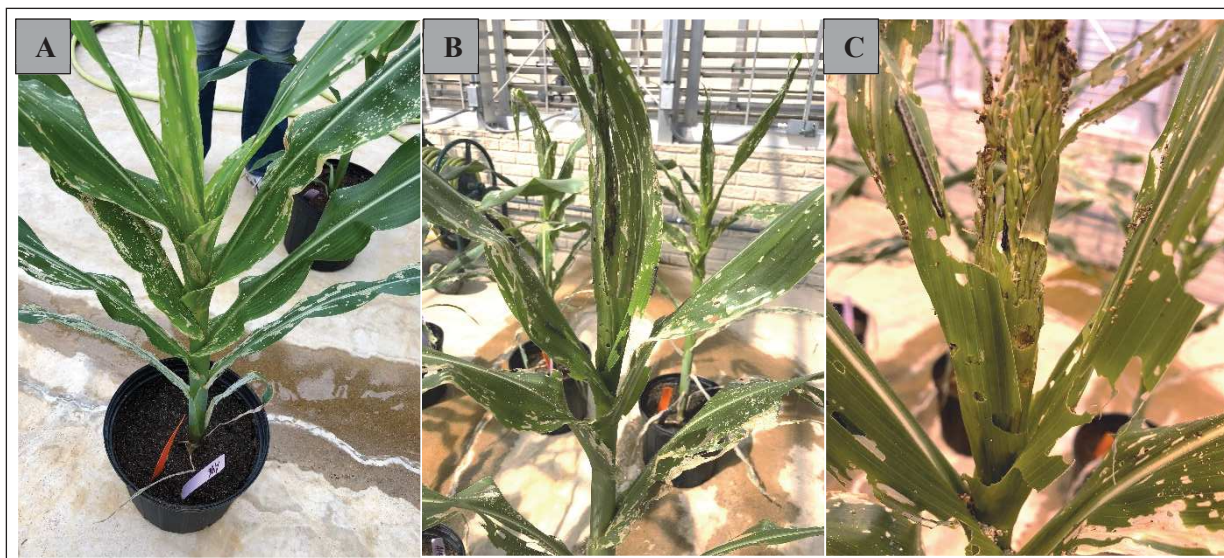


Figure 6: FAW damage on maize susceptible plants (A188) (Genective, 2019). A-B-C: 3 days - 7 days-13 days post infestation with FAW neonates respectively

2 The use of Bt and derivatives in control of corn insect pests

Used either in bioinsecticide formulations or to produce genetically resistant plants, the bacterium *Bacillus thuringiensis* (Bt) have long been the “leader” among crop protection options. This gram-positive rod-shaped bacterium secretes a variety of insecticidal proteins during its different growth phases. The vegetative growth phase is known for producing secreted insecticidal protein (Sip reassigned as Mpp by (Crickmore et al., 2020) (Table 1) and vegetative insecticidal proteins (Vip), whereas parasporal crystalline δ -endotoxins, such as cytolytic toxin (Cyt) and crystal toxin (Cry), are produced during sporulation and the vegetative stationary phase (Palma et al., 2014) (Figure 7). Sip proteins are probably pore forming proteins (Palma et al., 2014). Two Sip (Sip1Aa and Sip1Ab) proteins were identified to be active against some coleopterans such as WCR (Donovan et al., 2006; Sha et al., 2018). The Vip1 and Vip2 proteins act as binary toxins and are toxic to some members of the Coleoptera and Hemiptera whereas Vip3 has no sequence similarity to Vip1 or Vip2 and is toxic to a wide variety of members of the Lepidoptera (Chakroun et al., 2016). The last member of the Vip family, Vip4 has no identified insect target. To date, Vip3 proteins have become an alternative to delay resistance against Cry proteins. For instance, Vip3Aa20 is now commercialized in a pyramid maize event against fall armyworm. It has been demonstrated that Vip3Aa has a mode of action different from Cry proteins. The Cyt family is more specific to dipteran. Two modes of action have been reviewed for Cyt proteins by (Chattopadhyay and Banerjee, 2018). One suggested a pore formation scenario and the other one is known as a detergent model in which the protein

disrupts the lipid bilayer of the membrane (Chattopadhyay and Banerjee, 2018). Cry proteins remain the most studied among all Bt proteins. This might be related to their success in agricultural pest management but also in the control of vectors of human diseases such as malaria and dengue (Pardo-López et al., 2013). Here, we will put more details on the mode of action of Cry proteins and the potential reasons behind the appearance of resistance.

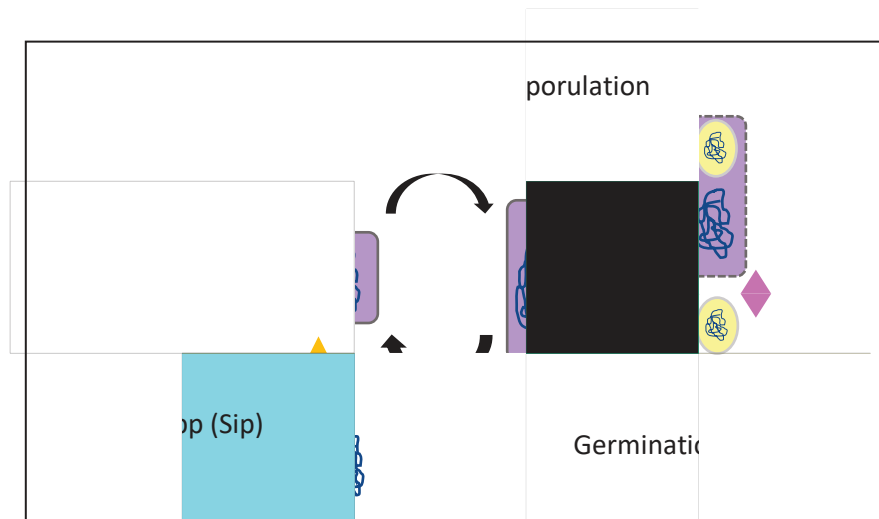


Figure 7: *Bacillus thuringiensis* growth phases. Sporulation occurs with a sporangium asymmetric division to produce two compartments: the mother cell and the prespore. The mother cell will then lyse to release a mature spore into the environment. During this phase 2 proteins parasporal crystalline endotoxins are produced: Cry and Cyt proteins. Spores are capable of quickly germinating and resuming vegetative growth in response to nutrients. During the cell division 2 proteins can be secreted /released into the medium: Mpp (Sip) and Vip proteins

Crystal proteins: Structure, mode of action and resistance

- Nomenclature:

The Bt pesticidal proteins nomenclature has been strictly based on amino acid sequence comparison. The Bt toxin nomenclature committee gave a four-rank name to each Bt pesticidal protein depending on its amino acid similarity with other Bt proteins from the same family. The first rank is represented by an Arabic number indicating a 45% similarity between two proteins. Then an uppercase and lowercase letter denote the second and the third ranks for 78% and 95% similarities, respectively, the fourth rank is denoted by an Arabic number for proteins that share more than 95% sequence identity (Palma et al., 2014). (Figure 8). This nomenclature has evolved and recently a new structure-based nomenclature has been proposed for Bt and non-Bt pesticidal proteins (Crickmore et al., 2020).

Cry2Ab2

Protein family (type) (Cry, Cyt, Sip, Vip)	Rank1 45%	Rank2 78%	Rank3 95%	Rank4 >95%
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Figure 8: Schematic representation of Bt insecticidal proteins based on a nomenclature system used by the Bt Toxin nomenclature Committee for δ -endotoxins (Cry and Cyt) and secretable (Vip and Sip) toxins.

Table 1: Classification groups within the revised pesticidal protein nomenclature. Conserved protein domains associated with each class are given along with examples and their protein database codes

Class	Previous classification	Conserved domain(s)	Description (PDB codes)
Cry	Cry	pfam03945, pfam00555, cd04085	Proteins originally isolated from <i>B. thuringiensis</i> crystals in which the active form normally consists of three domains. Examples include Cry1Aa (1CIY) and Cry3Aa (1DLC)
Cyt	Cyt	pfam01338	Cytolytic, normally single domain, proteins such as Cyt2Aa (1CBY)
Vip	Vip3	pfam12495, pfam02018	Multi-domain proteins originally identified as being V egetative I nsecticidal P roteins such as Vip3Bc (6VIV)
Tpp	Cry, Bin	pfam05431	Beta pore-forming pesticidal proteins containing the Toxin_10 (Bin-like) domain. Examples include Tpp35Aa (previously Cry35Aa 4JP0) and Tpp1Aa (previously BinA 5FOY)
Mpp	Cry, Mtx2, Sip	pfam03318	Beta pore-forming pesticidal proteins from the ETX/Mtx2 family. Examples include Mpp51Aa (previously Cry51Aa 4PKM) and Mpp2Aa (previously Mtx2)
Gpp	Cry	pfam06355	Aegerolysin like pesticidal proteins such as Gpp34Aa (previously Cry34 4JOX)
App	Cry, Pax, Xax, Yax		Predominantly alpha helical pesticidal proteins such as App6Aa (previously Cry6Aa 5KUD) and App1Ca (previously YaxA 6EK7)
Spp		pfam01289, pfam17440	Sphaericolysin like pesticidal proteins
Mcf		pfam12920	Proteins related to the « M akes C aterpillars F loppy » toxins originally described from <i>Photorhabdus</i> .
Mtx	Mtx1		Proteins related to the Mtx1 toxin (2VSE) originally isolated from <i>Lysinibacillus sphaericus</i>
Vpa	Vip2	cd00233	Proteins related to the ADP-ribosyltransferase active component of binary toxins such as Vip2 (1QS2) (from the Vip1 / Vip2 toxin)
Vpb	Vip1, Vip4	pfam07691, pfam03495, pfam17475, pfam17476	Proteins related to the b inding component of binary toxins such as Vip1 (6SMS), Vip4.
Pra	PirA		Proteins related to the <i>Photorhabdus</i> Insect-Related toxin A component.
Prb	PirB	pfam03945	Proteins related to the <i>Photorhabdus</i> Insect-Related toxin B component.
Mpf	PluMACPF GNIP	pfam01823	Pesticidal proteins that are part of the M embrane A ttack C omplex / P erforin superfamily.
Xpp			A holding class for pesticidal proteins with currently uncharacterized structures.

where known (Crickmore et al., 2020)

- Structure:

Most Cry proteins share a conserved structure with 3 domains: The domain I located in the N-terminus is a bundle of seven α -helices in which the central helix- $\alpha 5$ is hydrophobic and surrounded by six other amphipathic helices. This helical domain is in charge of membrane insertion and pore-formation. The domain II consists of three anti-parallel β -sheets with exposed loop regions. The domain III is a β -sandwich of 2 antiparallel β -sheets. Exposed regions in domain II and domain III are mostly involved in receptor binding and in specific interactions with different gut proteins (Figure 9) (Bravo et al., 2007).

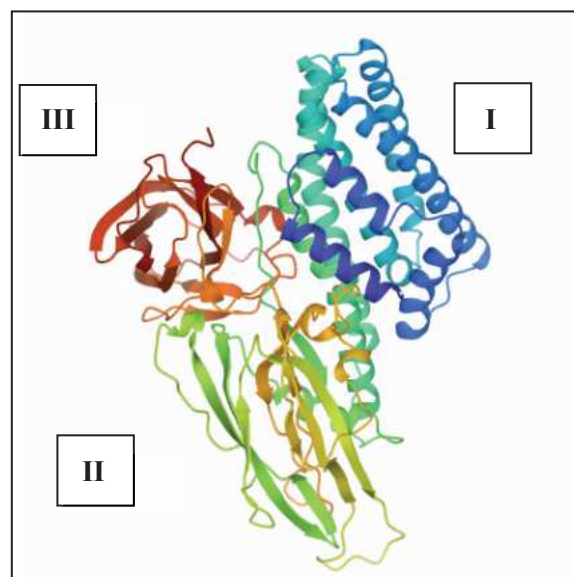


Figure 9: 3-domain structure of Cry1Aa protein from PDB ID 1CIY. I: N-terminal domain (α -helices); II: central core domain; III: C-terminal β -sandwich domain (PDB ID 1CIY)

- Mode of action:

Cry proteins are produced by Bt as insoluble crystal protoxins. Different modes of action have been proposed. The two most often discussed are the classical model, also known as “sequential binding model”, and a recent one, known as the “signaling pathway model” (Chattopadhyay and Banerjee, 2018; Heckel, 2020) (Figure 10). In both models, the ingested Cry protein is solubilized and activated in the insect midgut fluid under suitable physicochemical conditions. Cry proteins solubilization usually requires an alkaline pH. The protoxin activation consists in N-terminal end (and C-terminal end in case of long protoxin) processing by gut proteases (trypsin and chymotrypsin like proteases) to eventually form monomers (Bravo et al., 2007). Some protoxins can be active without processing. The

monomers will then interact specifically with receptors present on the midgut epithelial cells surface leading to cell death. To date, various insect midgut receptors, such as ABC transporters, glycosylphosphatidylinositol (GPI) anchored alkaline phosphatases (ALP), cadherin like proteins (CADR), and GPI anchored aminopeptidases (APN), have been described as a prerequisite for Cry proteins activity (Yudina et al. 2007).

In the Bravo model (Classic mode of action), the processed Cry protein oligomerizes to form a pre-pore complex. Once inserted, the membrane-spanning α -helix hairpins of the oligomer creates a small pore in the membrane. The pore formation allows the entrance of cations which causes an osmotic chock leading to cell lysis. Furthermore, a severe epithelium disruption affects the insect feeding behavior and enables bacteria (including Bt spores) existing in the gut content to leak out into the hemocoel, resulting in septicemia and insect death (Bravo et al., 2007; Chattopadhyay and Banerjee, 2018; Heckel, 2020).

The other alternate mode of action suggests that the Cry monomers bind specifically to a CADR receptor which activates a signal cascade pathway requiring the presence of Mg²⁺ ions. This activates G-protein which results in cyclic-AMP production by stimulating the adenylyl cyclase. The accumulation of cyclic-AMP activates protein Kinase-A that in turn leads to necrotic cell death without involvement of oligomer or pore formation (Zhang et al., 2006).

It has been discussed in (Heckel, 2020), that the second model (the signaling pathway model) should be interpreted as a complementary series of events that could take place when the classical mode of action cannot occur due to the absence of an essential non-CADR receptor. It has been also demonstrated that aquaporins (small, integral membrane proteins that mediate water transport across cell membranes) play an important role in promoting cell osmotic lysis in case of Cry intoxication. Thus, the scarcity of aquaporins in case of resistance may also limit the osmotic cell death model (Endo et al., 2017).

Many studies have focused on the mode of action of Cry proteins once they have reached the midgut epithelium. However, before that, Bt pesticidal proteins need to bypass the peritrophic matrix (PM). This matrix plays an important role in the food digestion process and serves as a barrier to protect the midgut epithelium against physical injury, chemical toxicity, and microbial infections from food contamination (Lehane, 1997; Hegedus et al., 2009; Weiss et al., 2014). It is a mucus-like layer made up mainly of chitin and some highly glycosylated proteins. Little is known about Cry proteins interactions with the PM. Valaitis and Podgwaite (2013) demonstrated that Cry1A could bind to highly glycosylated PM proteins in the Douglas fir tussock moth *Orgyia pseudotsugata*. This may be due to the recognition of glycosylic residues by the lectin-like folds present in two of the three domains of most Cry proteins.

Recently, it has been reported that chitin-binding proteins can be induced in vegetative Bt cells during their stay in the midgut alkaline fluid, promoting the Bt bacterium adhesion to the insect PM. This might be the first step of the Bt infection process (Qin et al., 2020). Besides, chitinases inhibitors were shown to reduce the toxicity of Bt strains suggesting that Bt secretes chitinases to facilitate the penetration of its toxins through the PM (Regev et al., 1996; Sampson and Gooday, 1998; Lertcanawanichakul et al., 2004; Ding et al., 2008). Rees et al., (2009) have proposed that one of the reasons behind *Agrotis ipsilon* and *Mamestra brassicae* larvae tolerance to Cry1Ac is that this protein was retained in the PM and then eliminated in the frass without interacting with midgut cells. Better knowledge on the interaction of Bt pesticidal proteins with the insect PM is critical for a full understanding of their mode of action and may provide some insight into the possibility of PM participation in resistance emergence.

- Resistance to Bt proteins (*Spodoptera frugiperda* and *Diabrotica virgifera virgifera*)

Resistance to Bt insecticidal proteins particularly the Cry proteins might theoretically emerge at any step of the mode of action discussed above. In fact, studies on resistant lab insect populations have demonstrated that different mechanisms could be involved in resistance establishment. Thus, resistance may occur when activation of Cry proteins by midgut proteases is altered or when Bt proteins are sequestered by glycolipid moieties or (Pardo-López et al., 2013). However, the most studied mechanism is the alteration of toxin binding to midgut receptors (Ferré and Van Rie, 2002). This might be related to a lack of functional receptors or to a reduction in receptors expression. For instance, a reduced expression of an alkaline phosphatase gene (SfmALP2) (Jakka et al., 2016a), as well as a mutation in an ABCC2 gene, were associated with resistance to Cry1Fa corn in FAW from Puerto Rico (Banerjee et al., 2017). A study conducted on suspected resistant FAW from Puerto Rico suggested that resistant Cry1Fa FAW show a partial cross resistance to Cry1A.105 and demonstrated that the ABCC2 gene mutation is responsible for this resistance (Flagel et al., 2018).

The mechanisms of Bt resistance have been extensively studied in lepidopteran insect pests (Heckel et al., 2007; Pardo-López et al., 2013; Banerjee et al., 2017). However, as seen above, field-evolved resistance has also been detected in WCR populations, probably due to the intensive deployment of Bt corns for WCR management. Thus, studies have been initiated to identify the WCR receptors involved in the mode of action of Bt insecticidal proteins as well as the mechanism behind the appearance of resistance. Recently, DvABCB1 was validated as a WCR receptor for Cry3 proteins. A reduced expression and alternative splicing of DvABCB1 transcripts were found associated to WCR resistance to mCry3A (Niu et al.,

2020). Besides, a comparative RNA-seq experiment conducted with WCR strains susceptible and resistant to eCry3.1Ab showed that two genes were up-regulated in resistant strains: an esterase (enzyme with a detoxification role) and a dynein heavy chain-like protein (cytoskeletal motor protein involved in intracellular transportation and the movement of chromosomes) (Zhao et al., 2019).

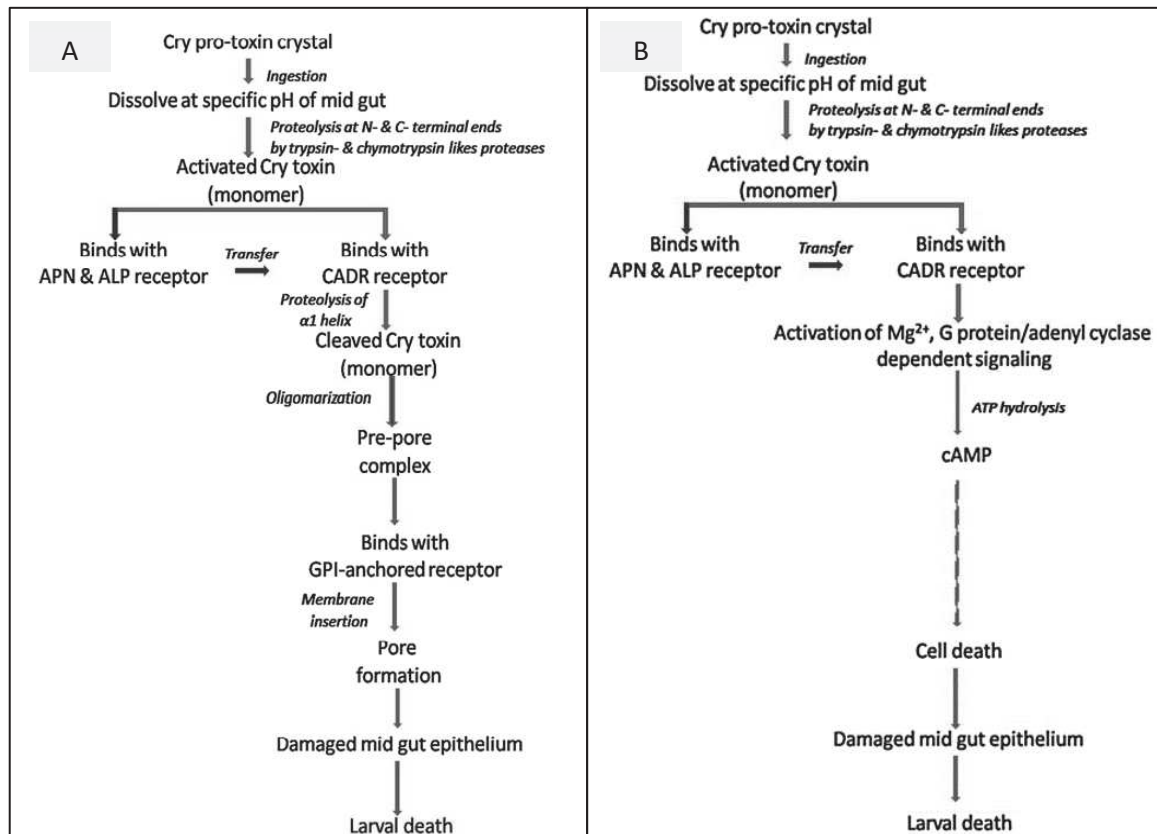


Figure 10: The two most known Cry proteins mode of action. A: the Sequential-binding model (classical mode of action) of Cry toxin showing toxin activation, binding with specific receptors, formation of pores, and subsequent death of pest insect larvae. B: The signaling pathway model of Cry toxin requiring attachment of Cry toxin to cadherin receptor which accelerates the events of intracellular apoptotic pathways (Chattopadhyay and Banerjee, 2018).

3 Potential of insect viruses in control of corn pests (alone or in complement to bacterial toxins)

Entomopathogenic viruses have been widely studied for their potential use in controlling insect pests either singly or in an integrated pest management program with other organisms or molecules. Baculoviruses are among the largest group of insect-attacking viruses and have been the leader in virus-based bioinsecticides with a predominance use against lepidopterans

(Valicente, 2019). Baculoviruses synergies with both conventional insecticides (Dáder et al., 2020) and other microorganisms (such as Bt) have been reported against some lepidopteran pests (Liu et al., 2011; Hesketh and Hails, 2015). Despite an increasing interest in using viruses for insect control in the last few years, little is known in the capacity of viruses to control coleopteran pests. Recently, Liu et al., (2021) have identified nudivirus-derived sequences from southern corn rootworm (SCR, *Diabrotica undecimpunctata howardi* Barber, and WCR) suggesting existence of viruses pathogenic to coleopteran corn pests that could be used in pest management.

Other virus families have potential in insect microbial control, for instance densoviruses, from the *Parvoviridae* family. One of them is currently being investigated for its activity against FAW. Junonia coenia densovirus (JcDV) is a non-enveloped virus with a 22 nm icosahedral capsid (Caspar and Klug, 1962). JcDV has an ambisense linear single-stranded DNA genome of about 6 kb encoding three non-structural proteins (NS1, NS2 and NS3) and four capsid proteins (VP1, VP2, VP3 and VP4) (Figure 11) (Jourdan et al., 1990; Bruemmer et al., 2005). The four VP proteins, which have a common C-terminal part, are present in the capsid with a stoichiometry of 1: 9: 9: 41 (VP1, VP2, VP3, VP4). VP4 is therefore the major protein of the capsid with the shortest sequence (Tijssen et al., 1976; Tijssen and Kurstak, 1981; Bruemmer et al., 2005). The JcDV viral genome is ambisense since it is composed of two strands with opposite open reading frames (ORFs) (Dumas et al., 1992). The "positive" strand harbors three open reading frames (named ORF2 to 4) that encode the NS proteins (Wang et al., 2013a). Their role in the JcDV viral cycle is not well known, with only a few data on the DNA helicase and nickase activities of NS1 (Ding et al., 2002) and the participation of NS3 in viral replication (Abd-Alla et al., 2004). The "negative" strand, contained a single ORF (ORF1) that encodes VP proteins (Shirk et al., 2007). Through analysis of its primary structure, a phospholipase domain (type A2 (PLA2)) was identified in VP1 protein, suggesting that this protein could be involved in the release of the viral genome into the cell nucleus, as shown for *Parvovirinae* (Zádori et al., 2001). This PLA2 domain is located in N-terminal of VP1 in JcDV, but its role during cell trafficking is still unknown (Zádori et al., 2001). Nuclear localization signals (NLS) were identified *in silico* for all VPs and could be used to import these proteins into the nucleus of the cell, in particular during virion assembly (Owens, 2013). A recent publication of Labadie et al., (2021), has demonstrated the critical role of VP proteins in JcDV infection and replication. VP4 has probably a role in virus-receptor interaction since the VP4 is the major capsid protein and the only one exposed outside the capsid. Several lines of evidence indicate that VP4 plays a role in JcDV entry into insect cells. First, modification of four amino acids of

VP4 protein was shown to affect the efficiency of the virus to cross the intestinal epithelium and to cause a delayed caterpillar mortality compared to wild-type JcDV (Multeau et al., 2012). Second, virus-like particles (VLPs) composed of VP4 alone are able to enter in *Lymantria dispar* cell line by endocytosis like the virions (Vendeville et al., 2009). Indeed, VP4 alone is sufficient to produce virus-like particles (VLP) morphologically comparable to JcDV particles but without the genetic material (Croizier et al., 2000).

JcDV efficacy has been proved against few lepidopterans including FAW larvae (Rivers and Longworth, 1972; Bergoin and Tijssen, 2008; Mutuel et al., 2010). Once ingested by the FAW caterpillar, JcDV travels to the midgut where it agglutinates on the surface of the first barrier: the peritrophic matrix. It has been demonstrated by (Pigeyre et al., 2019) that JcDV early infection is marked with an arrest of N-Acetylglucosamine secretion and a disruption in the integrity of the peritrophic matrix, which may allow viral particles to pass through. JcDV then penetrates by a caveolin-dependent endocytosis and crosses the columnar cells by transcytosis (Wang et al., 2013b). During its passage, JcDV was reported to disrupt the septate junctions (which are located near the apical region of the epithelium) between neighboring cells and considered as a biological barrier that controls the permeability of the paracellular transport pathway (Pakpour et al., 2013; Wang et al., 2013b). Finally, the virus leaves the cell by exocytosis and reaches the target tissues (hemocytes, trachea, epiderm) where it replicates (Mutuel et al., 2010; Gosselin-Grenet et al., 2015). Viral multiplication in these tissues blocks molting and causes suffocation of the caterpillars by obstruction of the tracheae, leading to the death of the host within 5 to 10 days after the initial ingestion (Figure 12) (Gosselin-Grenet et al., 2015).

JcDV's ability to transcytose through the gut epithelium has caught the interest of researchers. Kemmerer and Bonning (2018) have demonstrated that VP4 fused to eGFP (enhanced Green Fluorescent Protein) with a proline-rich linker can cross the gut epithelium by transcytosis. Then, they have recently claimed the possibility to use VP4 as a carrier for other insecticidal peptides (Bonning and Kemmerer, 2020). The patent deposited by these authors covered the possible use of these fusion proteins either in spray formulations or to produce genetically engineered plants. But to date, there has been no evidence of VP4 expression either singly or fused to insecticidal proteins in a plant system.

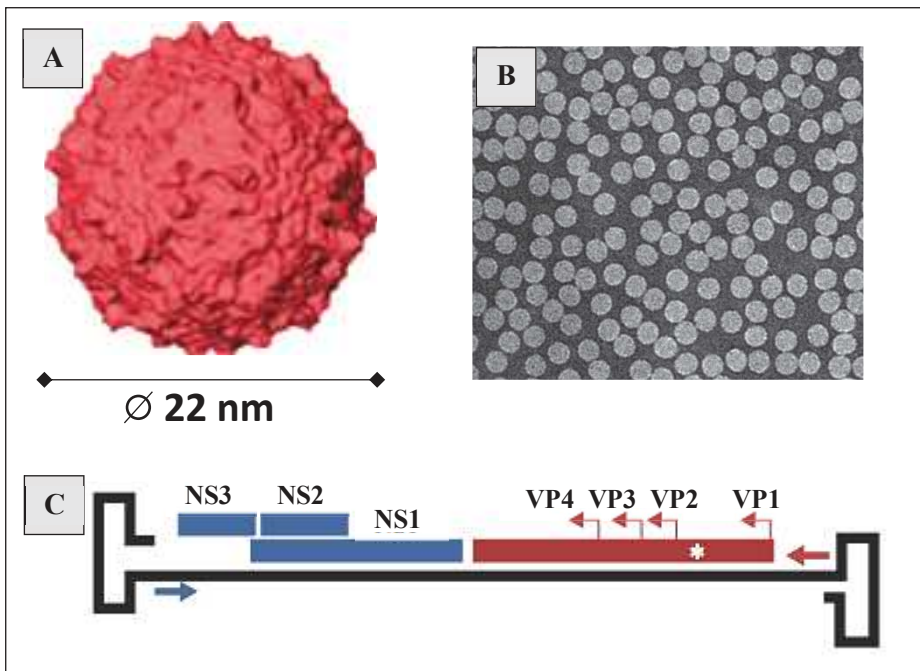


Figure 11: Structure and genomic organization of JcDV. A: Representation of the JcDV capsid with cryo-electron microscopy (cryo-ME) (Bruemmer et al., 2005). B: Transmission electron micrograph (TEM) of JcDV particles (UMR DGIMI). C: Schematic representation of the JcDV ambisense genome and the associated proteins. The terminal ends (terminal repeats inverted (ITR)) are fold into a hairpin structure that serves as a primer for viral replication. The arrows represent the promoters initiating the transcription of genes encoding non-structural proteins (NS, in blue) and the capsid proteins (VP, in red) (Gosselin-Grenet et al., 2015).

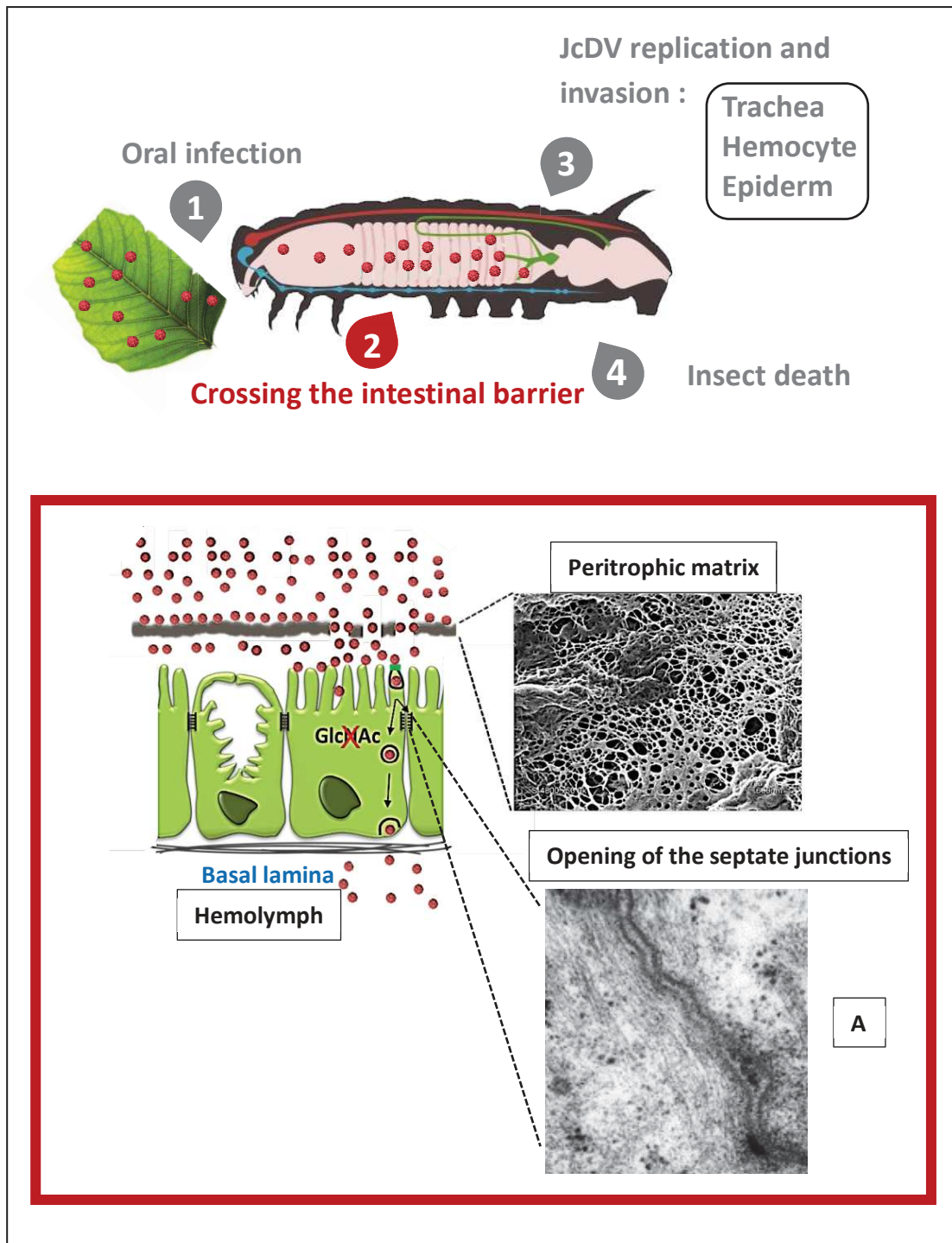


Figure 12: JcDV pathogenesis in *Spodoptera frugiperda* : Virus particles (in red) are ingested by the caterpillar and travel to the midgut where they concentrate on the peritrophic matrix leading to its destruction. This permeabilization would allow the viral particles to flow through the interperitrophic space. The particles then attach to the lipid rafts of the apical brush margins of the intestinal epithelium. JcDV then enters by a caveolin-dependent endocytosis and crosses the columnar cells by transcytosis. During its passage, JcDV generates a disorganization of the septate junctions (A: TEM image of a midgut epithelium disrupted septate junction of FAW larvae infected by JcDV from Wang et al., 2013b) , as well as an a halt in GlcNAc synthesis and / or its transport to microvilli (red cross) which would participate in the disruption of the peritrophic matrix. Finally, the virus particles leave the cell by exocytosis and reach the target tissues via hemolymph to replicate (Mutuel et al., 2010; Wang et al., 2013a; Wang et al., 2013b; Gosselin-Grenet et al., 2015; Pigeyre, 2019)

Part 2: Contributions

**CHAPTER 1: Identification of a new family of
insecticidal proteins against the Western Corn
Rootworm (WCR)**



1 Introduction

Insect resistance to various type of insecticides requires seed companies to constantly search for new agents/molecules with a new mode of action. As noted in the state-of-the-art (see page 8), the Western Corn Rootworm (WCR) remains one of the most economically important maize pests due to its adaptabilities to pest management tools. A victim of its own success, pesticidal proteins from the soil bacteria *Bacillus thuringiensis* have been the focus of research for decades. However, due to the appearance of resistance, scientists and corn industries have turned to new microbial resources to control WCR larvae.

This chapter describes the research strategy we followed to identify and characterize novel family of pesticidal proteins active against the WCR. The identification of new "candidate" proteins active against lepidopterans and coleopterans is carried out *in silico* by searching for sequences with homologies to existing toxins from bacterial genomes. Within the framework of the project, entitled DIETETIC, partially financed by the SEMAE (French Interprofessional Organisation for Seeds and Plants), 2500 bacterial genomes have been sequenced. "Candidate" proteins were then selected based on homology with known insecticidal proteins present in our internal database and other public databases such as UniProt (<https://www.uniprot.org/>). Following that, these "candidates" were produced as recombinant proteins either in *Escherichia coli* or *Pseudomonas fluorescens* production systems. The crude lysates are tested on first instar (L1) larvae of the various susceptible strains of target insects using the diet overlay bioassay technique described by Graser and Walters, (2016). Larval mortality is assessed after 3 and 5 days after infestation. These routine screening tests are conducted between Biogemma and CABI (our partner in Hungary) for WCR, at Biogemma for ECB, and at Genective (USA) for other lepidopteran species. If a "candidate" protein shows activity against a susceptible strain of mentioned species, it will be tested against resistant strains to commercial toxins to check whether the mode of action is different or not. Genective has access to different maize insect pest resistant strains. Once the level of toxicity is sufficient, especially on strains resistant to commercial toxins, the candidate toxin is classified as "active" protein *in vitro*. The next step consists in evaluating its activity *in planta*. Thus, "active proteins" will be expressed transiently in *Nicotiana benthamiana* by agroinfiltration or stably in maize via transgenesis using a constitutive promoter. The activity of total protein leaf extracts is tested within the same bioassay technique described above. Once the toxic activity is validated on plant extracts, the entomotoxic activity of maize plants against various insects is assessed in

two steps: first in the greenhouse, then in the field. These trials are conducted in the United States by Genective.

2 A novel binary toxin from *Chryseobacterium arthrosphaerae* controls *Diabrotica virgifera virgifera* via a different mode of action to existing commercial toxins (article)

Based on the strategy outlined above, we report in this article the identification of a new family of binary pesticidal proteins active against WCR larvae. These proteins were all found in several strains of *Chryseobacterium* species. We concentrated our study on a couple of proteins called GDI0005A/GDI0006A found in the same operon from *Chryseobacterium arthrosphaerae* genome. They were chosen for their similarities to a class of binary pesticidal proteins (PIP) described in a patent from Pioneer (Gruver et al., 2016). The activity consists of significant mortality and sublethal effects (stunting) on susceptible WCR larvae. When tested on WCR strains resistant to commercially available *Bacillus thuringiensis* pesticidal proteins (Cry3Bb1 and Gpp34Ab1/Tpp35Ab1), significant weight loss was observed. This suggests that GDI0005A/GDI0006A might have a different binding site implying a new mode of action. Two different *Chryseobacterium arthrosphaerae* strains one isolated from the Sahara Desert (ZSTG2175) and the other one from pill millipede *Arthrosphaera magna* (DSM25208), carry identical sequences of GDI0005A/GDI0006A. Bacterial lysates of both strains were significantly active against rootworms opening broad prospects for biocontrol applications.

PLOS ONE

A novel binary pesticidal protein from *Chryseobacterium arthrosphaerae* controls *Diabrotica virgifera virgifera* via a different mode of action to existing commercial pesticidal proteins

--Manuscript Draft--

Submitted : 04/10/2022

1 **A novel binary pesticidal protein from *Chryseobacterium arthrosphaerae***
2 **controls *Diabrotica virgifera virgifera* via a different mode of action to**
3 **existing commercial pesticidal proteins**

4
5 **Pesticidal protein from *Chryseobacterium arthrosphaerae* and**
6 ***Diabrotica virgifera virgifera***

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31
32 Abstract

33 The Western Corn Rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte) remains
34 one of the economically most important maize pests due to its adaptive capabilities to pest
35 management options. To date, commercial pesticidal proteins, used either as biopesticides or in
36 transgenic hybrid maize, come from different strains of a single bacteria species *Bacillus*
37 *thuringiensis* (*Bt*). The efficacy of these proteins has faltered since the appearance of resistant
38 rootworms. In this study, we report the discovery of a new family of binary pesticidal proteins
39 isolated from the *Chryseobacterium*, active against WCR larvae. In diet bioassays, GDI0005A
40 and GDI0006A recombinant proteins caused drastic weight loss in WCR larvae. These effects
41 were found in WCR colonies susceptible as well as colonies resistant to Cry3Bb1 and

42 Cry34Ab1/Cry35Ab1 (reassigned Gpp34Ab1/Tpp35Ab1) which reveals that the new binary
43 insecticidal proteins may not share the same binding sites as those commercially deployed
44 proteins and therefore representing a new mode of action. Several *Chryseobacterium* species
45 appeared to carry GDI0005A and GDI0006A proteins. Bacteria carrying these binary proteins
46 showed good insecticidal activity against larvae in favor of their use as biocontrol agents against
47 WCR. This paves the way for diverse approaches towards the development of novel pest
48 management strategies urgently needed against rootworms.

49

50 **Introduction**

51 Worldwide, Maize (*Zea mays* L.) is one of the most extensively grown staple cereal
52 crops since it provides an important nutritional source for humans and animals and is used to
53 produce biofuel [1]. However, maize production is constrained by extreme climatic events,
54 pests and diseases [2,3]. Insect pests cause a significant reduction in maize yield [4,5]. The
55 western corn rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte, 1868) (Coleoptera:
56 Chrysomelidae), is amongst the most serious pest of maize in the USA [6,7]. Both larvae and
57 adults are responsible for more than a cost of \$1 billion in the USA each year, through
58 management costs and yield losses [6,8,9] as well as in central Europe [10]. Most crop losses
59 from WCR are due to larval feeding on maize roots leading to plant lodging [6]. Crop rotations
60 or the application of insecticides were the major approaches to mitigate rootworm damage
61 before the commercialization of genetically modified maize in 2003 [11,12].

62 During the last decade, pesticidal proteins derived from the bacterium *Bacillus*
63 *thuringiensis* (*Bt*) Berliner have been successfully used to control rootworms [13] through
64 expression in maize roots. The first *Bt* maize commercialized against maize rootworm larvae
65 produced the *Bt*-derived proteins Cry3Bb1 (MON863 event in 2003) [14]. Subsequently several
66 pesticidal proteins have been used either singly or as pyramids, including Cry34Ab1/Cry35Ab1
67 (reassigned as Gpp34Ab1/Tpp35Ab1 by the Bacterial Pesticidal Protein Resource Center
68 [BPPRC]) [15], mCry3A, eCry3.1Ab and Cry3Bb1 pesticidal proteins [16–19]. Nevertheless,
69 despite some success of the use of *Bt* maize for managing maize rootworm, field-evolved
70 resistance has been detected against the currently used of commercially available pesticidal
71 proteins of either Cry3A/B or Gpp34Ab1/Tpp35Ab1 family type. The appearance of resistant
72 rootworms to Cry3Bb1 [20], which are cross resistant to mCry3A [21], and the emergence of
73 Gpp34Ab1/Tpp35Ab1 resistant populations [22], have shown that rootworms can relatively

74 quickly adapt to such bacterial modes of actions and that resistance management strategies like
75 “refuge in the bag” may not be always sufficient to delay resistance [23,24].

76 Thus, finding agents or ingredients with new mode of actions (MOA) against rootworm
77 is the current challenge to plant protection and maize breeding industry. New approaches
78 involving RNA interference targeting WCR genes were recently tested [25]. For example, *in*
79 *planta* expression of double-stranded RNAs encoding the WCR gene DvSnf7, a component of
80 the ESCRT-III complex (endosomal sorting complex required for transport), shows promising
81 results [26,27]. It has been associated with some commercial Cry pesticidal proteins to generate
82 new pyramid maize hybrids (MON 87411 expressing Cry3Bb1 + DvSnf7 and SmartStax PRO
83 expressing Cry3Bb1, Gpp34Ab1/Tpp35Ab1 and DvSnf7) [28,29]. Also, biocontrol approaches
84 found their niche, such as the use of entomopathogenic nematodes against WCR larvae in
85 Europe. Microbial biopesticides have also been intensively studied for biocontrol purposes, but
86 none yet commercialized. The exceptions are microbial plant biofertilizers/biostimulants, such
87 as different *Pseudomonas* or *Bacillus* species that may also have some pesticidal “side” effects.
88 Additionally, non-*Bt* proteins activity from *Chromobacterium*, *Brevibacillus* and *Pseudomonas*
89 species [30–33] have shown success to reduce WCR root damage in GM maize in the field but
90 so far, they are not in commercial products.

91 In the perspective of discovering novel pesticidal proteins against WCR for the current
92 project, thousands of bacteria genomes from different origins were sequenced. Then, candidate
93 pesticidal proteins were searched for based on their similarities with known proteins. Lastly,
94 artificial diet-based bioassays were performed to evaluate their pesticidal activity. Here, we
95 report the discovery of a family of pesticidal proteins (GDI0005A & GDI0006A) present in
96 several *Chryseobacterium* species and having an activity against WCR larvae. Insect bioassays
97 showed that like Gpp34Ab1/Tpp35Ab1, the pesticidal activity against WCR is a two-
98 component system. We demonstrated that the two proteins together are active against field-
99 evolved rootworms populations resistant to Cry3Bb1 and to Gpp34Ab1/Tpp35Ab1, suggesting
100 a new MOA. Our findings could pave the way for diverse strategies towards the development
101 of novel pest management approaches urgently needed against rootworms.

102 Results

103 Discovery of novel pesticidal proteins

104 Two candidates (GDI0005A and GDI0006A) were identified in a *Chryseobacterium*
105 *arthrosphaerae* strain (ZSTG2175), isolated from Sahara desert and whose 16S rRNA

106 sequences had 99% identity to that of *C. arthrosphaerae* CC-VM-7 (NCBI:txid651561; [34],
107 isolated from the faeces of the pill millipede *Arthrosphaera magna* Attems [35]). The two
108 proteins were present on a same operon and were found to have low identity of less than 35%
109 to pesticidal proteins of the PIP45 and PIP74 families, described in the patent application
110 [36,37]. The two candidates were named by the Bacterial Pesticidal Protein Resource Center
111 (BPPRC) XppXX and XppYY (submission in progress) due to their uncharacterized structure.

112 GDI0005A showed 33%, 36%, and 34% identity to PIP-45Aa-1, PIP-45Ga-1 and PIP-
113 74Aa1 respectively (Table 1), whereas GDI0006A shares 27%, 28% and 28% identity to PIP-
114 45Aa-2, PIP-45-Ga-2 and PIP-74Aa-2 respectively (Table 2). Despite the low level of identity,
115 several conserved amino acids are conserved along the sequence alignment suggesting that all
116 members could belong to a similar structural family of proteins (S1 and S2 Figs.). All members
117 of
118 the PIP-45 and PIP-74 protein families were described as active when associated in pairs. Each
119 of the two components were encoded by genes found in the same operon of the original bacteria
120 (S3 Fig). Among them, some pairs were claimed to be active against WCR larvae, for instance,
121 PIP-74Aa-1 and PIP-74Aa-2 from *Pseudomonas rhodesiae* or PIP-45Aa1 and PIP-45Aa2 from
122 *Pseudomonas brenneri* [36]. Conversely, despite belonging to the same family of proteins, no
123 WCR activity was reported for PIP-45-Ga1 and PIP-45-Ga-2 isolated from *Cellvibrio japonicus*
124 [36].

125 GDI0005A and GDI0006A genes are organized in an operonic structure with a 4 bp
126 overlap, like PIP45 members, which strengthened the hypothesis of a binary protein with
127 insecticidal activity (Fig 1A). The GDI0005A and GDI0006A genes are 1743 and 1404
128 nucleotides in length (including a stop codon) resulting in full-length proteins of 580 and 467
129 amino acids with a theoretical molecular weight of 64.4 kDa and 52.3 kDa, respectively.

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136 **Table 1:** Percentage of AA identity between members of the PIP45, PIP75 and GDI0005A
 137 protein families. Multiple Alignment with free end gaps were produced using Blosum62 matrix
 138 on Geneious Prime software. Information on the origin of protein sequences are listed in the S1
 139 Table.
 140

141

1	GDI0005 A	GDI0103 A	GDI0175 A	GDI0177 A	GDI0179 A	GDI0181 A	GDI0183 A	GDI0185 A	GDI0187 A	PIP- 45Aa-1	PIP- 45Ad-1	PIP- 45Ba-1	PIP- 45Bd-1	PIP- 45Be-1	PIP- 45Ca-1	PIP- 45Cb-1	PIP- 45Ea-1	PIP- 45Ga-1	PIP- 74Aa-1
GDI0005 A	100	32	76	76	76	72	74	81	89	33	32	33	32	33	33	32	31	36	34
GDI0103 A		100	32	33	33	34	33	33	33	36	36	34	34	34	34	34	33	30	34
GDI0175 A			100	93	86	84	84	76	76	32	31	32	32	33	33	32	29	36	33
GDI0177 A				100	87	84	84	76	77	32	32	33	33	33	33	33	29	36	33
GDI0179 A					100	84	84	76	76	33	33	34	34	34	33	33	31	36	34
GDI0181 A						100	82	74	74	32	31	32	32	32	33	32	30	36	33
GDI0183 A							100	73	75	32	32	33	33	33	33	33	31	36	33
GDI0185 A								100	82	32	32	33	32	33	33	32	30	36	34
GDI0187 A									100	33	33	33	33	33	33	33	32	37	33
PIP-45Aa- 1										100	97	89	88	87	78	77	60	33	31
PIP-45Ad- 1											100	87	88	86	77	76	59	32	30
PIP-45Ba- 1												100	96	84	77	77	60	33	30
PIP-45Bd- 1													100	84	77	76	59	33	30
PIP-45Be- 1														100	75	73	58	32	31
PIP-45Ca- 1															100	95	61	33	31
PIP-45Cb- 1																100	59	32	30
PIP-45Ea- 1																	100	31	30
PIP-45Ga- 1																		100	35
PIP-74Aa- 1																			100

142 **Table 2:** Percentage of AA identity between members of the PIP45, PIP75 and GDI0006A
 143 protein families. Multiple Alignment with free end gaps were produced using Blosum62 matrix
 144 on Geneious Prime software. Information on the origin of protein sequences are listed in the S1
 145 Table.
 146

2	GDI0006 A	GDI0104 A	GDI0176 A	GDI0178 A	GDI0180 A	GDI0182 A	GDI0184 A	GDI0186 A	GDI0188 A	PIP- 45Aa- 2	PIP- 45Ad- 2	PIP- 45Ba- 2	PIP- 45Bd- 2	PIP- 45Be- 2	PIP- 45Ca- 2	PIP- 45Cb- 2	PIP- 45Ea- 2	PIP- 45Ga- 2	PIP- 74Aa- 2
GDI0006 A	100	29	70	69	68	67	68	77	90	27	27	28	28	27	29	29	30	28	28
GDI0104 A		100	29	29	28	29	29	29	29	28	28	28	29	29	28	28	31	28	27
GDI0176 A			100	98	87	85	89	70	70	27	27	28	29	28	29	29	29	27	30
GDI0178 A				100	87	84	89	70	70	28	28	28	28	28	29	29	29	27	30
GDI0180 A					100	84	87	70	69	26	27	27	28	27	28	28	28	28	28
GDI0182 A						100	84	69	67	27	27	27	28	27	28	28	29	27	29
GDI0184 A							100	69	69	27	27	28	28	28	29	29	29	28	28
GDI0186 A								100	78	27	27	28	28	27	29	29	29	28	28
GDI0188 A									100	27	27	27	28	28	28	29	29	28	28
PIP- 45Aa- 2										100	94	84	84	77	66	66	46	27	25
PIP- 45Ad- 2											100	84	83	78	67	67	46	27	25
PIP- 45Ba- 2												100	96	75	66	66	47	28	25
PIP- 45Bd- 2													100	75	66	67	47	29	25
PIP- 45Be- 2														100	68	67	46	28	25
PIP- 45Ca- 2															100	93	45	29	26
PIP- 45Cb- 2																100	44	29	25
PIP- 45Ea- 2																	100	28	25
PIP- 45Ga- 2																		100	24
PIP- 74Aa- 2																			100

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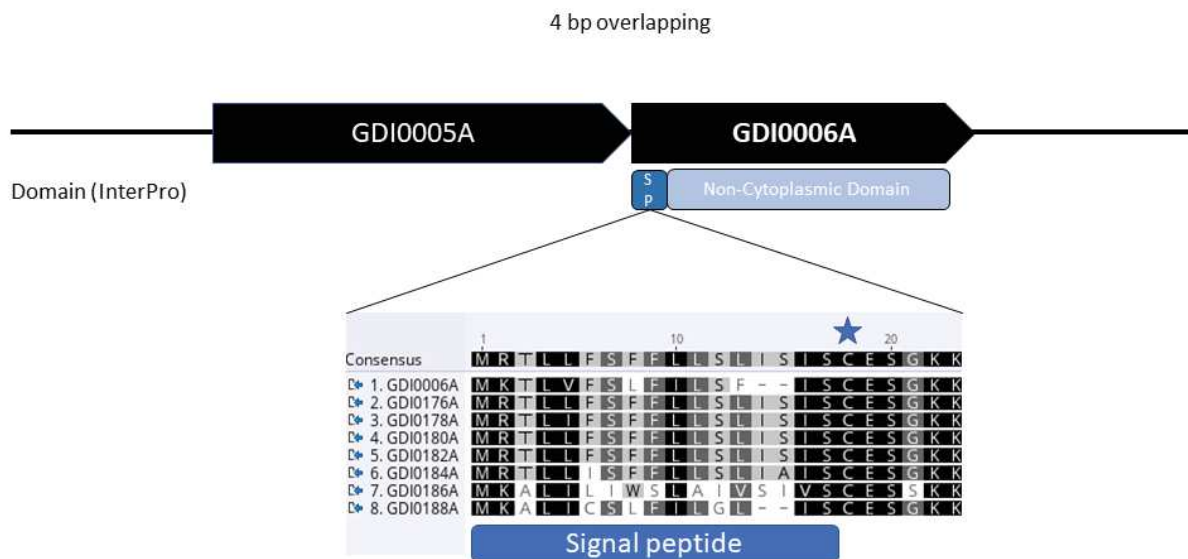
148 A scan for matches against the InterPro database [37] did not detect any known domain
149 or motif for GDI0005A. In contrast, GDI0006A contains a non-cytoplasmic domain predicted
150 by Phobius-1.01 (<https://phobius.sbc.su.se>) that covers the entire protein apart from an N-
151 terminal signal peptide (SP) found in prokaryotic lipoproteins (Fig 1B). Based on the SignalP-
152 5.0 software (<http://cbs.dtu.dk/services/SignalP>), the SP is predicted to have a size of 18 amino
153 acids (AA) with a cleavage site between the serine 15 and the cysteine 16. The first AA of the
154 mature protein is therefore a cysteine, which is a lipoprotein feature [38]. The rest of the protein
155 is predicted as a non-cytoplasmic domain suggesting that the protein might be a membrane-
156 associated protein. These results strengthen the hypothesis of a lipoprotein anchored in the outer
157 membrane of the original bacteria that could explain the lower yield of GDI0006A compared
158 to GDI0005A when produced in *Escherichia coli*. No matches with any known 3-D protein
159 structure for either protein was found while using the Phyre2 web portal for protein modeling,
160 prediction and analysis [39].

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162 **GDI0005A and GDI0006A are binary pesticidal proteins** 163 **having WCR activity**

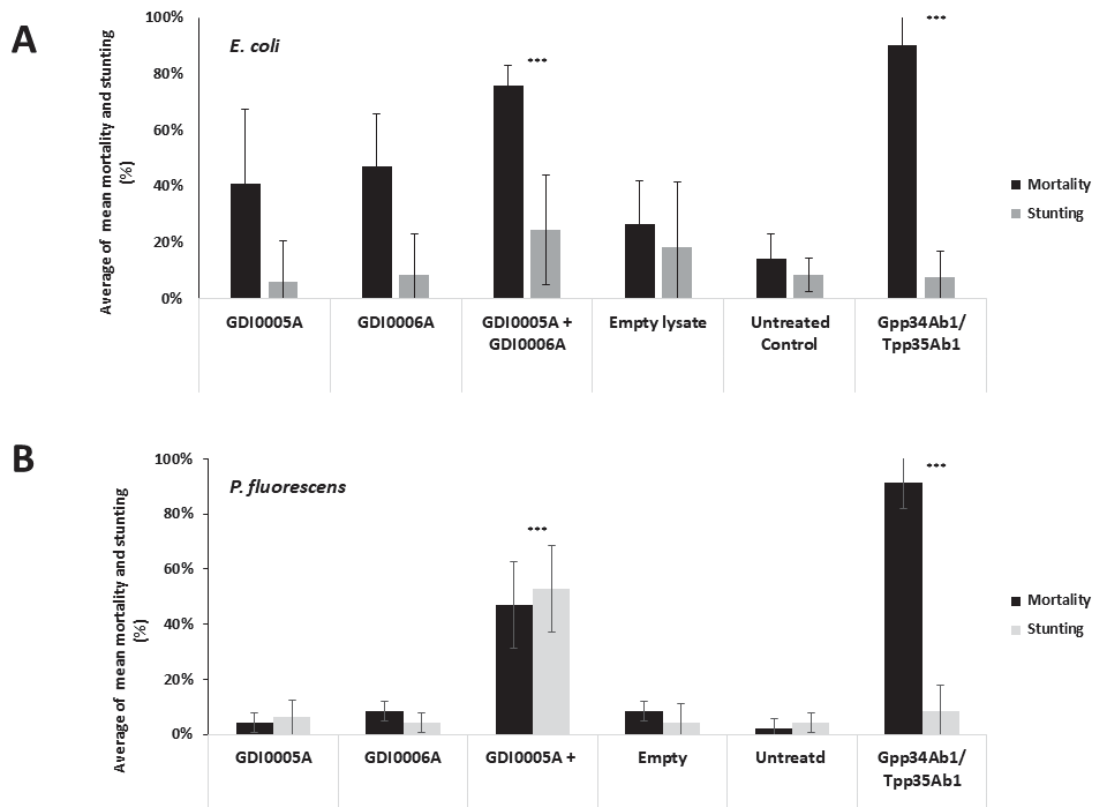
164 Finding a pair of proteins with low similarity with PIPs and encoded by genes in the
165 same operon as it is the case of GDI0005A and GDI0006A is not sufficient to predict that they
166 will be active against WCR. The potential WCR activity of GDI0005A in association with
167 GDI0006A was therefore tested to confirm the information obtained from sequence analysis.
168 GDI0005A and GDI0006A were produced as recombinant proteins in *E. coli* and *Pseudomonas*
169 *fluorescens* expression systems. Recombinant proteins were detected from crude lysates by
170 western blot. Bioassays with artificial diet overlaid with crude lysates from *E. coli* and *P.*
171 *fluorescens* containing GDI0005A and GDI0006A revealed significant mortality of WCR
172 neonates whereas surviving larvae were all stunted (Fig 2). In contrast, individual crude lysates
173 containing either GDI0005A or GDI0006A alone did not show any activity (Fig 2)
174 demonstrating that both GDI0005A and GDI0006A are required for full activity against WCR
175 larvae.

176 The binary pesticidal proteins GDI0005A and GDI0006A were tested against the major
177 lepidopteran maize pests: European corn borer (ECB) *Ostrinia nubilalis* (Hubner), fall
178 armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith), southwestern corn borer (SWCB)
179 *Diatraea grandiosella* Dyar, and corn earworm (CEW) *Helicoverpa zea* (boddie), using a diet
180 overlay assay. No significant activity was detected against any of these insects (S2 Table).



181

182 **Fig 1. Characteristics of the GDI0005A and GDI0006A operon**
 183 **from *C. arthrosphaerae* ZSTG2175 genome. A)** The two GDI0005A and GDI0006A open
 184 reading frames overlap by 4 nucleotides. **B)** Sequence overview of the signal peptide from all
 185 GDI00006A protein family members. The blue star indicates the potential cleavage site
 186 between the serine and the cysteine amino acid predicted by SignalP5.0 algorithm.



187

188 **Fig 2. Activity of GDI0005A and GDI0006A proteins** produced in *Escherichia coli* (A) and
 189 *Pseudomonas fluorescens* (B) against WCR neonates (USDA ARS non-diapause susceptible
 190 colony) 5 days after infestation using artificial diet overlay bioassays. Activity was evaluated
 191 by assessing the percentage of mortality (black boxes) and the percentage of stunting (grey
 192 boxes). Each WCR larvae was fed on ~200 μ L of WCR artificial diet overlaid with 20 μ L of
 193 bacterial lysate. Bacterial lysates contained GDI0005A, GDI0006A or both proteins
 194 (GDI0005A + GDI0006A) mixed. Negative controls consisted of diet overlaid with lysates
 195 from bacteria transfected with *E. coli* empty strain (empty lysate) and diet overlaid with
 196 sterilized water (untreated control). The positive control consisted of diet overlaid with lysates
 197 from bacteria transfected with a plasmid expressing the commercial Gpp34Ab1/Tpp35Ab1
 198 binary proteins. For each experiment, a total of 48 WCR neonate larvae were analyzed.
 199 Statistical analyses were conducted using Fisher exact test comparing each sample results
 200 (mortality and stunting) to the empty lysate. The untreated control was only used to evaluate
 201 the quality and reliability of each test. P-values of less than 0.05 were considered significant (*
 202 $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The error bars show the standard error of the mean (SEM).

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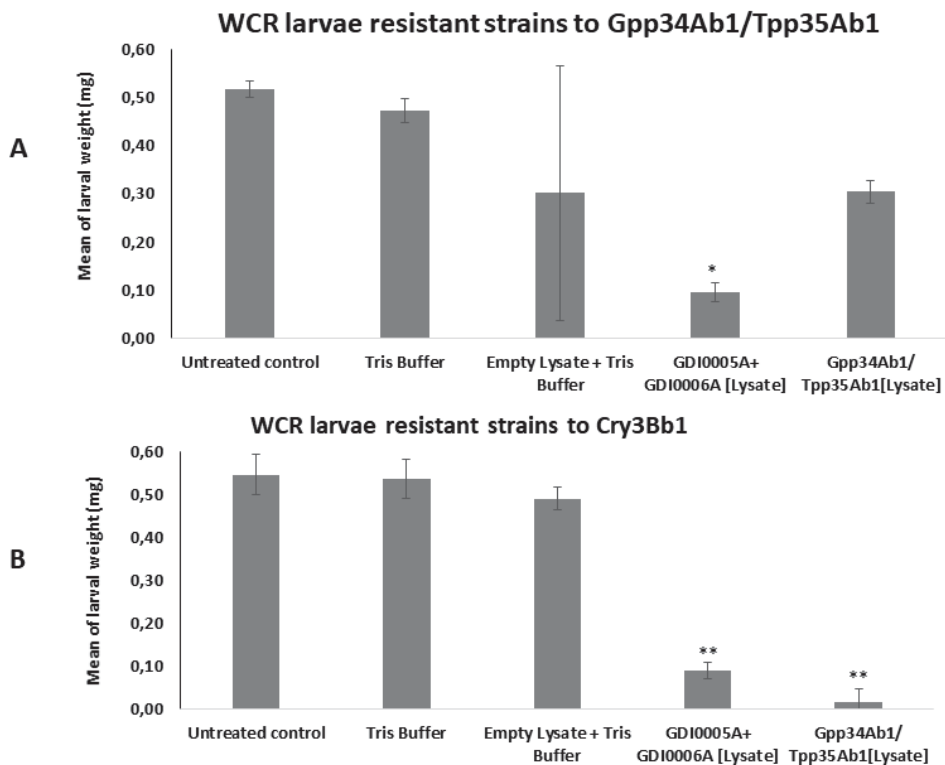
GDI0005A and GDI0006A have a putative novel Mode of Action (MOA)

To determine whether our binary pesticidal proteins are novel to current commercial proteins Gpp34Ab1/Tpp35Ab1 and Cry3Bb1, the crude lysates of recombinant GDI0005A and GDI0006A proteins from *E. coli* were tested against field evolved WCR colonies resistant to Gpp34Ab1/Tpp35Ab1 and Cry3Bb1. In bioassays with susceptible WCR colonies, we have observed some variability in the level of mortality caused by GDI0005A and GDI0006A. To measure more precisely the effect on resistant populations, we measured the weight of larvae after 5 days exposure to the crude lysate containing the pesticidal proteins.

We have used a WCR Gpp34Ab1/Tpp35Ab1 resistant population that presents an incomplete resistance to Gpp34Ab1/Tpp35Ab1 toxins as reported by [40]. As expected in bioassays, the lysate of Gpp34Ab1/Tpp35Ab1 recombinant proteins that is used as a positive control cause only a partial loss of weight (0.3 mg versus 0.5 mg) of Gpp34Ab1/Tpp35Ab1 resistant colonies. On the contrary, the same sample caused nearly a complete weight loss (<0.02 mg versus 0.5 mg) for Cry3Bb1 resistant colonies (Fig 3). When the lysate of GDI0005A and GDI0006A recombinant proteins was tested, a significant high weight loss (<0.06 mg versus 0.5 mg) was observed on both Gpp34Ab1/Tpp35Ab1 and Cry3Bb1 resistant strains (S4 Fig.). These results suggest that there is no cross-resistance between GDI0005A/GDI0006A and the two commercial *Bt* pesticidal proteins against WCR. Thus, GDI0005A and GDI0006A may not share the same binding sites as Gpp34Ab1/Tpp35Ab1 or Cry3Bb1 which could imply a new MOA.

GDI0005A/GDI0006A homologs from *Chryseobacterium* species have WCR activity

To investigate these novel pesticidal proteins, a BlastP search against public (Uniprot) and proprietary databases was conducted leading to the identification of 7 homologs to GDI0005A ranging from 72% identity (GDI0181A) to 89% identity (GDI0187A) and to GDI0006A ranging from 67% identity (GDI0182A) to 90% identity (GDI0188A), respectively (Table 1).



234
 235 **Fig 3. Activity of GDI0005A and GDI0006A proteins produced in *E. coli* on resistant WCR**
 236 **larvae using diet overlay bioassay. A.** WCR strain resistant to the insecticidal proteins
 237 Gpp34Ab1/Tpp35Ab1. **B.** WCR strain resistant to the insecticidal protein Cry3Bb1. Each
 238 WCR larvae was exposed to ~200 μ L of WCR artificial diet overlaid with 20 μ L of bacterial
 239 lysate containing (GDI0005A + GDI0006A). Negative controls consisted in diet overlaid with
 240 lysates from bacteria transfected with *E. coli* empty strain (empty lysate), Tris buffer and
 241 sterilized water (untreated control). The positive control consisted in diet overlaid with lysates
 242 from bacteria transfected with a plasmid expressing the commercial Gpp34Ab1/Tpp35Ab1
 243 binary proteins. For each experiment, a total of 24 WCR neonate larvae were analyzed. 5 days
 244 after treatment, mortality was assessed, and larvae were weighed as a pooled group per each
 245 column of each treatment. One-way analysis of variance (ANOVA) and Tukey's HSD post-hoc
 246 test were used to assess significant differences among the treatments. P-values of less than 0.05
 247 were considered significant ** $p < 0.01$. The error bars show the standard deviation.

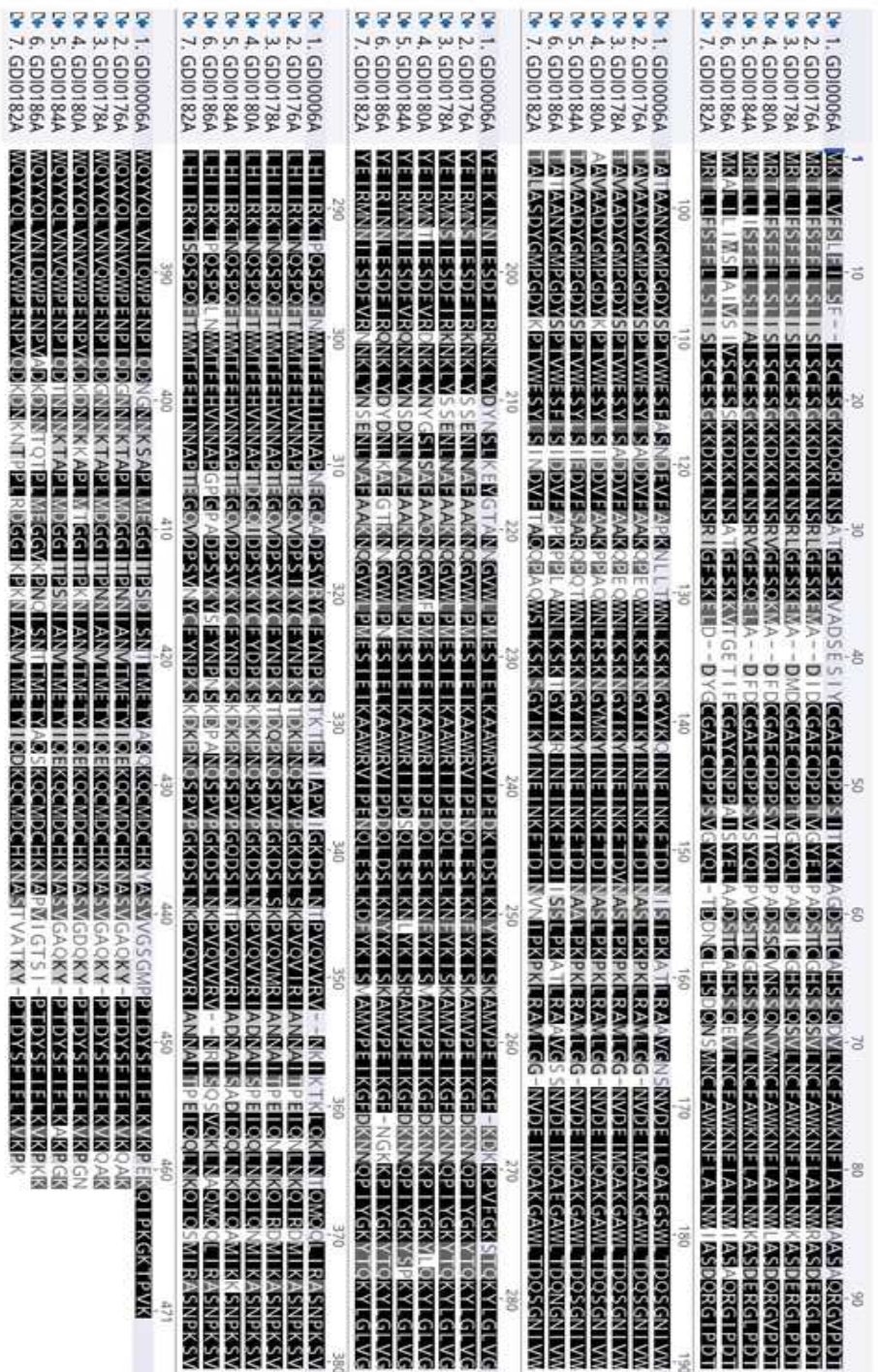
248 These homologs were all found in *Chryseobacterium* species isolated from highly
249 diverse environments such as soil, plant rhizosphere, raw chicken, and a lactic acid beverage
250 (Material & Methods, Table 3). Figs 4 and 5 show the protein sequence alignments of
251 GDI0005A and GDI0006A with their homologs and the corresponding phylogenetic trees. The
252 differences are mainly located in the N- and C-terminal parts of the protein,
253 GDI0185A/GDI0186A proteins being the most divergent. The SP and the non-cytoplasmic
254 domain detected in GDI0006A previously were also detected in all GDI0006A-like sequences.
255 A C-terminal extension (Cter) of 10 amino acids was only detected in the GDI0006A sequence.
256

257 **Cross-activity between GDI0005A and GDI0006A** 258 **components from different origins**

259 PIP-45 pesticide proteins are binary toxins having an activity when a member of the
260 PIP-45-1 family is associated with the corresponding member of the PIP-45-2 family (*i.e.* found
261 in the same strain). However, members of the PIP-45-1 family have been shown to be active on
262 WCR when associated with different members of the PIP-45-2 family [36] which are not from
263 the same strains. We tested whether a similar situation would apply to the GDI0005A and
264 GDI0006A binary proteins. We set up a series of cross-experiments between GDI0005A and
265 different GDI0006A-like proteins. All the pairs were tested for WCR insecticidal activity in
266 diet assays as described above. Whereas two combinations (GDI0005A/GDI0180A and
267 GDI0005A/GDI0182A) did not show any activity, all other combinations,
268 GDI0005A/GDI0176A, GDI0005A/GDI0178A, GDI0005A/GDI0184A and
269 GDI0005A/GDI0186A provided WCR activity (Fig 7). Therefore, as with PIP-45, different
270 sources of GDI0006A from *Chryseobacterium* species can provide pesticidal activity with
271 GDI0005A.
272

273 **The N-terminal sequence is essential for the activity of** 274 **GDI0006A**

275 The N-terminal signal peptide of 18 AA and the C-terminal extension of 10 AA present
276 in GDI0006A and all GDI0006A homologs were evaluated for their role in the activity of
277 GDI0006A. Six variants (named V1 to V6) of GDI0006A with or without the SP and CTer
278 sequences were generated (Fig 8A).



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Fig 5. Protein alignment of GDI0006A homologs. (A) GDI0006A and homologous protein sequences were aligned using Geneious Multiple alignment tool with the Blosum 62 cost matrix. Identical amino acids are indicated in black; conserved amino acids in Grey; all the others in white. (B) Phylogenetic tree of GDI0006A homologs built with the Neighbor-joining method using Jukes-Cantor Genetic Distance model (Geneious Tree Builder from Geneious)

291 All constructs contained a His-tag fused to their N- or C-terminus for detection of the
292 recombinant protein by western-blot using an anti-histidine antibody. GDI0006A was present
293 in all bacterial lysates but in variable amounts depending on the construct (Fig 8B). The lack of
294 signal in the sample containing the complete GDI0006A produced with a N-term His-tag (row
295 6, Fig 8B) reinforce the hypothesis that the native GDI0006A is normally processed through
296 the cleavage of its SP (deletion of the His-tag during the cleavage process).

297 All the bacterial lysates were then tested associated with GDI0005A on WCR larvae
298 using diet overlay bioassays. The lysates obtained from V1 to V4 constructs where SP was
299 deleted were all inactive, whereas those from V5 and V6 harboring SP showed activities (Fig
300 8C). Finding an activity for both V5 and V6 lysates indicates that the position of the His-tag
301 had no effect on the protein activity. Overall, these results demonstrate that the N-terminal SP,
302 presumably required for targeting to the membrane, appears to be essential for the activity of
303 the GDI0006A protein. On the contrary, the Cter sequence has no impact on the activity of the
304 protein, as shown by the results with GDI6 homologs that do not have a Cter sequence
305 extension.

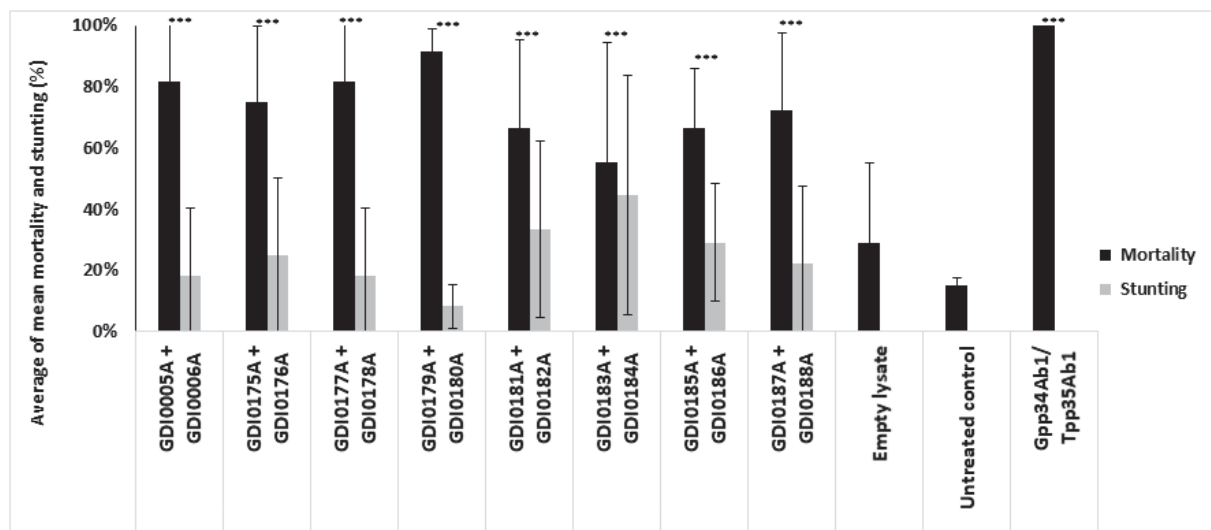
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307 **Activity of *C. arthrosphaerae* cultured strains against WCR** 308 **larvae**

309 The original *C. arthrosphaerae* strain isolated from the Sahara Desert (ZSTG2175) and
310 the strain isolated from pill millipede (DSM25208) were tested for their activity against WCR.
311 A PCR assay from liquid cultures confirmed the presence of the genes encoding GDI0005A
312 and GDI0006A in both strains and sequencing of the PCR products confirmed that the two
313 strains carry identical sequences (S3 Table.).

314 Two independent experiments were conducted with each strain grown in rich medium.
315 The supernatant and lysates from the pellets of both cultures were tested for their activity on
316 WCR's larvae. Experiments revealed that most samples from ZSTG2175 or DSM25208
317 bacteria caused mortality and stunted growth in WCR neonates within 5 days (Fig 9) except for
318 two samples from DSM25208. However, the ZSTG2175 strain overall showed statistically
319 significantly more activity than the DSM25208 strain with both supernatant and pellet samples.

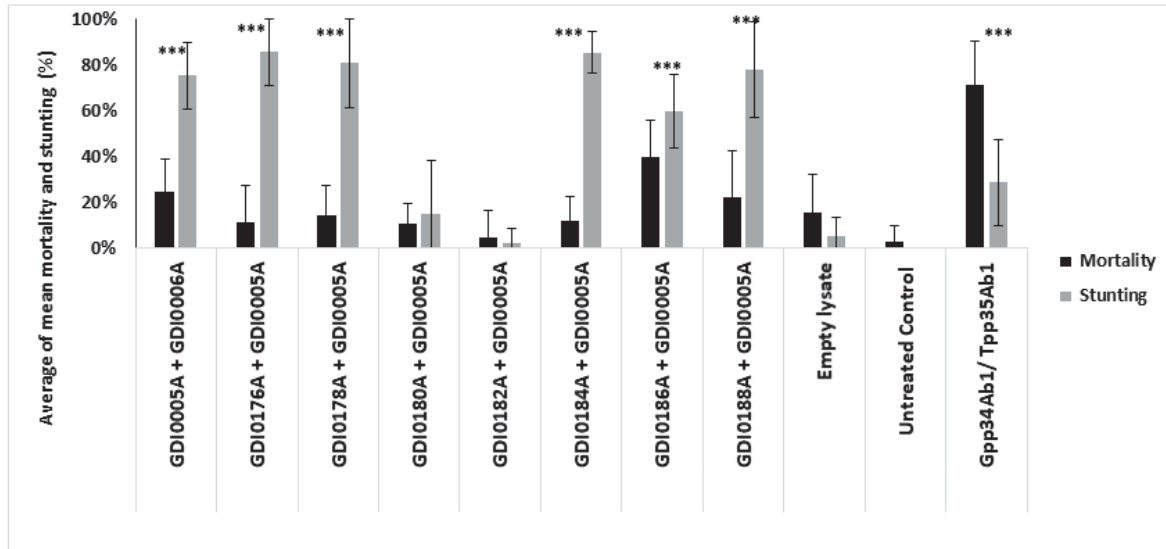
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323 **Fig 6. Activity of different pairs of GDI0005A/GDI0006A** proteins produced in *E. coli* on
 324 WCR susceptible neonates 5 days after infestation using diet overlay bioassay. Different
 325 GDI0005 and GDI0006A homologs are compared. Activity was evaluated by assessing the
 326 percentage of mortality (black boxes) and the percentage of stunting (grey boxes). Each WCR
 327 larvae was exposed to ~200 μ L of WCR artificial diet overlaid with 20 μ L of bacterial lysate
 328 mixtures containing (GDI0005A + GDI0006A) or (GDI0005A homologs + GDI0006A
 329 homologs). Negative controls consisted of diet overlaid with lysates from bacteria transfected
 330 with *E. coli* empty strain (empty lysate) and diet overlaid with sterilized water (untreated
 331 control). The positive control consisted of diet overlaid with lysates from bacteria transfected
 332 with a plasmid expressing the commercial Gpp34Ab1/Tpp35Ab1 binary proteins. For each
 333 experiment, a total of 48 WCR neonate larvae were analyzed. Statistical analyses were
 334 conducted using Fisher exact test comparing each sample results (mortality and stunting) to the
 335 empty lysate. The untreated control was only used to evaluate the quality and reliability of each
 336 test. P-values of less than 0.05 were considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p <$
 337 0.001). The error bars show the standard error of the mean (SEM).

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Fig 7. Cross activity between component I (GDI0005A and homologues) and Component II (GDI0006A and Homologues). Activity was evaluated on WCR neonates 5 days after infestation by assessing the percentage of mortality (black boxes) and the percentage of stunting (grey boxes). Each WCR larvae was exposed to ~200 μ L of WCR artificial diet overlaid with 20 μ L of bacterial lysate mixtures containing (GDI0005A + GDI0006A) or (GDI0005A combined with GDI0006A homologs). Negative controls consisted of diet overlaid with lysates from bacteria transfected with *E. coli* empty strain (empty lysate) and diet overlaid with sterilized water (untreated control). The positive control consisted of diet overlaid with lysates from bacteria transfected with a plasmid expressing the commercial Gpp34Ab1/Tpp35Ab1 binary proteins. For each experiment, a total of 48 WCR neonate larvae were analyzed. Statistical analyses were conducted using Fisher exact test comparing each sample results (mortality and stunting) to the empty lysate. The untreated control was only used to evaluate the quality and reliability of each test. P-values of less than 0.05 were considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The error bars show the standard error of the mean (SEM).

356 Discussion

357 Insect pressure in areas of the world where maize is grown extensively is very high [41].
358 Depending on the climatic conditions, it can have a major impact on yield and food security
359 [2]. WCR is one of the most damaging insects of the maize crop in North America. Genetically
360 engineered maize expressing Cry3 proteins (including Cry3Bb, mCry3A and eCry3.1Ab) and
361 Gpp34Ab1/Tpp35Ab1 from *B. thuringiensis* are currently available on the market to manage
362 WCR populations. However, the ability of WCR to develop resistance to these commercially
363 deployed pesticidal proteins is impacting the efficiency of these products [42]. Thus, finding
364 candidates with new MOAs is crucial to develop new products. Whereas many studies still
365 focus on *B. thuringiensis* to find new pesticidal proteins, several novel pesticidal proteins from
366 non-*Bt* strains were identified in the last five years. This includes IPD072Aa, a small protein of
367 86 amino acids purified from *Pseudomonas chlororaphis* [32], Mpf3Aa1 (formerly GNIP1a)
368 from *Chromobacterium piscinae* having a Membrane Attack Complex/Perforin (MACPF)
369 domain that is also present in proteins involved in animal immune response [31], PIP-47Aa
370 from *Pseudomonas mosselii*, which does not share domains with other known proteins [33] and
371 Mpp75Aa protein homologs belonging to the Etx/MTX2 family from *Brevibacillus*
372 *laterosporus* [30]. All these 4 proteins kill WCR larvae in laboratory assays with high efficiency
373 and demonstrate selectivity for a few coleopteran species. In all these studies, transgenic maize
374 plants expressing these novel pesticidal proteins showed significant protection from root
375 damage by WCR demonstrating that proteins from other species could be as efficacious as *Bt*
376 pesticidal proteins in controlling major insect pests such as WCR.

377 In this study, we demonstrated that GDI0005A and GDI0006A from *C. arthrosphaerae*
378 exhibit a WCR activity when both proteins were ingested by WCR larvae. These proteins
379 belong to a large protein family that was first identified by Gruver et al. [36] as a two-
380 component system having the pesticidal activity (S1 Fig). These proteins were identified in
381 many Proteobacteria such as *Pseudomonas* species (*P. putida*, *P. monteilii*, *P. brenneri*, *P.*
382 *fluorescens*) from the soil or water, but also in other genera like *Burkholderia*, *Cellvibrio*,
383 *Paracoccus* in addition to *Chryseobacterium* (this study). The pesticidal activity is found in
384 several clades in the dendrogram, although the level of similarity between clades is low.
385 According to our study and in addition to the results of Gruver et al.[36], no activity against
386 lepidopteran maize pests such as CEW, FAW, ECB, SWCB was detected. Thus, the pesticidal
387 activity of GDI0005A/GDI0006A could be limited to the certain coleopteran species, but
388 further testing is needed to define the spectrum of activity more precisely.

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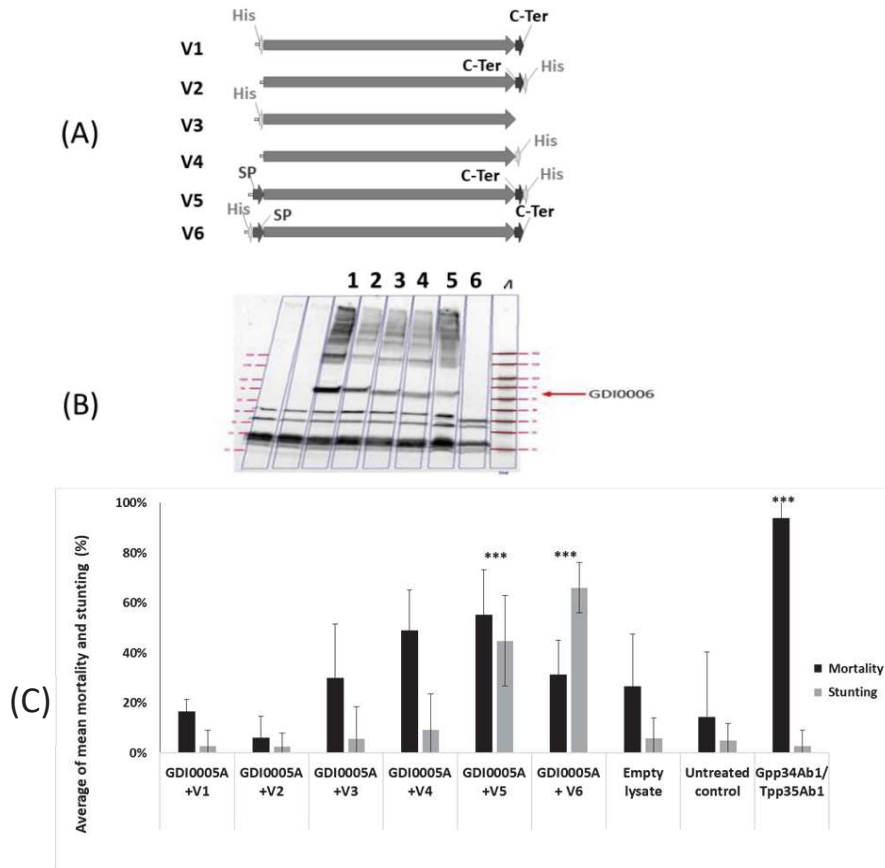
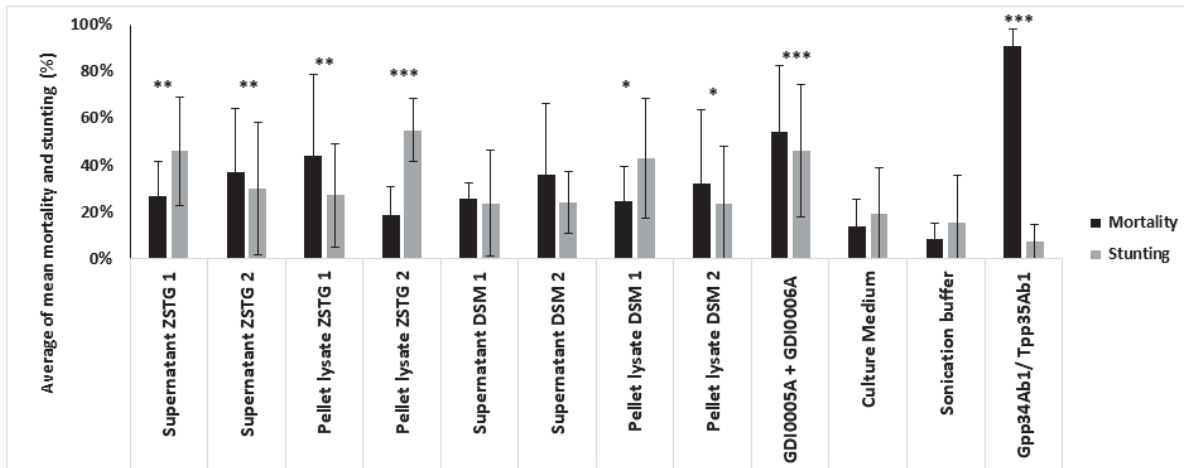


Fig 8. Expression and activity of GDI0006A. **A)** Representation of GDI0006A new constructs. V1: signal peptide (SP) deleted, His-tag at the N-terminus; V2: signal peptide deleted and His-tag at C-terminus; V3: signal peptide deleted, C-terminal extension (C-term) deleted, and His-tag at N-terminus; V4: signal peptide deleted, C-terminal extension deleted and His-tag at C-terminus; V5: full protein with a His-tag at the C-terminus; V6: full protein with a His-tag at the N-terminus (construct used in the bioassays of this work). **B)** Western blot analysis of GDI0006A new constructs expression using anti-His antibody. The lane 1 corresponds to the construct V1, lane 2 to construct V2, lane 3 to construct V3, lane 4 to construct V4, lane 5 to construct V5 and lane 6 to construct V6. **C)** Activity of GDI0006A new constructs (V1 to V6) mixed with GDI0005A on WCR neonates 5 days after infestation using diet overlay bioassay. Activity was evaluated by assessing the percentage of mortality (black boxes) and the percentage of stunting (grey boxes). Each WCR larvae was exposed to ~200 μ L of WCR artificial diet overlaid with 20 μ L of bacterial lysate mixtures containing (GDI0005A + GDI0006A different constructs). Negative controls consisted of diet overlaid with lysates from bacteria transfected with *E. coli* empty strain (empty lysate) and diet overlaid with sterilized water (untreated control). The positive control consisted of diet overlaid with lysates from bacteria transfected with a plasmid expressing the commercial Gpp34Ab1/Tpp35Ab1 binary proteins. For each experiment, a total of 48 WCR neonate larvae were analyzed. Statistical analyses were conducted using Fisher exact test comparing each sample results (mortality and stunting) to the empty lysate. The untreated control was only used to evaluate the quality and reliability of each test. P-values of less than 0.05 were considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The error bars show the standard error of the mean (SEM).



437 **Fig 9. Assessment of the activity of 2 strains of *C. arthrosphaerae* (ZSTG2175 and**
 438 **DSM25208) on WCR neonates.** Activity was evaluated 5 days after infestation using diet
 439 overlay bioassay by assessing the percentage of mortality (black boxes) and the percentage of
 440 stunting (grey boxes). Each WCR larvae was exposed to ~200 μ L of WCR artificial diet
 441 overlaid with 20 μ L of supernatant or pellet lysates of both strains (ZSTG 1 and DSM 1: first
 442 replicate of ZSTG2175 and DSM25208; ZSTG 2 and DSM 2: second replicate of ZSTG2175
 443 and DSM25208). Negative controls consisted of diet overlaid with culture medium and
 444 sonication buffer. The positive control consisted of diet overlaid with lysates from *E.*
 445 *coli* transfected with a plasmid expressing the commercial Gpp34Ab1/Tpp35Ab1 binary
 446 proteins. For each experiment, a total of 48 WCR neonate larvae were analyzed. Statistical
 447 analyses were conducted using Fisher exact test comparing each sample results (mortality and
 448 stunting) to the culture medium. P-values of less than 0.05 were considered significant (* $p <$
 449 0.05; ** $p <$ 0.01; *** $p <$ 0.001). The error bars show the standard error of the mean (SEM).

450 Although we have identified GDI0005A and GDI0006A homologs in several
451 *Chryseobacterium* species, not all members of this genus possess these genes indicating that
452 they are probably part of the nonessential genome of *Chryseobacterium*. This is typical to genes
453 encoding nonessential function for the survival of the microorganism like pesticidal proteins
454 (absence of pesticidal encoding genes in the list of essential genes in DEG database/
455 <http://www.essentialgene.org>). However, they may be involved in the adaptation of the
456 bacterium to its ecological niche and to its fitness.

457 To commercialize maize plants expressing pesticidal proteins with an activity against a
458 maize pest, a prerequisite is to determine whether the MOA of the pesticidal proteins is different
459 to the MOA of an existing commercial product. Apart the RNAi mode, only two different MOA
460 of microbial pesticidal proteins are present in commercial WCR products: one from the binary
461 pesticidal protein Gpp34Ab1/Tpp35Ab1 and the other from different Cry3 variants [43].
462 GDI0005A/ GDI0006A recombinant proteins when overlaid in the WCR diet, induced a
463 significant weight loss in populations resistant to Gpp34Ab1/Tpp35Ab1 and Cry3Bb1 (Fig 3).
464 Even if the level of mortality caused by GDI0005A/GDI0006A was low in these experiments,
465 these results suggests that the binary pesticidal proteins are active against resistant strains and
466 could represent an additional component to complement the WCR control arsenal. Additional
467 studies such as competition experiments using brush border membrane vesicles (BBMV) could
468 confirm whether this new family of binary toxins have a different binding site on mid-gut cell
469 membranes compared to other commercial toxins [44].

470 Bacteria of the genus *Chryseobacterium* [45] can be found in various environments such
471 as soil [46], rhizosphere [47], water (either wastewater or freshwater) [48]; chicken [49], fish
472 [50], insect gut [51,52] and faeces of millipedes [35]. Most strains are agriculturally important
473 showing efficacy as plant growth-promoting rhizobacteria [53]. As an example, biocontrol
474 activity of *Chryseobacterium* strains ISE14 and KJ9C8 against the plant pathogen
475 *Phytophthora capsici* (causative agent of *Phytophthora* blight) [54,55] was described.

476 Pesticidal activities against plant pests were described in several types of non-*Bt*
477 bacteria. Cell lysates and crude protein extracts of *P. mosselii* and *P. chlororaphis* isolates
478 show a strong activity against WCR [32,33] that led to the identification of at least one causal
479 agents PIP47Aa and IPD072Aa respectively. However, activity of *Chryseobacterium* strains
480 against plant insects pests has only been described once [56]. In their study, *Chryseobacterium*
481 strains Rz1.2-5A associated with avocado root showed entomopathogenic activity against
482 laboratory-reared avocado thrips *Scirtothrips perseae* with an efficiency similar to the chemical

483 control. No molecules or proteins associated with this activity were described. In our study, we
 484 found that several strains of *Chryseobacterium* contain in their genome proteins with an activity
 485 against WCR larvae. The use of *Chryseobacterium* strains in biocontrol could be envisaged
 486 based on our studies showing that some cultivated *C. arthrosphaerae* strains are active against
 487 WCR larvae. Even if the link between the protein and the strain activity has not been established
 488 yet these results provide significant insight to develop further studies on this species to control
 489 coleopteran pests.

490 Materials and methods

491 List and origin of the strains assessed

492 **Table 3:** Detailed Information on bacterial strains described in the paper

493

Strain name	Available strains (ID Collection)	Binary Toxin 1	Binary Toxin 2	Strain Storage	Sources
<i>Chryseobacterium arthrosphaerae</i>	ZSTG2175	GDI0005A	GDI0006A	LEMIRE	Isolated from the Sahara Desert (W. Achouak, Lemire, CEA Cadarache)
<i>Chryseobacterium arthrosphaerae</i> CC-VM	DSM 25208 ^T	GDI0005A1	GDI0006A1	PROTEUS	Jeong, J.-J., Lee, D.W., Park, B., Sang, M.K., Choi, I.-G., and Kim, K.D. "Chryseobacterium cucumeris sp. nov., an endophyte isolated from cucumber (<i>Cucumis sativus</i> L.) root and emended description of <i>Chryseobacterium arthrosphaerae</i> ." <i>Int. J. Syst. Evol. Microbiol.</i> (2017) 67:610-616.
<i>Chryseobacterium carnipullorum</i>	DSM 25581 ^T	GDI0175A	GDI0176A	"PROTEUS	Raw chicken from a poultry processing plant in Bloemfontein in South Africa (Charimba et al., 2013)
<i>Chryseobacterium shigense</i>	DSM 17126 ^T	GDI0177A	GDI0178A	"PROTEUS	Lactic acid beverage (Shimomura et al., 2005)
<i>Chryseobacterium</i> sp. OV279 (CFB group bacteria)	Not available	GDI0179A	GDI0180A	NA	Populus root rhizosphere NCBI Uniprot
<i>Chryseobacterium</i> sp. YR203	Not available	GDI0181A	GDI0182A	NA	Populus root rhizosphere NCBI Uniprot
<i>Chryseobacterium kwangjuense</i>	JCM 15904 ^T	GDI0183A	GDI0184A	"PROTEUS	Root of a pepper plant <i>Capsicum annuum</i> in Kwangju in Korea (Sang et al., 2013)
<i>Chryseobacterium</i> sp. OV705	Not available	GDI0185A	GDI0186A	NA	Populus root rhizosphere NCBI Uniprot

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500 **Medium and cultivation conditions of *Chryseobacterium*** 501 **strains**

502 *Chryseobacterium* strains were grown aerobically at 30 °C using a rich medium
503 (Overnight Express™ Instant LB Medium – Novagen). Cultures were centrifuged for 20 min
504 at 4000 rpm. Cell pellets were resuspended in a sonication buffer (50 mM Tris, 150 mM NaCl
505 pH 7.5) and sonicated on an ice bath for 3 min using 500W sonicator probe (Fisherbrand
506 FB505AEUK-220)

507 **DNA extraction and sequencing**

508 Genomic DNA was extracted from overnight cultures and quantified by Picogreen
509 measurement. The quality was checked with an agarose gel electrophoresis analysis. The
510 genomic DNA samples were normalized before being fragmented using Adaptative Focused
511 Acoustics technology. Illumina compatible PCR free libraries were produced from each initial
512 DNA sample. Each library was individually barcoded with a unique double index strategy and
513 then quantified by qPCR measurement before being pooled. 96 libraries were pooled together
514 and sequenced with an Illumina paired-end 2x100 bases strategy. Roughly 50 Mb were
515 produced per pool. Read sequences from each sample were both adapted, and quality trimmed
516 with the tool Cutadapt version 1.8.3 (<https://pypi.org/project/cutadapt/>). Trimmed reads were
517 further assembled with the de novo assembler SPADES version 3.5.0
518 (<http://cab.spbu.ru/software/spades/>), and protein coding genes were predicted with tool
519 Prodigal version 2.6.3 (<https://github.com/hyattpd/Prodigal>). Predicted protein sequences were
520 annotated based on homology to sequences in public databases. Genomes were also analyzed
521 using the MicroScope platform [57].

522 **Protein analysis tools**

523 Multiple sequence alignments and conserved domain searches were carried out using
524 Geneious Prime 2021.1.1 (<https://www.geneious.com>) appropriate tools such as InterProScan
525 [58] and Signal 5.0 [59]. Prediction of proteins 3D structure were carried out with Phyre2
526 software [39].

527 **Recombinant protein expression in *E. coli***

528 All GDI sequences and Cry34Ab1/Cry35Ab1 (Gpp34Ab1/Tpp35Ab1) are listed in S1
529 Table. They were obtained from genome sequencing or public database and adapted to *E. coli*
530 codon use and synthesized by GenScript Biotech (Leiden, Netherlands). The corresponding
531 DNA fragments were cloned using Electra Protocol

532 (https://www.atum.bio/catalog/reagents/electra#_electra-overview) into pD441-NH vector
533 (ATUM, Newark, California, USA) under the control of IPTG-inducible T5 promoter. Cloning
534 was performed in *E. coli* BL21DE3 strain suitable for protein expression. The GDI0006A
535 variants were obtained by PCR from the original construct, for versions with a C-terminus tag,
536 cloning was performed in pD441-CH vector (ATUM, Newark, California, USA). All the
537 plasmids were verified by sequencing. Protein expression was performed overnight at 30 °C in
538 Staby Switch auto inducible expression medium (DelphiGenetics, Charleroi, Belgium) under
539 agitation at 200 rpm in a Infors HT Multitron Pro incubator (Infors AG, Bottmingen,
540 Switzerland).

541 **Recombinant protein expression in *Pseudomonas fluorescens***

542 The bacterial strain and plasmid used in this study were described in [60]. *P. fluorescens*
543 KOB2Δ1, is the *alkB* gene knockout mutant of *P. fluorescens* CHA0 and the plasmid pCom10,
544 is the pCom8 plasmid modified to carry kanamycin resistance instead of gentamycin resistance.
545 The recombinant cells of *P. fluorescens* KOB2Δ1 transformed with the pCOM10 plasmid were
546 grown in Luria-Bertani (LB) medium with kanamycin (60 µg/mL) at 30 °C. Synthetic genes
547 were codon optimized for *P. fluorescens* and cloned in pCOM10 between *NdeI* and *BamHI*
548 sites and plasmids were checked by sequencing. The recombinant cells were transformed by
549 electroporation (Eppendorf-electroporator 2510, 1600V) with 500 ng of each plasmid.
550 Transformations were plated on LB agar plates containing kanamycin and glucose (1%). A
551 glycerol-stock was prepared for each construction. Expression cultures were performed in LB
552 medium with kanamycin (60 µg/mL) at 30 °C to reach OD600 of 0.4-0.6. Then cultures were
553 induced with 0.05% (w/v) dicyclopropyl ketone (DCPK) and incubated 20 h at 30 °C. Cells
554 pellets were resuspended in buffer Tris-HCl 20 mM pH8, NaCl 150 mM, DTT 1 mM. Cells
555 were lysed mechanically by using lysing matrix E (silica and glass beads) and in a FastPrep-
556 24TM 5G (MP BiomedicalsTM).

557

558 **Strains rearing and larval diet**

559 WCR (EPPO code DIABVI) were mass-reared following the procedures of [61–63]. A
560 non-diapause colony (USDA ARS, Bookings, USA) was used to infest artificial diet-based
561 bioassay with neonates (<24h old) (see procedures below). The population is considered
562 susceptible to most insecticides or novel agents as it had not been exposed to any of those, and
563 therefore resistance is considered unlikely [64,65]. Insect colonies resistant to Cry3Bb1 and

564 Gpp34Ab1/Tpp35Ab1 are described in Ludwick et al. (2018). As described, these insect strains
565 initially evolved some level of resistance in the field, were crossed to a nondiapausing strain,
566 and thereafter were continuously selected on seeds expressing Cry3Bb1 or
567 Gpp34Ab1/Tpp35Ab1 in USDA-ARS facilities in Columbia, MO USA.

568 For susceptible strains, two weeks prior the bioassays, soil dishes with freshly laid eggs
569 had been removed from WCR adult rearing cages to allow sufficient incubation time until egg
570 hatch. Eggs were washed with cool tap water with <0.01% NaOCl through a 300 µm mesh
571 sieve. Around 5000 eggs were transferred to sterilized, slightly moist river sand (< 200 µm
572 grains) in Petri dishes. They were incubated at 24 ± 2 °C in darkness for 8 to 12 days until
573 hatching started. One the day before a bioassay, the ready-to-hatch eggs were again washed and
574 sieved. Eggs were then again placed on sterile moist sand onto slightly moist tissue paper into
575 a dish to allow clean hatching conditions of new neonates and their use for bioassays. For
576 resistant strains, the methods of Ludwick et al. [66] and Huynh et al. [67] were used.

577 For work with susceptible strains, WCR diet was prepared one day prior to the bioassay.
578 The diet was prepared under semi-sterile conditions following methods described previously
579 [67–69]. A commercial southern corn rootworm diet (Frontier #F9800B, Frontier Scientific
580 Ltd., USA) plus additions of maize root powder and food color were used to make the WCR
581 diet. To prepare 100 ml of diet, 13.8 g of the #F9800B diet was ground and added to 88 ml fluid
582 agar consisting of 1.5 g agar (CAS 9002-18-0, Chejeter, Japan) and deionized water. After
583 blending and cooling to 55 °C to 60 °C, 0.75 g ground lyophilized maize roots (GLH5939
584 Pioneer, USA, or Phileaxx RAGT, Hungary) were added into the diet mixture, followed by the
585 addition of 0.1 g green food colour for better larval observation (Les Artistes, France). Then,
586 1.7 to 1.8 ml 10% (w/v) KOH were added to reach a pH between 6.2 and 6.5. This mix was
587 blended again, and then stirred at 50 °C to 55 °C. Then, 190 µl diet solution was pipetted into
588 each 330 µl well of 96-well plates filling each to around 2/3rd. Then, the 96-well plates with
589 diet were allowed to dry in a laminar flow cabinet for 45 minutes, and then stored at 3 °C to 5
590 °C overnight. Diets for work with resistant strains followed Man et al. (2019).

591 **Insect bioassay *in vitro***

592 WCR bioassays on susceptible strains were conducted in 96-well plates by adding 20
593 µL of cleared liquid lysate of different *E. coli* or *P. fluorescens* clones to each well. Each
594 treatment was applied to 8 wells of each plate per bioassay. Each treatment-dose combination
595 was tested in at least two biological replicates. Order of treatments were shifted every other

596 plate to avoid edge effects. Plates were dried for 1 to 1.5 h, and then cooled for 1 h in a fridge
597 at 3 °C to 5 °C. One neonate larva was placed per well using a fine artist brush. A fast-moving,
598 healthy-looking larva was chosen, and lifted from the end of abdomen with the brush, moved
599 towards a well surface, and allowed to crawl off the brush onto the diet. Larvae were not placed
600 in treatment column order but rectangular to avoid systemic errors. After every 12th larva, the
601 brush was cleaned in 70% ethanol followed by sterile tap water. The filled plate was closed
602 with an optically clear adhesive qPCR seal sheet (#AB-1170, Thermo Scientific, USA or
603 #BS3017000, Bioleader, USA) allowing data assessments without opening the plate. Four to
604 five holes were made with flamed 00-insect pins into the seal per well to allow aeration. The
605 plates were incubated at 24 ± 2 °C and 50 to 70% relative humidity (RH) in the dark in a
606 ventilated incubator (Friocell 22, MMM Medcenter, Munich, Germany) for 5 days. Bacterial
607 lysate containing Gpp34Ab1/Tpp35Ab1 (<https://www.rcsb.org/>) was used as a positive control.
608 Cleared empty lysate, which was a bacterial lysate produced in the same conditions and from
609 the same bacterial strain containing the same expression vector as the other lysates but without
610 the gene encoding the pesticidal protein, served as the negative control. Insecticidal activity
611 was scored as dead, stunted (survived with no growth), no activity (growth equal to controls)
612 after 3 and 5 days. Statistical analyses were determined using Fisher exact test comparing each
613 sample result to the negative control. Results were considered positive if larval mortality and
614 stunting were statistically higher than in the negative control and recorded at 3- and 5-days post
615 treatment. Stunting was qualitatively assessed as an indicator for sublethal effects in
616 comparison to the size and form of larvae in the negative controls. Feeding was assessed
617 through observing food remains, frass, and diet in the larval gut to assure that diet and a
618 treatment had been ingested.

619 WCR bioassays on resistant strains (Gpp34Ab1/Tpp35Ab1 and Cry3Bb1) were
620 conducted at the Plant Genetics Research Unit, USDA/ARS, in Columbia, Missouri, USA, with
621 similar methods used as for susceptible WCR strain except for the following points. The diet
622 used in the bioassays was WCRMO2 [67]. The experiment consisted of a series of 3 plates
623 totaling 3 x 8 wells (column) (3 replicates per sample) and 24 larvae per treatment and controls
624 (N=24). Five days after treatment, mortality was assessed, and experimental plates were placed
625 in a -20°C freezer (Thermo Fisher Scientific TSE 400A, Marietta, GA) overnight. The next day
626 the plates were removed and thawed to room temperature and the total weights of surviving
627 larvae from each treatment was recorded in a Sartorius micro scale (model # MSE6-6S-000-
628 DM, Sartorius Corporate, Göttingen, Germany) and then divided by the number of individuals

629 to calculate mean larval weight per 8-well replicate. One-way analysis of variance (ANOVA)
630 and Tukey's HSD post-hoc test were used to assess significant differences among the
631 treatments.

632 Lepidopteran bioassays were conducted in 96-well plates by adding 20 μ L of crude
633 lysate to 200 μ L diet (General Purpose Lepidoptera Diet Frontier Scientific F9772 diet for ECB,
634 FAW and CEW, and Southwestern Corn Borer Diet, Frontier Scientific F0635 plus Vitamin
635 Mix, Frontier Scientific F0717 and Neomycin Sulfate, Sigma N1876). The diet was dispensed
636 into a 96-well plate using an 8-channel pipette (Finni pipette Multistepper 4540500 Thermo
637 Fisher, Marietta, GA), delivering 200 μ l into each well and allowed to cool. Then, 20 μ l of
638 lysate was dispensed (Socorex Acura 826 XS 20-200 μ l, Ecublens, Switzerland) onto each well
639 of the diet plate as directed in the experimental plan. The wells were dried in a laminar flow
640 hood for approximately 1 hour or until all inoculated wells were dried. All lepidopteran insects
641 used in these experiments were received in eggs. All lepidopteran eggs were hatched in a 29
642 $^{\circ}$ C, 60% RH, 0:24L:D growth chamber (Convicon A1000, Manitoba, Canada). Eight wells per
643 replicate and 3 replicates per treatment were infested with a single larva for each experiment
644 providing 24 datapoints per experiment for each sample. After infesting each plate 2 holes were
645 poked into the sealing film using a 000-insect pin and plates were placed in the growth chamber
646 (Convicon A1000, Manitoba, Canada) at 26 $^{\circ}$ C, 50% RH, 0:24 L:D for 5 days. Insecticidal
647 activity was scored as dead, stunted (survived with no growth), no activity (growth similar to
648 negative controls) after 5 days. After scoring, the experimental plates were placed in a -80 $^{\circ}$ C
649 freezer (Thermo Fisher Scientific TSE 400A, Marietta, GA) overnight. The next day the plates
650 were removed and thawed to room temperature and the total weights (A&D GR-200 Analytical
651 Balance, Columbia, MD) for each sample replicate were collected and then divided by the
652 number of individuals to calculate mean larval weight per 8-well replicate. Statistical analyses
653 were determined using Fisher exact test comparing each sample results to the negative control.
654 P-values of less than 0.05 were considered significant.

655 All information on the origin of the insect strains are described in S3 table

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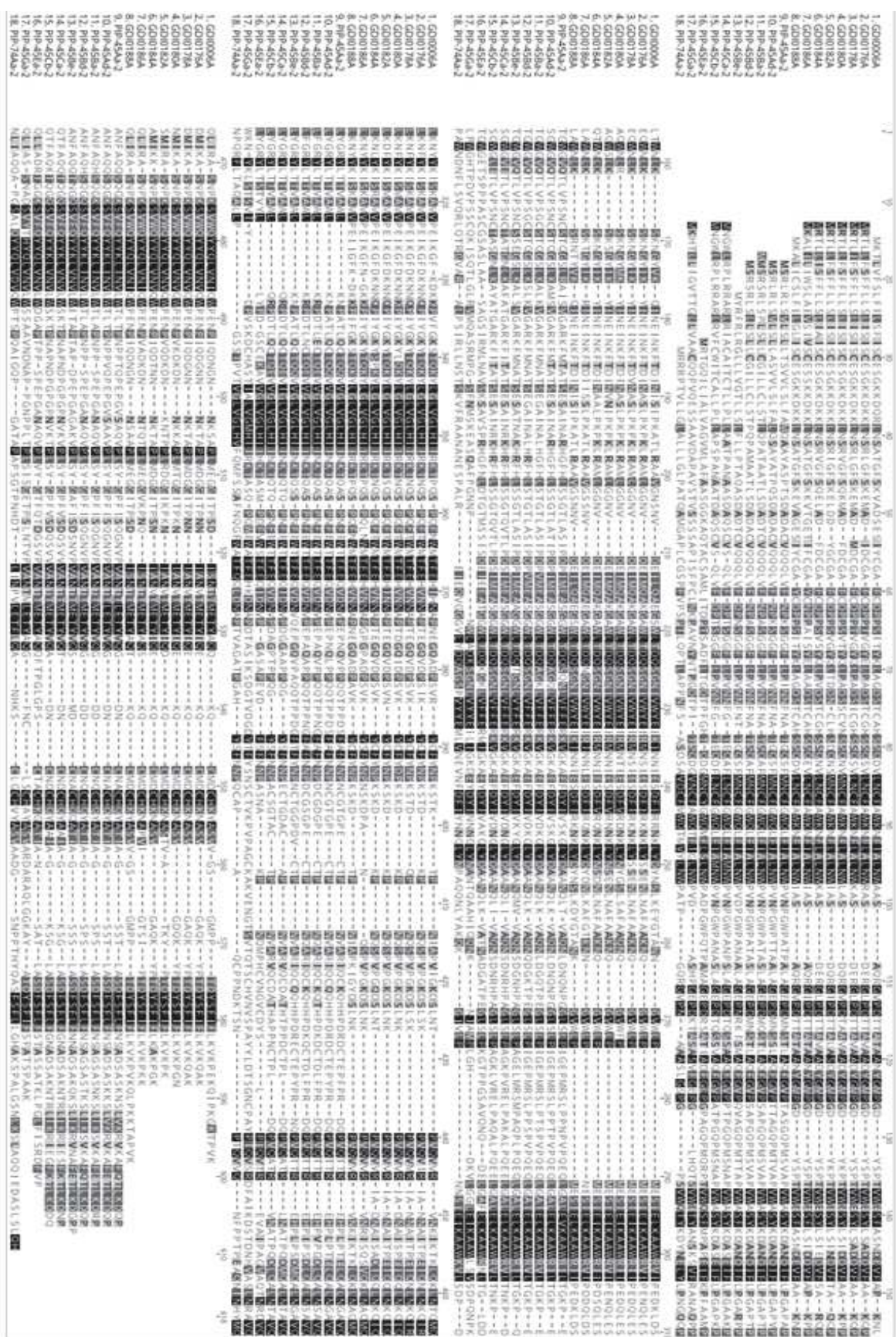
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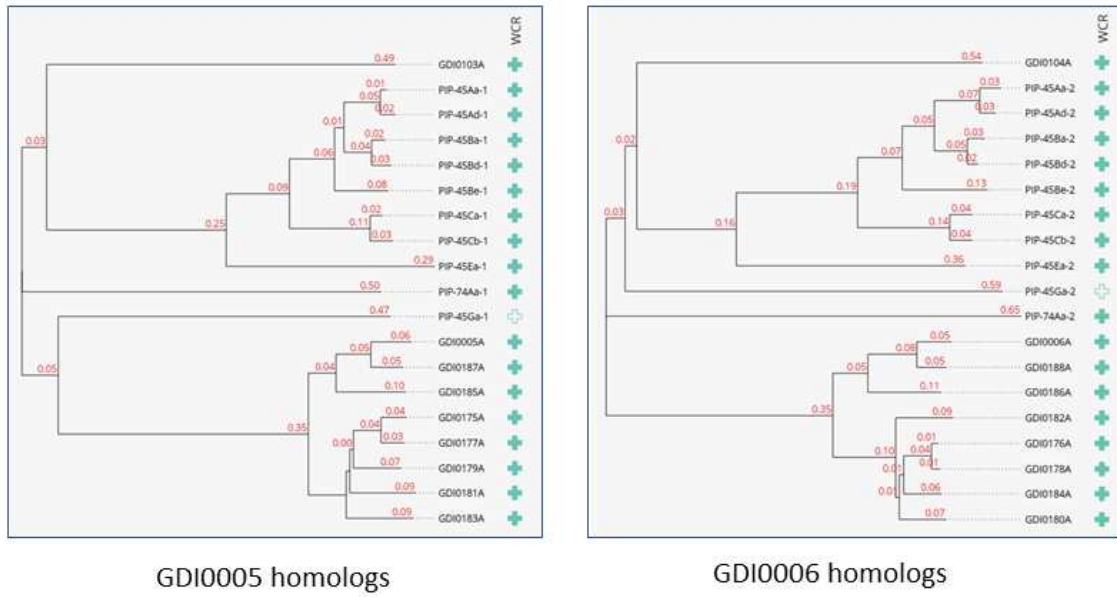
891 **Supporting Information**

892 **Supplementary data**

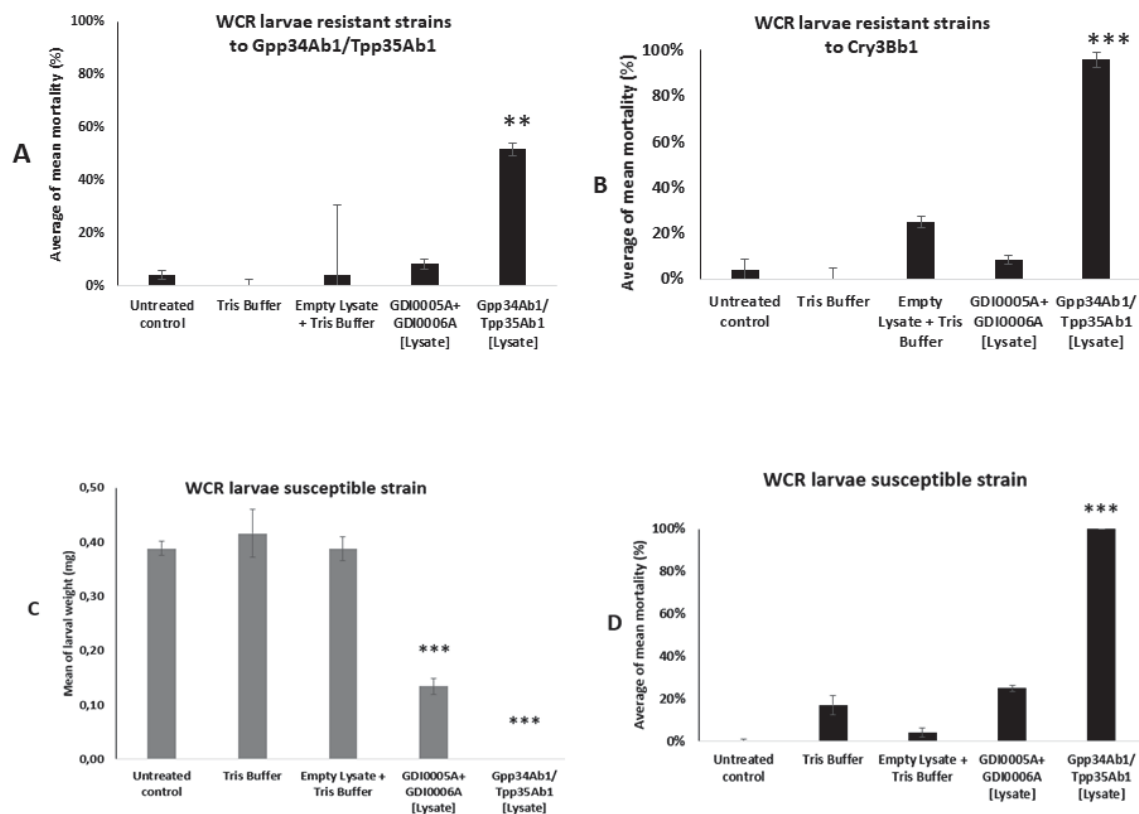


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Figure S1: B) Multiple AA sequence alignment of component 2 of PIP45, PIP75 and GDI protein families. All the information about the sequences are present in the table 2. GDI proteins are from this study. PIP sequences are from patent WO2016/114973



900 **Figure S2:** Activity of GDI0005A and GDI0006A homologs as pair against WCR.
 901 Phylogenetic tree was built with the Neighbor-joining method using Jukes-Cantor Genetic
 902 Distance model. Full cross indicates an insecticidal activity against WCR, Empty cross
 903 indicates a non-activity against WCR (Geneious Tree Builder from Geneious)
 904
 905



906

907 **Figure S3:** Activity of GDI0005A and GDI0006A and commercial toxins
 908 Gpp34Aa1/Tpp35Ab1 and Cry3Bb1 on WCR susceptible and resistant strains to commercial
 909 Bt Toxins Gpp34Aa1/Tpp35Ab1 and Cry3Bb1. A, B, and D describe the mortality and C)
 910 describe the larval weight obtained in with the binary toxins GDI0005A&GDI0006A and
 911 commercial toxins Gpp34Aa1/Tpp35Ab1.
 912

913 **Table S1:** GDI0005A/GDI0006A lysates activity against some lepidopteran maize pests using
 914 diet overlay bioassay: ECB: European corn borer *Ostrinia nubilalis*, FAW : fall armyworm
 915 *Spodoptera frugiperda* ; SWCB : southwestern corn borer *Diatraea grandiosella*; CEW : corn
 916 earworm *Helicoverpa zea*. Statistical analyses were determined using Fisher exact test
 917 comparing each sample results to the negative control. P-values of less than 0.05 were
 918 considered significant.
 919

	GDI0005A + GDI0006A			Positive control			Empty lysate	
	Stunting (%)	Mortality (%)	p-value	Stunting (%)	Mortality (%)	p-value	Stunting (%)	Mortality (%)
ECB	0	9,4 ± 3,1	0.23	0	100	1.09 E-18	0	0
FAW	6	0	0.67	50 ± 12,5	25 ± 12,5	4.4 E-7	12,5 ± 6,2	0
CEW	4,1 ± 4,1	22,2 ± 14,6	0.18	nd	nd	nd	4,1 ± 4,1	4,1 ± 4,1
SWCB	0	4,1 ± 4,1	1	nd	nd	nd	4,1 ± 4,1	0

920

3 Protein characterization of the binary toxins: a prerequisite to determine the *in vitro* efficiency of the toxins

To further assess the level of pesticidal activity of GDI0005A/GDI0006A, the median lethal concentration (LC50) must be determined. Therefore, we set out to purify GDI0005A and GDI0006A proteins to perform these bioassays. As mentioned in the article, no protein domain was identified in GDI0005A via *in silico* approaches as well as in its homologs. It was concluded that GDI0005A is most likely present in the cytoplasm. However, GDI0006A and its homologous sequences were all found to have a type II lipoprotein N-terminal signal peptide and a conserved cysteine (Cys) residue. The proteins are predicted to be secreted via the Sec translocon machinery and processed by signal peptidase II (SPaseII) after the transfer of a diacylglyceride to the cysteine sulphhydryl group. Further translational modifications, such as the insertion of a fatty acid to the amino-terminal diacylglyceryl-Cys residue, are required to ensure the mature proteins' anchoring to the outer membrane (Braun and Hantke, 1974; Nakayama et al., 2012; Sankaran and Wu, 1995). Consequently, we expected to have difficulties in purifying this family of membrane proteins.

3.1 GDI0005A/GDI0006A purification at the AFMB lab

A first purification test of GDI0005A and GDI0006A and their homologs was performed in the laboratory of Renaud Vincentelli (AFMB, Marseille, France, see material and methods section). Constructs with both His-Tag fusion and/or fused to maltose binding protein (MBP), known for enhancing proteins solubility, were used (Kapust and Waugh, 1999; Korepanova et al., 2007). In addition, constructs with only the His tag were also tested for GDI0005A and GDI0006A. Tags were put at the N terminus of each protein. As expected, GDI0005A (both His and His-MBP tags) and its homologs have been successfully purified. In contrast, there have been some difficulties with GDI0006A proteins apart from GDI0176A and GDI0178A, two homologues of GDI0006A. Western blots using His antibodies and Bradford analysis show an unexplainable variation between the wash and elution fractions. With the exception of GDI0176A and GDI0178A, a significant amount of protein appears to be lost during the washing steps. Among all proteins, the molecular weight of GDI0178A appears to be slightly higher than expected (Figure 13, Table 2). Therefore, GDI0005A and GDI0176A were chosen to produce specific polyclonal antibodies due to their better yield and correct size.

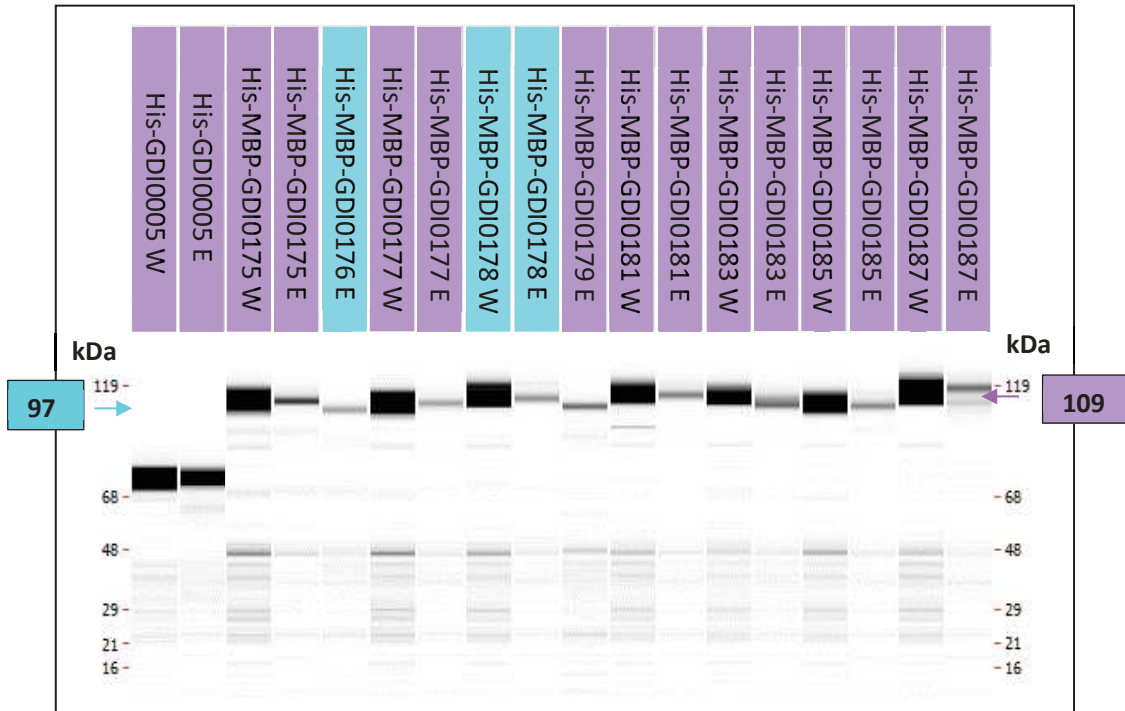


Figure 13: Visualization of the successfully purified proteins detected by an anti-His antibody (Western Blot): in purple GDI0005A and its homologs; in blue two homologs of GDI0006A. W : Wash fraction; E: Elution fraction

Table 2: Concentrations of the wash (W) and the elution (E) fractions of the successfully purified proteins at the AFMB lab: in purple GDI0005A and its homologs; in blue two homologs of GDI0006A.

Protein	Concentration W (mg/ml)	Concentration E (mg/ml)	Theoretical molecular weight (kDa)
His-GDI0005A	7,20	3,58	65,6
His-MBP-GDI0175A	6,10	1,10	109,2
His-MBP-GDI0176A	0,00	0,80	97
His-MBP-GDI0177A	6,10	0,55	109,2
His-MBP-GDI0178A	4,55	0,50	97
His-MBP-GDI0179A	0,00	1,12	109
His-MBP-GDI0181A	5,60	0,92	109
His-MBP-GDI0183A	5,80	1,28	109
His-MBP-GDI0185A	5,90	0,92	109,7
His-MBP-GDI0187A	4,70	1,00	109,7

3.2 GDI0005A/GDI0006A purification at Limagrain

3.2.1 GDI0006A purification

As mentioned previously, difficulties were encountered while purifying GDI0006A. It has been shown that the N-terminal signal peptide is crucial for the activity and potentially the folding of the protein (see article). Thus, putting the tags at the N-terminal position was not the best option since the signal peptide would potentially be cleaved resulting in the loss of the tag that is used for its purification. This might be one of the reasons behind GDI0006A purification failure. Before focusing on the purification improvement process, optimization of protein expression should be investigated.

3.2.1.1 Could Lemo21(DE3) enhance GDI0006A expression?

E. coli BL21(DE3) is the most commonly used strain to express recombinant proteins. To improve the expression of the GDI0006A protein, we choose a derivative strain called Lemo21(DE3) designed to improve challenging proteins expression including toxic and membrane proteins (Giacalone et al., 2006; Schlegel et al., 2012). We successfully transformed Lemo21(DE3) and BL21(DE3) with GDI0006A. Transformation and gene insertion were checked by real time-PCR using specific primers for amplification of 5' and 3' ends as shown in Figure 14. Bacterial lysates containing GDI0006A from both BL21(DE3) and Lemo21(DE3) were compared. SDS page and Western blot analysis using specific antibodies did not show any significant difference between both bacterial lysates for GDI0006A production, then BL21 was used in next experiments (Figure 15). Moreover, our protein seems to be mostly present in the pellet fractions which confirm the hypothesis of a non-soluble protein (Figure 15).

3.2.1.2 Testing different methods for efficient protein extraction

Since GDI0006A is probably a membrane protein, detergents are needed to help remove the protein from the membrane. Choosing the best surfactants that are compatible with downstream chromatographic strategies and preserve the structure and function of the native protein remains a major challenge.

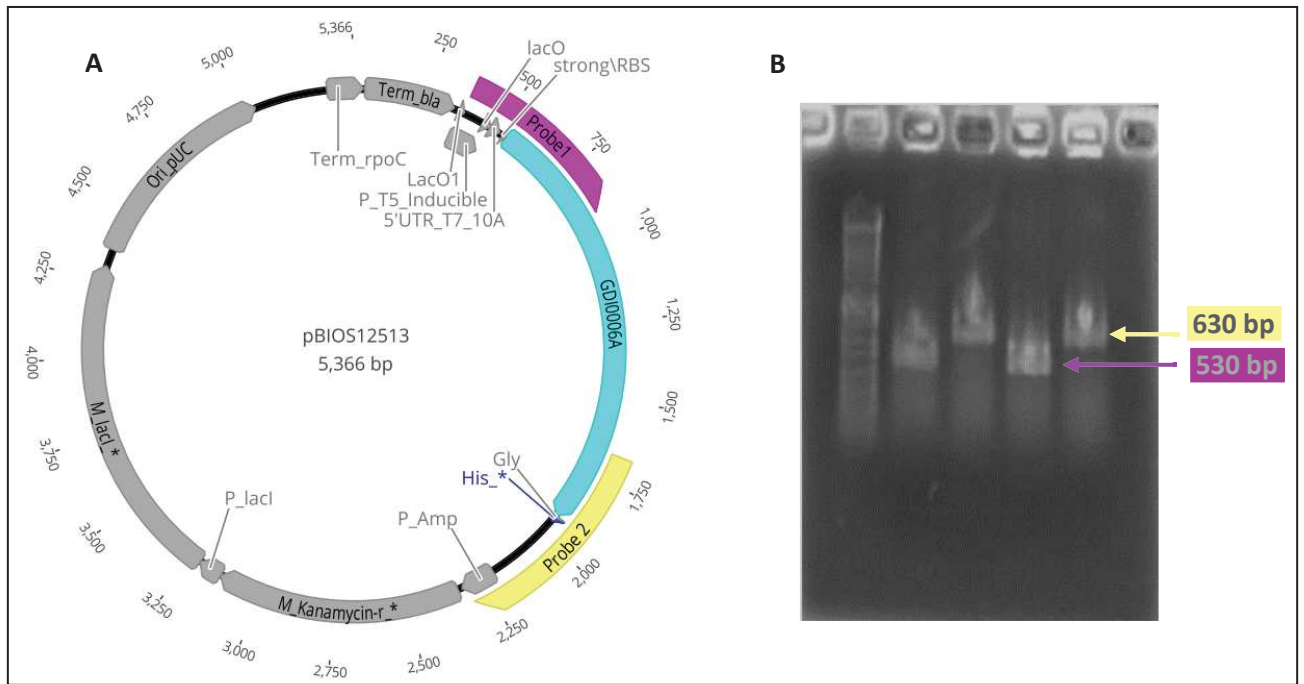


Figure 14: A: Representation of the plasmid containing GDI0006A for Lemo21(DE3) transformation. **B:** PCR verification of Lemo21 (DE3) transformation

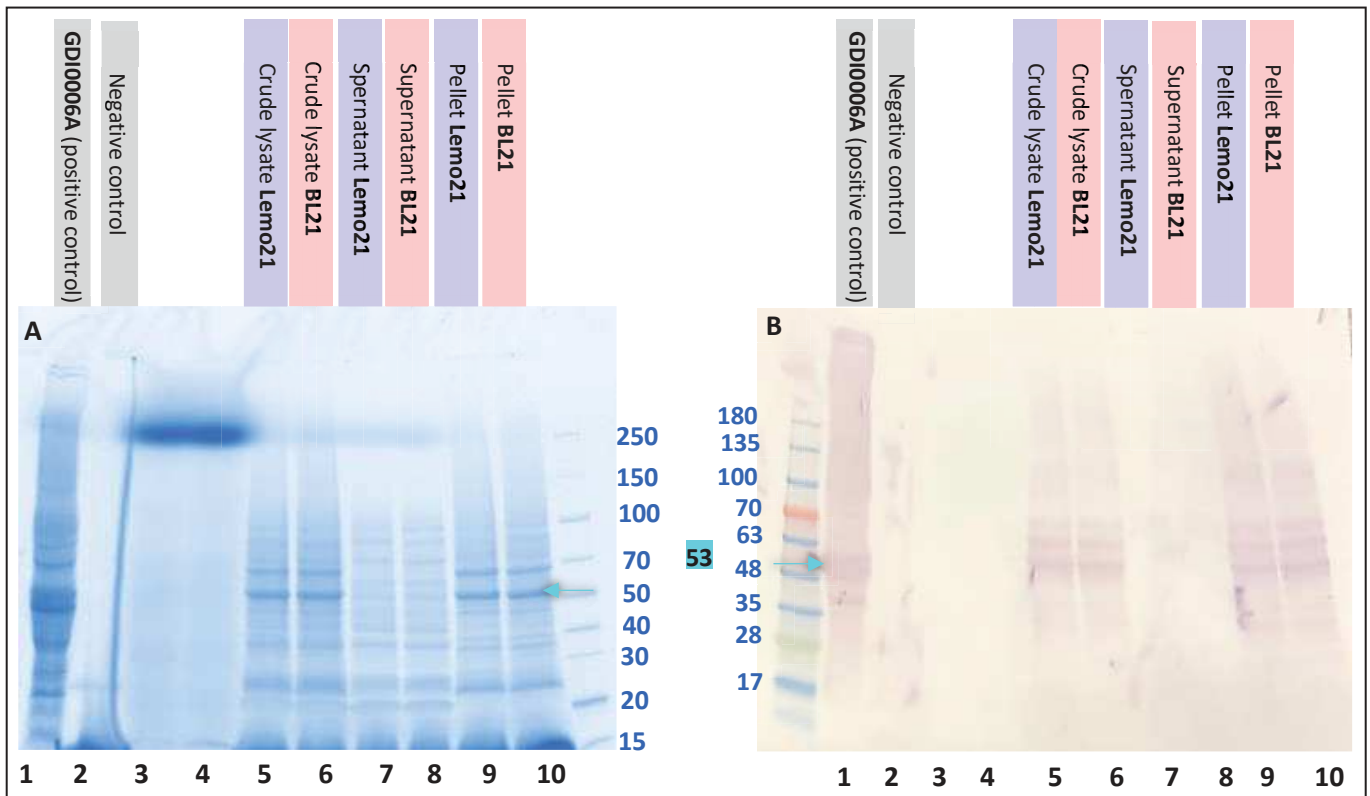


Figure 15: GDI0006A expression comparison using two *E. coli* strains BL21 (DE3) (red) and Lemo21 (DE3) (purple). The samples were loaded on SDS-page gel (A) and then transferred to a PVDF membrane and immunoblotted using specific antibody anti-GDI0006A-like. Protein detection was performed by adding a chromogenic substrate for a colorimetric detection (B)

Cell lysis using BugBuster Protein Extraction Reagent (Bugbuster)

Recombinant *E. coli* BL21(DE3) cells expressing GDI0006A were harvested chemically using Bugbuster (BBU) protein extraction reagent. Proteins were solubilized in the same buffer. The sample was dialyzed against Tris NaCl buffer to ensure compatibility with the chromatography resin column. Centrifugation and filtration through 0,22 µm membrane are needed to eliminate impurities. According to protein analysis, a significant GDI0006A quantity was lost after centrifugation and filtration, resulting in purification failure (Figure 16).

Protein extraction using non-denaturing detergents

- Zwitterionic detergent (CHAPS)

3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate CHAPS is a non-denaturing zwitterionic detergent known for its capacities to solubilize membrane proteins (Cladera et al., 1997). After sonication of the cells, proteins were solubilized in CHAPS buffer. Western blots using specific GDI0006A antibodies show 2 bands at 52 kDa (expected size) and 40 kDa suggesting partial degradation of the protein with a size of 12 kDa. Protein solubility has been markedly enhanced since we didn't lose it after centrifugation and filtration (lanes 3, 4&5, Figure 17). Nevertheless, the protein present in the final purification fraction has a molecular weight of 40 kDa (lanes 6&7, Figure 16) which is lower than the theoretical molecular weight of GDI0006A.

- Non-ionic detergent (DDM)

n-Dodecyl β-D-maltoside (DDM) is a non-ionic gentle detergent known to improve membrane proteins stability (Rouse et al., 2013). When used, DDM seems to effectively solubilize GDI0006A. However, the same 2 bands are observed with CHAPS detergent, with the sample containing GDI0006A after sonication of the cells, centrifugation and filtration (lanes 3&4, Figure 17) leading to the purification of a protein of about 40 kDa (lane 9, Figure 18) similar to the one found with the CHAPS detergent.

3.2.2 GDI0005A purification

GDI0005A purification, as seen at AFMB lab, was much easier than purifying GDI0006A. His-tagged GDI0005A from recombinant *E. coli* BL21 (DE3) was successfully purified at the correct size (66 kDa) with an appropriate purity level (Figure 19).

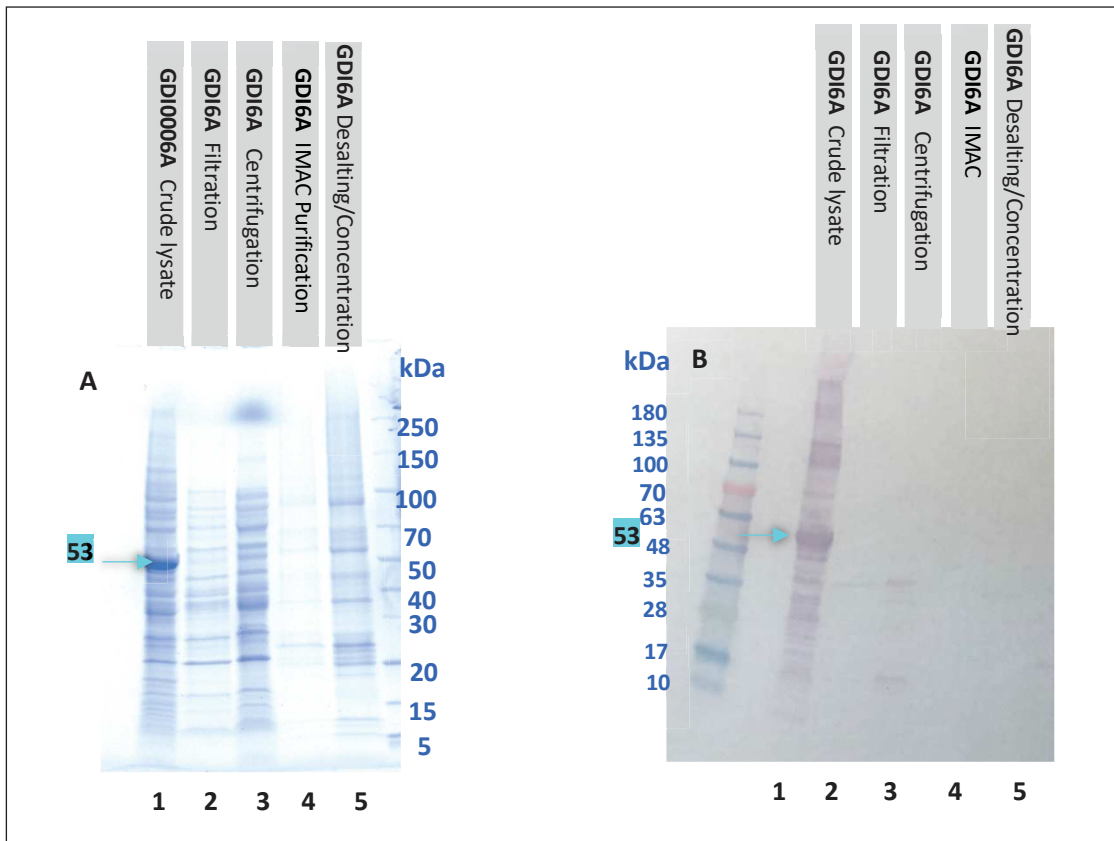


Figure 16: GDI0006A purification test using Bugbuster (BBU) protein extraction reagent. The samples were loaded on SDS-page gel (A) and then transferred to a PVDF membrane and immunoblotted using specific antibody anti-GDI0006A-like. Protein detection was performed by adding a chromogenic substrate for a colorimetric detection (B)

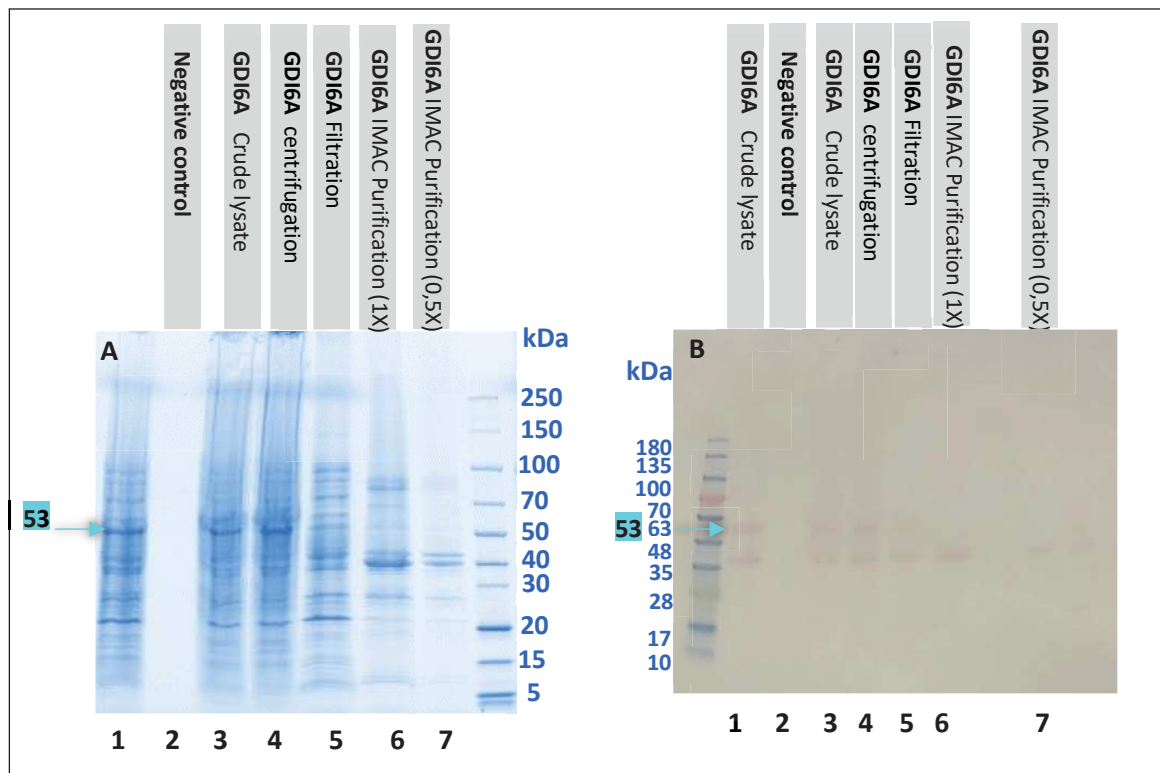


Figure 17: Purified GDI0006A fractions (using CHAPS detergent) analysis. The samples were loaded on SDS-page gel (A) and then transferred to a PVDF membrane and immunoblotted using specific antibody anti-GDI0006A-like. Protein detection was performed by adding a chromogenic substrate for a colorimetric detection (B)

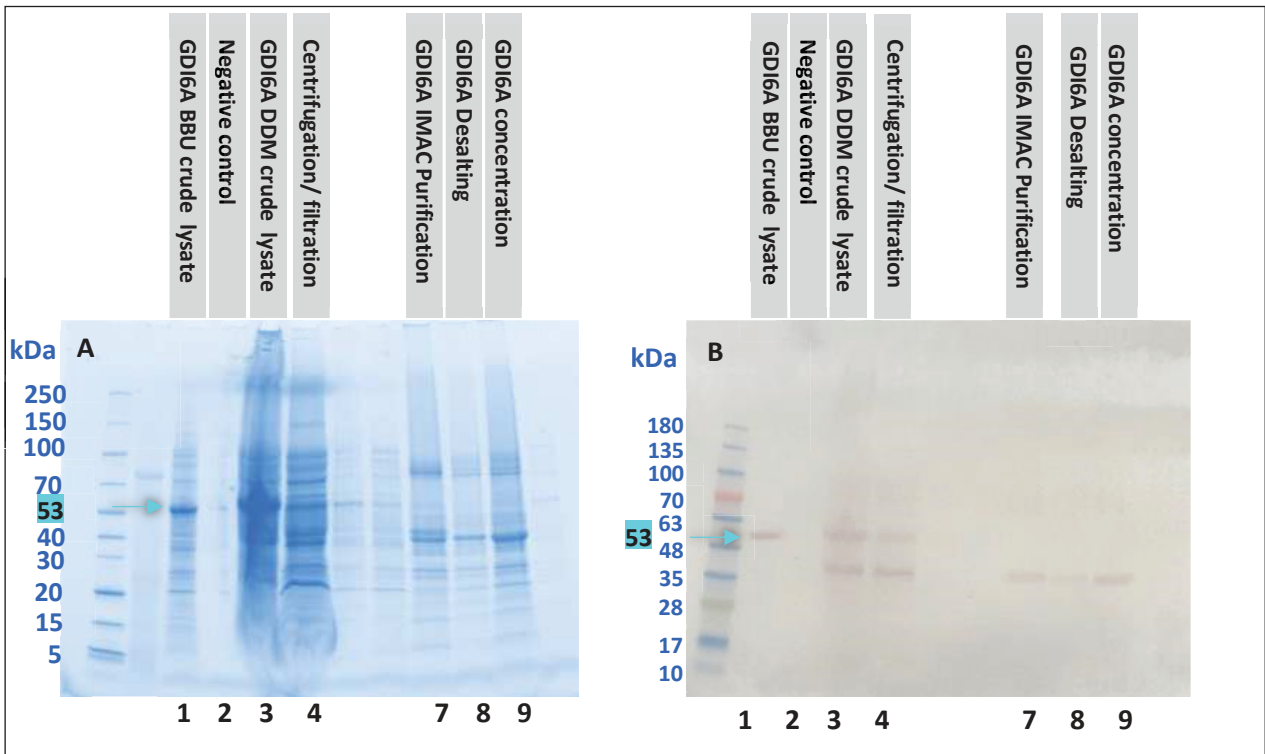


Figure 18: Purified GDI0006A fractions (using DDM detergent) analysis. The samples were loaded on SDS-page gel (A) and then transferred to a PVDF membrane and immunoblotted using specific antibody anti-GDI0006A-like. Protein detection was performed by adding a chromogenic substrate for a colorimetric detection (B)

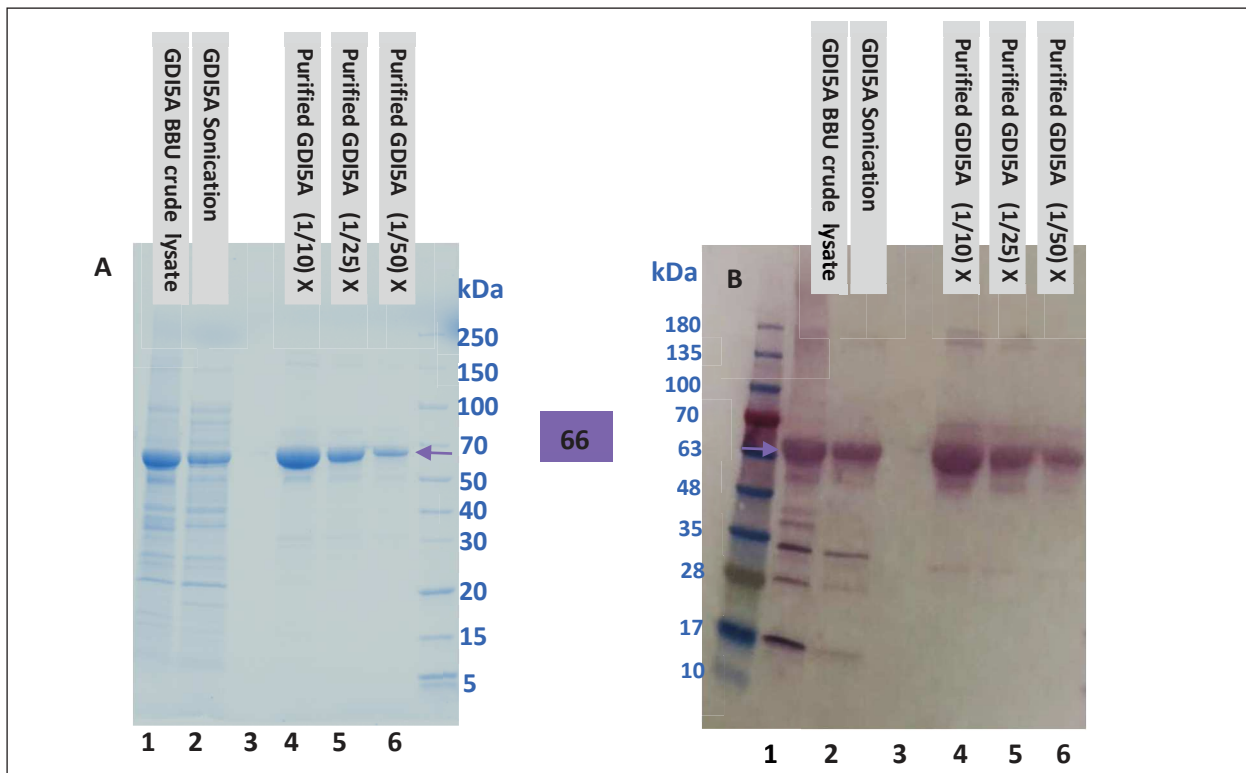


Figure 19: GDI0005A purification analysis : The purified fraction were loaded into SDS-page gel (A) and then transferred to a PVDF membrane and immunoblotted using specific antibody anti-GDI0005A. Protein detection was performed by adding a chromogenic substrate for a colorimetric detection (B).

Evaluation of the activity of the purified GDI0005A/GDI0006A against WCR larvae

The GDI0006A protein purified with DDM detergent and the GDI0005A, GDI0176A, and GDI0177A proteins purified at AFMB laboratory were tested for activity against WCR larvae using diet overlay bioassay method (see material and methods). A 1:25 and 1:7 (W:W) ratio was used for the binary pairs GDI0006A/GDI0005A and GDI0176A/GDI0177A respectively. As it can be observed in Figure 20, GDI0005A/GDI0006A appeared to be active against rootworms. A similar activity has been noticed with bacterial lysates reported in the article where GDI0005A/GDI0006A exhibits a significant mortality and stunting. These results demonstrate that the 40 kDa purified protein is probably the active form of GDI0006. Besides, a small amount of GDI0006A seems to be sufficient to interact with GDI0005A. The main activity of purified GDI0176A/GDI0177A consists in affecting larval growth and development. Since each sample was prepared using different buffer, we subtracted the background of mortality and stunting of each negative control to compare GDI0005A/GDI0006A *E. coli* lysates with purified GDI0005A/GDI0006A and GDI0176A/GDI0177A. As expected, activity of purified GDI0005A/GDI0006A was similar to *E. coli* lysate containing both proteins. Moreover, GDI0176A/GDI0177A activity was significantly different from GDI0005A/GDI0006A at both forms.

Difficulties encountered with GDI0006A purification have slowed the progress of LC50 studies. Now that the active form seems to be the 40 kDa peptide, the challenge will be to purify a highly concentrated protein to run the LC50 experiments.

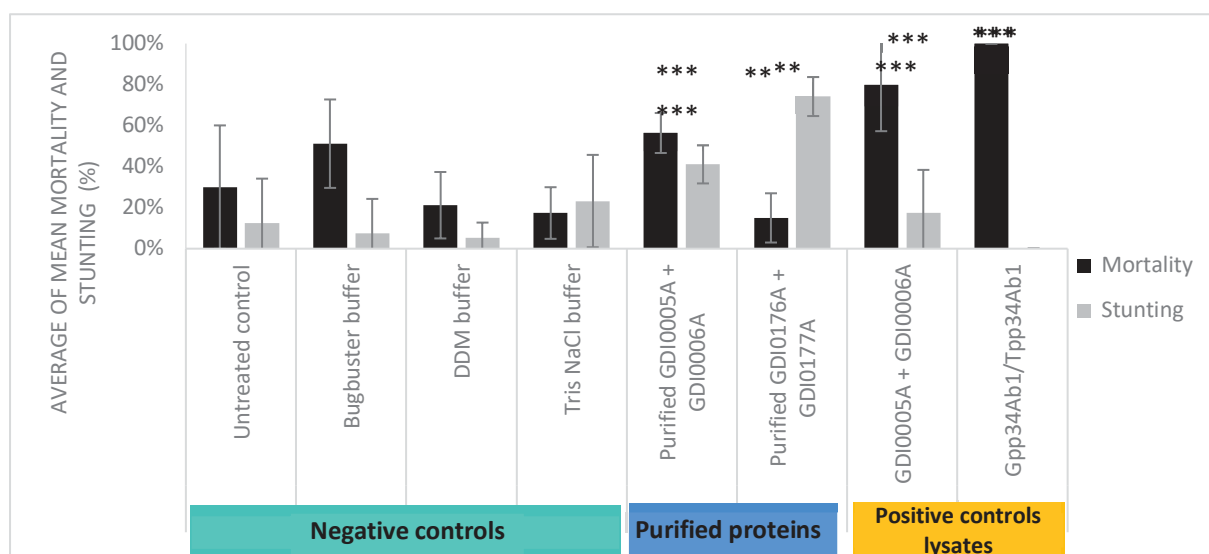


Figure 20: Activity of GDI0005A/ GDI0006A and GDI0176A/GDI0177A purified proteins against WCR neonates (USDA ARS non-diapause susceptible colony) 5 days after infestation using artificial diet overlay bioassays. Activity was evaluated by assessing the percentage of mortality (black boxes) and the percentage of stunting (grey boxes). Each WCR larvae was exposed to ~200 μ L of WCR artificial diet overlaid with 20 μ L of purified proteins. Negative controls consisted of diet overlaid with purification buffers, lysates from bacteria transfected with *E. coli* empty strain (empty lysate) and diet overlaid with sterilized water (untreated control). The positive controls consisted of diets overlaid with lysates from bacteria transfected with plasmids expressing GDI0005A and GDI0006A, Gpp34Ab1/Tpp35Ab1 extracted with Bugbuster buffer. GDI0006A was purified using DDM buffer, GDI0005 and GDI0176/GDI0177 were purified in Tris-NaCl. For each experiment, a total of 48 WCR neonate larvae were analyzed. Statistical analyses were conducted using Fisher exact test comparing each sample results (mortality and stunting) to the empty lysate. Then, background of mortality and stunting of each negative control was subtracted to compare GDI0005A/GDI0006A *E. coli* lysates with purified GDI0005A/GDI0006A and GDI0176A/GDI0177A using Tukey HSD test. The untreated control was only used to evaluate the quality and reliability of each test. P-values of less than 0.05 were considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The error bars show the standard error of the mean (SEM).

4 Evaluation of the expression and activity of binary toxins *in planta*

4.1 Expression of the binary toxins in Tobacco transient expression system

Aiming to study GDI0005A/GDI0006A expression and activity in a plant expression system, we used the agroinfiltration approach to transiently produce these proteins in *Nicotiana benthamiana* (NB) leaves. This technique allows a rapid and inexpensive response for the expression of proteins of interest *in planta* (Rodríguez et al., 2005). GDI0005A/GDI0006A as well as their homologs were expressed separately under the control of the constitutive viral promoter of Cassava Vein Mosaic Virus (CsVMV) to highly express the proteins (Figure 21) (Oyelakin et al., 2015). Molecular constructions and transformation into *Agrobacterium* strains were done by the Limagrain's TEV laboratory headed by Laurent Beuf. 7 days after infiltration of NB leaves with *Agrobacterium tumefaciens* carrying the genes of interest, total proteins were extracted, and samples were used to conduct Western blots analysis and bioassays against WCR larvae. As shown in Figure 22, GDI0005A and its homologs were highly expressed at the correct size (around 66 kDa). In contrast, GDI0006A homologs expression was not a success. GDI0176A, GDI0178A and GDI0184A are the most expressed but not at the expected size (>55 kDa) whereas GDI0006A was above the detection limits.

To overcome the difficulty to express GDI0006A, we decided to target GDI0006A to different cellular organelles (the endoplasmic reticulum, the chloroplast, the nucleus, the protein bodies, the apoplast and the peroxisome) by fusing the protein with specific targeting signals from these compartments (Table 3). The same promoter was used for all constructs. The level of GDI0006A expression, detected by WB using specific antibodies in the different organelles, is summarized in Table 4. Constructs targeted to the nucleus and the chloroplast, show a phytotoxicity of the protein in these compartments (Figure 23). Unexpected variation in GDI0006A expression in the different cell compartments was noticed among the replicates. Reliable results were seen with the protein was targeted to the peroxisome or to the apoplast, but the size of the protein was slightly higher than expected.

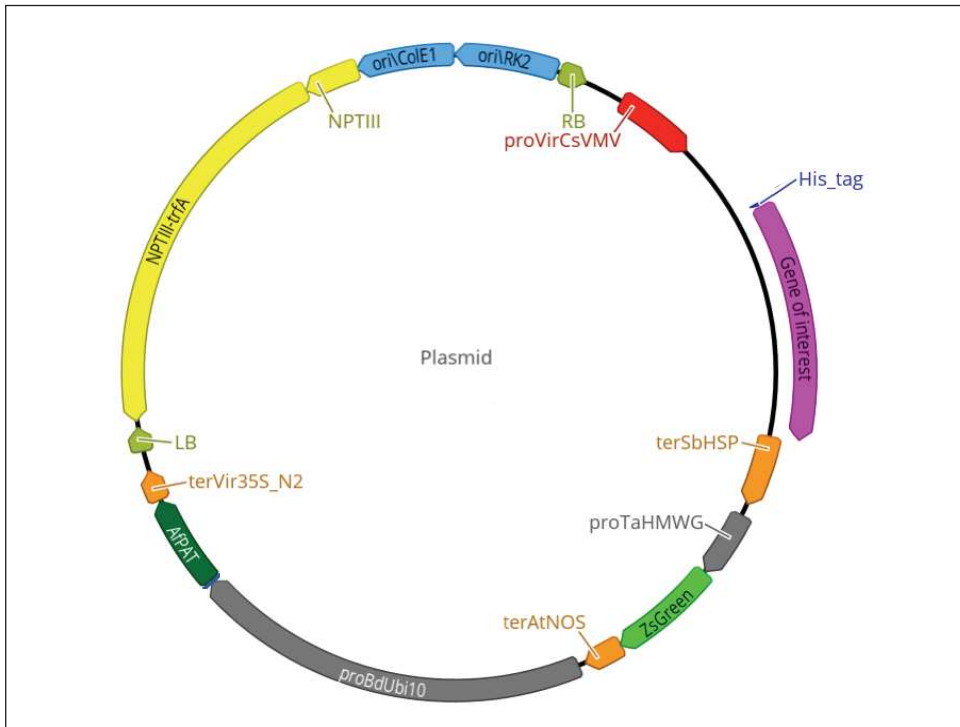


Figure 21: Schematic representation of the plasmid used in *Nicotiana Benthamiana* transfection. The T-DNA region of the vector has the gene of interest driven by the promoter viral proVirCsVMV (Cassava Vein Mosaic Virus), SynZsGreen a gene coding for the Green Fluorescence Protein, AfPat conferring resistance to glufosinate (herbicide) is under the control of the promoter rpoBdUBI10 (Ubiquitine) and NptIII antibiotics resistance gene for *Agrobacterium tumefaciens* selection.

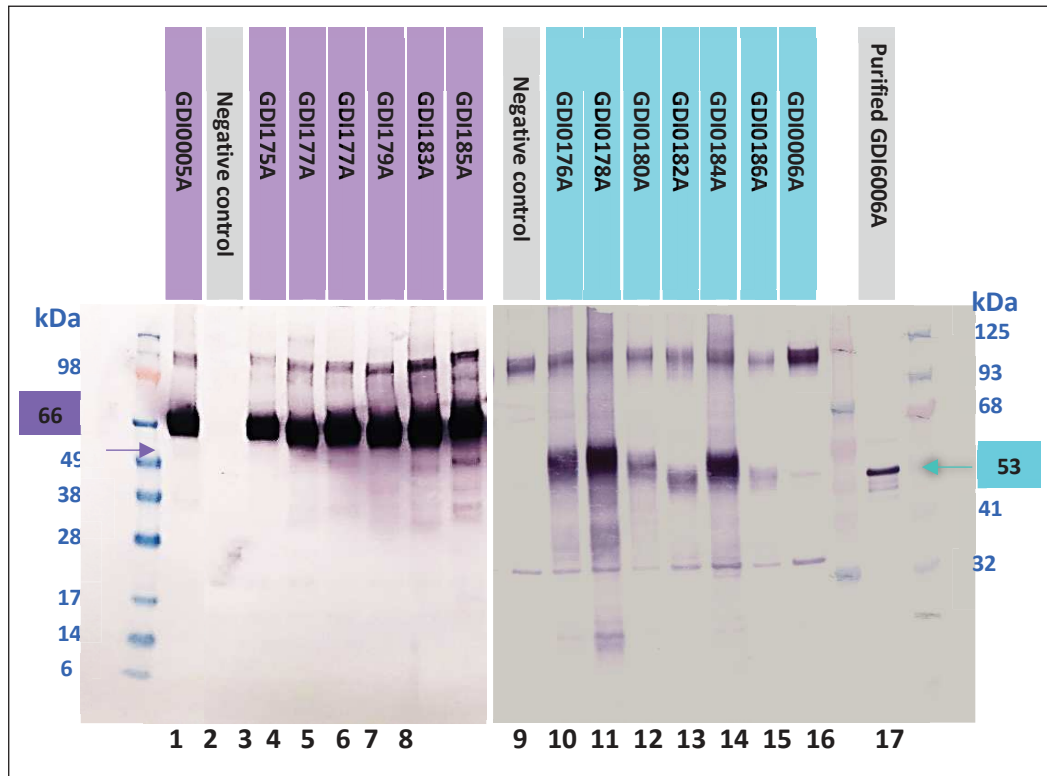


Figure 22: *Nicotiana benthamiana* leaf extracts expressing GDI0005A or its homologs (in purple) and GDI0006A or its homologs (in blue) analysis using western blot with anti-His tag antibody. Protein detection was performed by adding a chromogenic substrate for a colorimetric detection

Table 3: Features of GDI0006A constructs with target signals in different plant cell compartments

Characteristics	Cell compartment
- [proVirCsVMV_intOsActin_His_GDI0006A - G_SKL_STOP_terSbHSP] -	Peroxisome
- [proVirCsVMV_intOsActin_zera_CRWlinker_His_GDI0006A - G_STOP_terSbHSP] -	Protein body
- [proVirCsVMV_intOsActin_BAASS_His_GDI0006A- G_KDEL_STOP_terSbHSP] -	Endoplasmique reticulum
- [proVirCsVMV_intOsActin_BAASS_His_GDI0006A - G_STOP_terSbHSP] -	Apoplast
- [proVirCsVMV_intOsActin_ZmRCActp_His_GDI0006A - G_STOP_terSbHSP] -	Chloroplast
- [proVirCsVMV_intOsActin_SV40NLS_His_GDI0006A_ - G_STOP_terSbHSP] -	Nucleus

Table 4: Summary table of GDI0006A expression in different plant organelles

(+) : low protein detection
 + : acceptable protein detection
 ++ : high protein detection
 E: expected size
 NE: not-expected size

toxicity

		GDI0006A native	GDI0006A_ peroxysome	GDI0006A_ Protein body	GDI0006A_ Endoplasmic reticulum	GDI0006A_ Apoplast	GDI0006A_ Chloroplast	GDI0006A_ Nucleus
Replicate 1	Expression	(+)	+	++	-	+	(+)	-
	Size	E	NE	E	-	E	NE	-
Replicate 2	Expression	+	++	+	++	+	-	++
	Size	NE	NE	Degradation	NE	NE	-	NE
Replicate 3	Expression	++	++	-	+	++	+	(+)
	Size	NE	NE	-	NE	NE	Degradation	NE

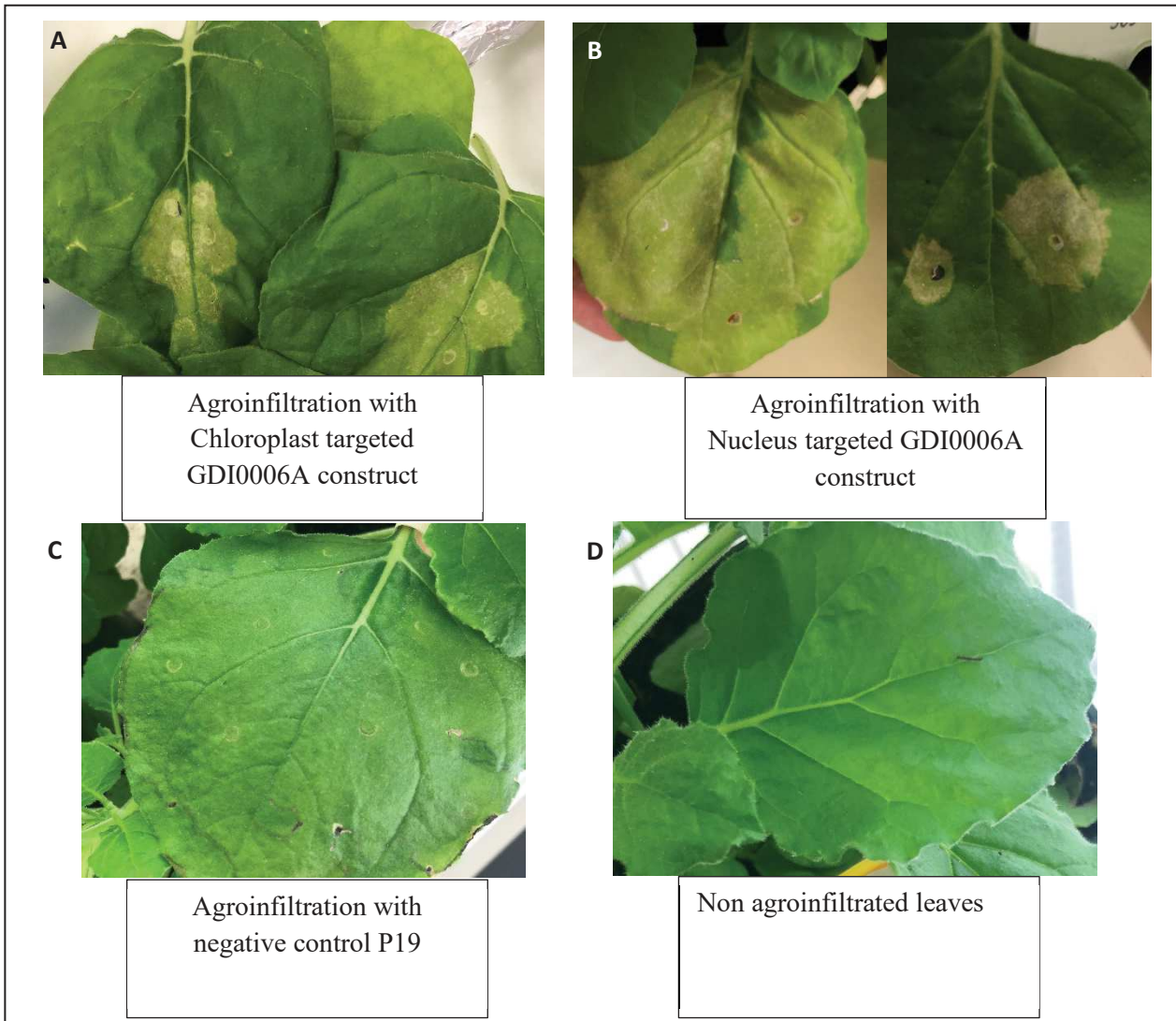


Figure 23: *Nicotiana benthamiana* Leaves observation 7 days after co-infiltration with *Agrobacterium tumefaciens* expressing P19 (protein for RNA silencing) and carrying genes coding for: A: GDI0006A constructions targeted in the Chloroplast, B: GDI0006A constructions targeted in the Nucleus. The negative control is *Nicotiana benthamiana* leaves agroinfiltrated with P19.

4.1.1 Activity of GDI0005A/GDI0006A leaf extracts

Evaluation of the activity of GDI0005A/GDI0006A proteins in tobacco leaves is performed using a crude extraction of the proteins from these leaves. We found that control extracts were toxic to WCR larvae. We hypothesized that this toxicity was due to small molecules from secondary leaf metabolism such as nicotine or terpenes. To test this hypothesis, we performed dialysis of the control samples using 2K MWCO Dialysis Cassettes. The results demonstrate that the dialysis is very effective in reducing the toxic effect contained in tobacco leaf extracts (Figure 24).

Data from Figure 24 (A) show no significant toxicity of extracts containing GDI0005A/GDI0006A proteins or homologous pairs against WCR larvae when their expression is targeted in the cytoplasm. These results were confirmed by our collaborator at CABI (Hungary) (Figure 25 (B)). Protein extracts containing GDI0005A + GDI0006A expressed in different cell compartment (peroxisome, apoplast, protein body) show an activity that is weaker than the positive control (GDI0005/GDI0006 from *E. coli* lysates) (Figure 26). These samples were selected for bioassay regarding their expression level. Because of the above-mentioned expression variability, these results must be confirmed for all replicates.

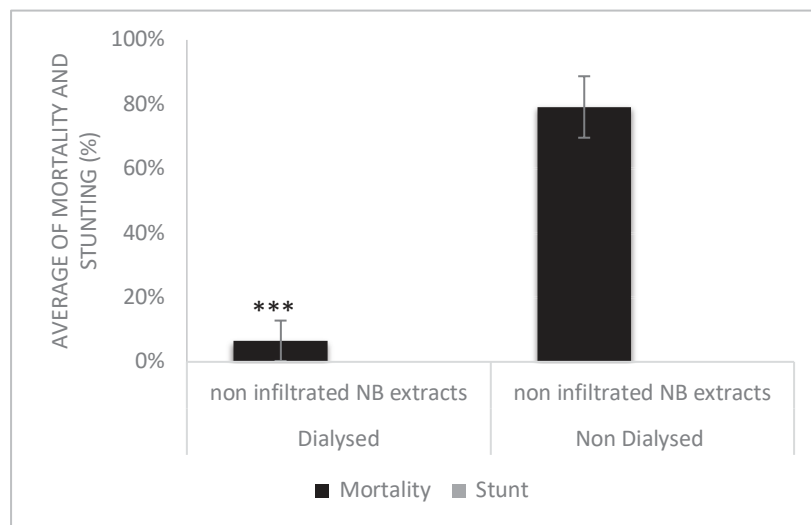


Figure 24: Activity comparison of non-dialyzed and dialyzed non infiltrated *Nicotiana benthamiana* leaves against WCR neonates (USDA ARS non-diapause susceptible colony) 5 days after infestation using artificial diet overlay bioassays. Activity was evaluated by assessing the percentage of mortality (black boxes) and the percentage of stunting (grey boxes). Each WCR larvae was exposed to ~200 μ L of WCR artificial diet overlaid with 20 μ L of leaf extracts. For each experiment, a total of 48 WCR neonate larvae were analyzed. Statistical analyses were conducted using Fisher exact test comparing both sample results (mortality and stunting). P-values of less than 0.05 were considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The error bars show the standard error of the mean (SEM)

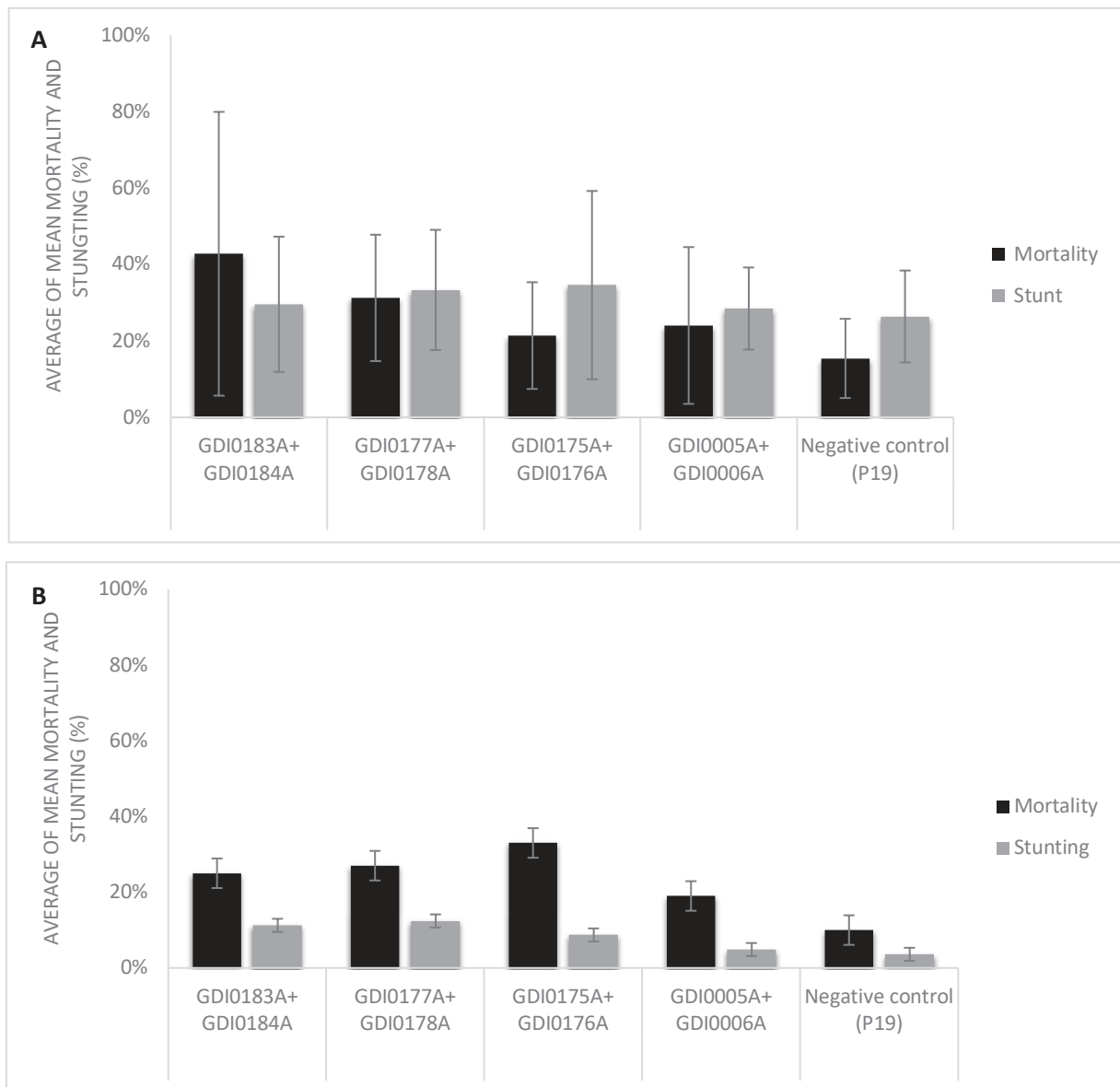


Figure 25: Evaluation of the activity of GDI0005A/ GDI0006A and 3 homologs expressed transiently in *Nicotiana benthamiana* leaves against WCR neonates (USDA ARS non-diapause susceptible colony) 5 days after infestation using artificial diet overlay bioassays and conducted in two different labs: **A) (CABI-Hungary), **B**) (Limagrain-France). Activity was evaluated by assessing the percentage of mortality (black boxes) and the percentage of stunting (grey boxes). Each WCR larvae was exposed to ~200 μ L of WCR artificial diet overlaid with 20 μ L of dialyzed total protein leaf extracts mixtures containing GDI0005A+GDI0006A. Negative control consisted of diet overlaid with dialyzed total protein leaf extracts containing the P19 protein an RNA silencing suppressor. For each experiment, a total of 48 WCR neonate larvae were analyzed. Statistical analyses were conducted using Fisher exact test comparing each sample results (mortality and stunting) to the negative control. P-values of less than 0.05 were considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The error bars show the standard error of the mean (SEM).**

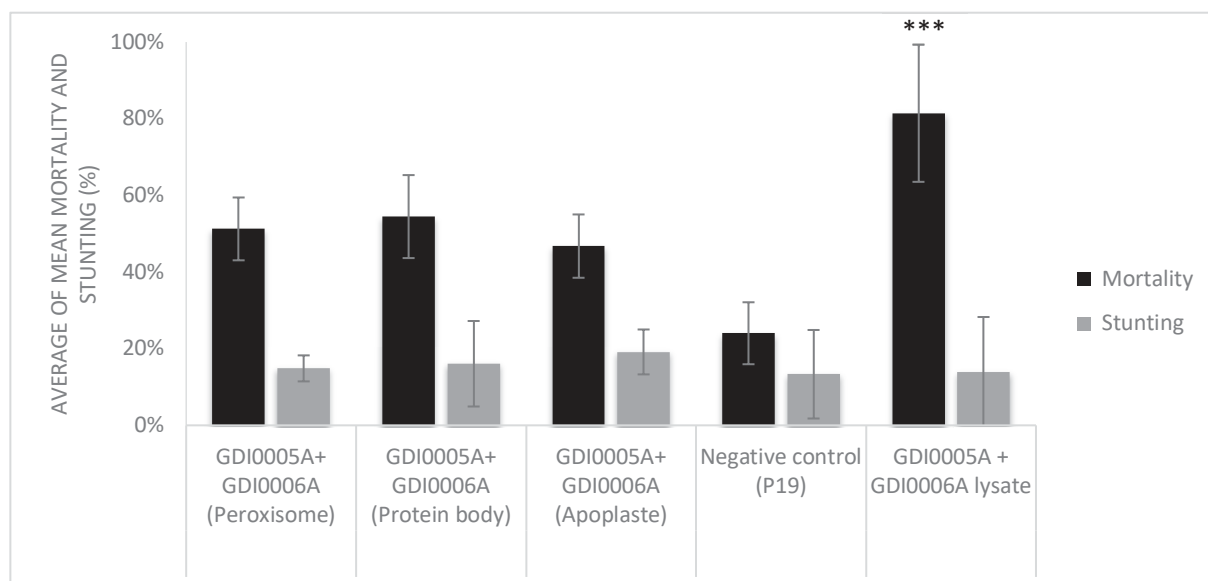


Figure 26: Activity of GDI0006A targeted constructs expressed transiently in *Nicotiana benthamiana* leaves against WCR neonates (USDA ARS non-diapause susceptible colony) 5 days after infestation using artificial diet overlay bioassays. Activity was evaluated by assessing the percentage of mortality (black boxes) and the percentage of stunting (grey boxes). Each WCR larvae was exposed to ~200 μ L of WCR artificial diet overlaid with 20 μ L of dialyzed total protein leaf extracts mixtures containing GDI0005A+GDI0006A different targeted constructs (Peroxisome, Protein body, Apoplaste). Negative control consisted of diet overlaid with dialyzed total protein leaf extracts containing P19 protein an RNA silencing suppressor. For each experiment, a total of 48 WCR neonate larvae were analyzed. Statistical analyses were conducted using Fisher exact test comparing each sample results (mortality and stunting) to the negative control. P-values of less than 0.05 were considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The error bars show the standard error of the mean (SEM).

4.2 Expression of GDI0005A/GDI0006A in maize

Transgenic maize expressing our pesticidal binary proteins was produced from immature embryos by the genetic engineering platform of Biogemma (GEP) using *Agrobacterium tumefaciens*. A binary plasmid having both His-tagged GDI0005A and GDI0006A under the control of a strong constitutive promoter CsVMV (Figure 27) was used. As a control, transgenic maize expressing the commercial Gpp34Ab1 and Tpp35Ab1 gene under the same promoter (CsVMV) as described in Figure 27 were produced. The first generation of transformed plants (T0) was checked for the level of transgene expression by RT-qPCR in both leaves and roots extracts using specific primers. The best plants exhibiting a high level of expression of both genes, were selected for insect bioassays as well as self-pollination to produce T1 plants. No phytotoxic phenotype has been noticed among all plants. Western blots with anti-His against GDI0005A from leaves and roots revealed a high level of expression for GDI0005A but the size of the protein observed on the gel is lower than expected (expected size 66 Kda). The difference in the total quantity of proteins in leaves and roots could be attributable to the level of water content of roots and leaves. Since the roots contain more water than leaves, extracted proteins in the same volume are more concentrated in leaves. Even though proteins were 10X more diluted on the root samples, GDI0005A was still detected, indicating that the protein is extensively expressed in both tissues (Figure 28 (A)). Both Gpp34Ab1 and Tpp35Ab1 were expressed at the correct molecular weight 15 kDa and 45 kDa respectively (Figure 28 (B)). T1 plants containing genes coding for GDI0005A/GDI0006A were also analyzed using specific antibodies anti-GDI0005A and anti GDI0006-like. In contrast to T0 plants, selected plants were found to exhibit high levels of GDI0005A expression at the correct molecular weight (Figure 29). GDI0006A was not detected in both T0 and T1 plants even though the gene is well expressed. It could suggest that GDI0006A is under the limit of detection by Western blot or that the translation is not so efficient.

4.2.1 Activity check of transformed GDI0005A/GDI0006A plants

Protein extracts from T0 leaves were tested for activity using diet overlay bioassay method against WCR larvae at Limagrain's lab (France) and CABI's lab (Hungary). Contradictory results were found while comparing both data. Activity of plant 1 and the trend of plant 3 seen in Limagrain's results were not confirmed at CABI's lab. Plant 2 trend have been observed at both labs. Gpp34Ab1/Tpp35Ab1 plants are not active against rootworms (Figure 30).

T1 root trainer assays have been conducted at Genective lab (Weldon). Figure 31 represents the average of rating per event. Plants with injury score from 0 to 0,5 are considered resistant. Susceptible plants score ranges between 1.5 and 3. For all T1 plants, there has been no evidence of significant resistance comparing to the negative control (Wild type). Hence, the low level of expression in those plants of GDI0006A could explain the absence of activity.

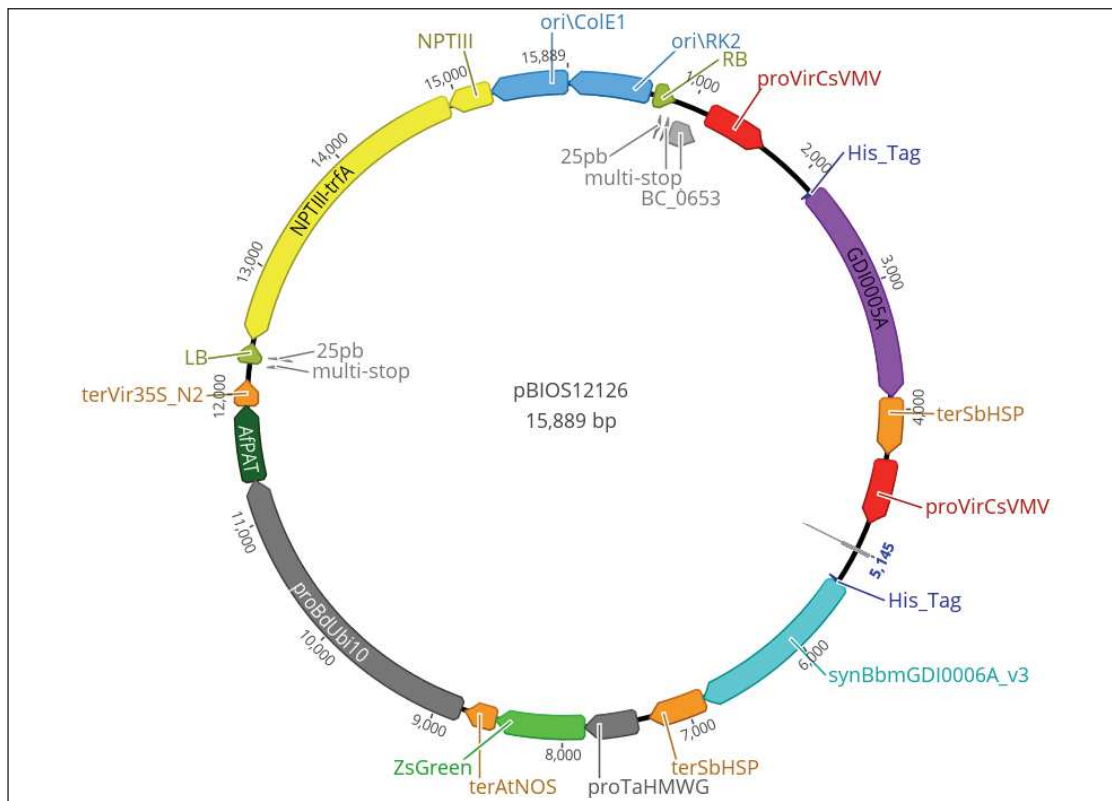


Figure 27: Schematic representation of the plasmid used in maize transgenesis for the coexpression of both GDI0005A and GDI0006A. The T-DNA region of the vector has the first gene of interest GDI0005A driven by the Cassava Vein Mosaic Virus (CsVMV), the second gene of interest GDI0006A also driven by the CsVMV promoter, ZsGreen a gene coding for green fluorescence protein, AfPAT conferring resistance to herbicides and NPTIII Kanamycin resistance gene for *Agrobacterium tumefaciens* selection. RB and LB indicate the right and the left T-DNA borders.

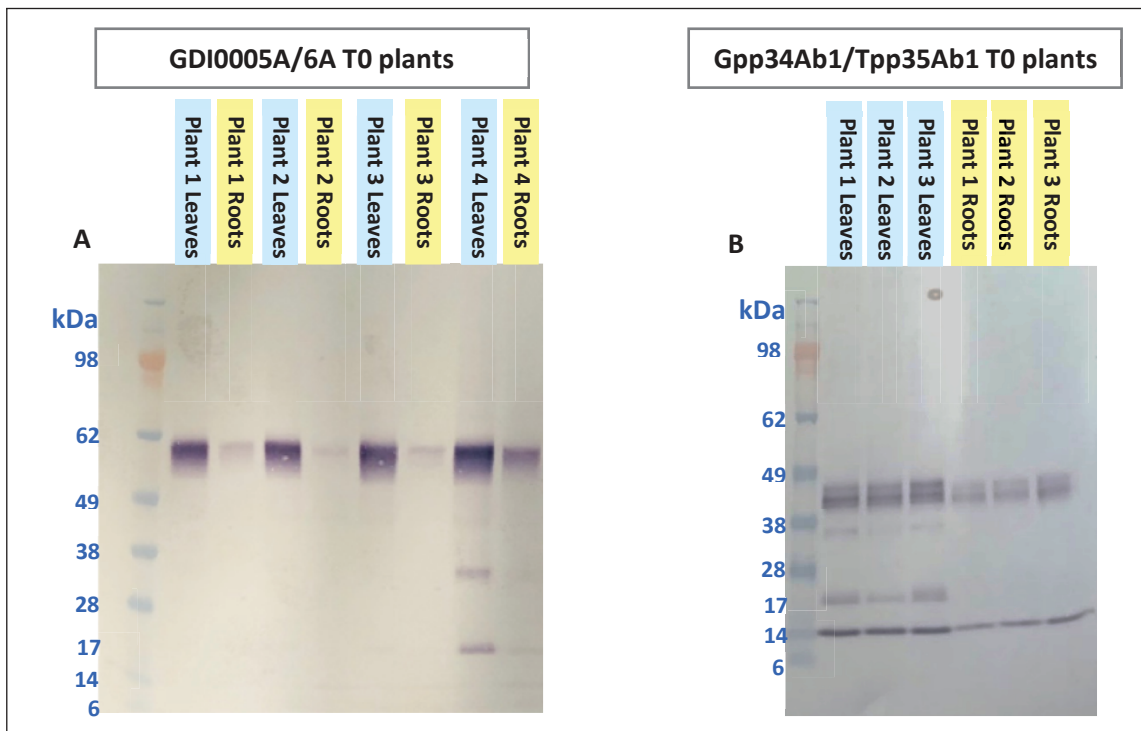


Figure 28: Maize protein extracts expression analysis using western blot with anti-His antibody. A: GDI0005/GDI0006A T0 leaves and roots total proteins extracts and B: Gpp34Ab1/Tpp35Ab1T0 leaves and roots total proteins extracts. Protein detection was performed by adding a chromogenic substrate for a colorimetric detection

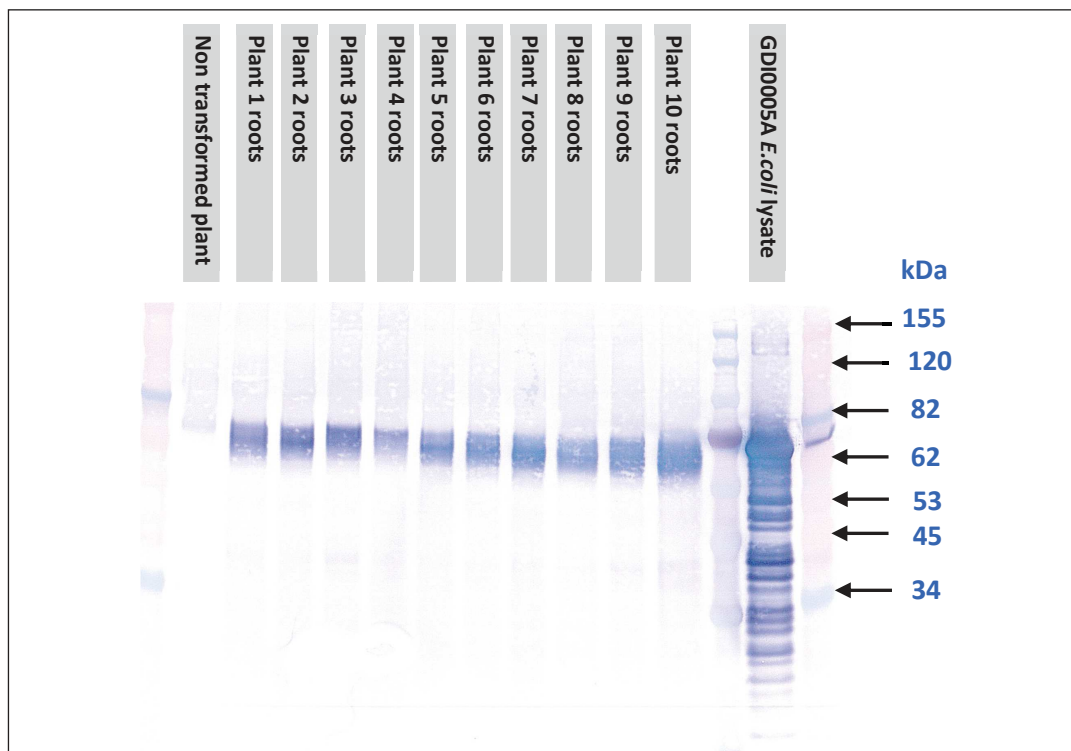


Figure 29: GDI0005A expression analysis in T1 maize plants root extracts using western blot (with anti-GDI0005A antibody). Protein detection was performed by adding a chromogenic substrate for a colorimetric detection.

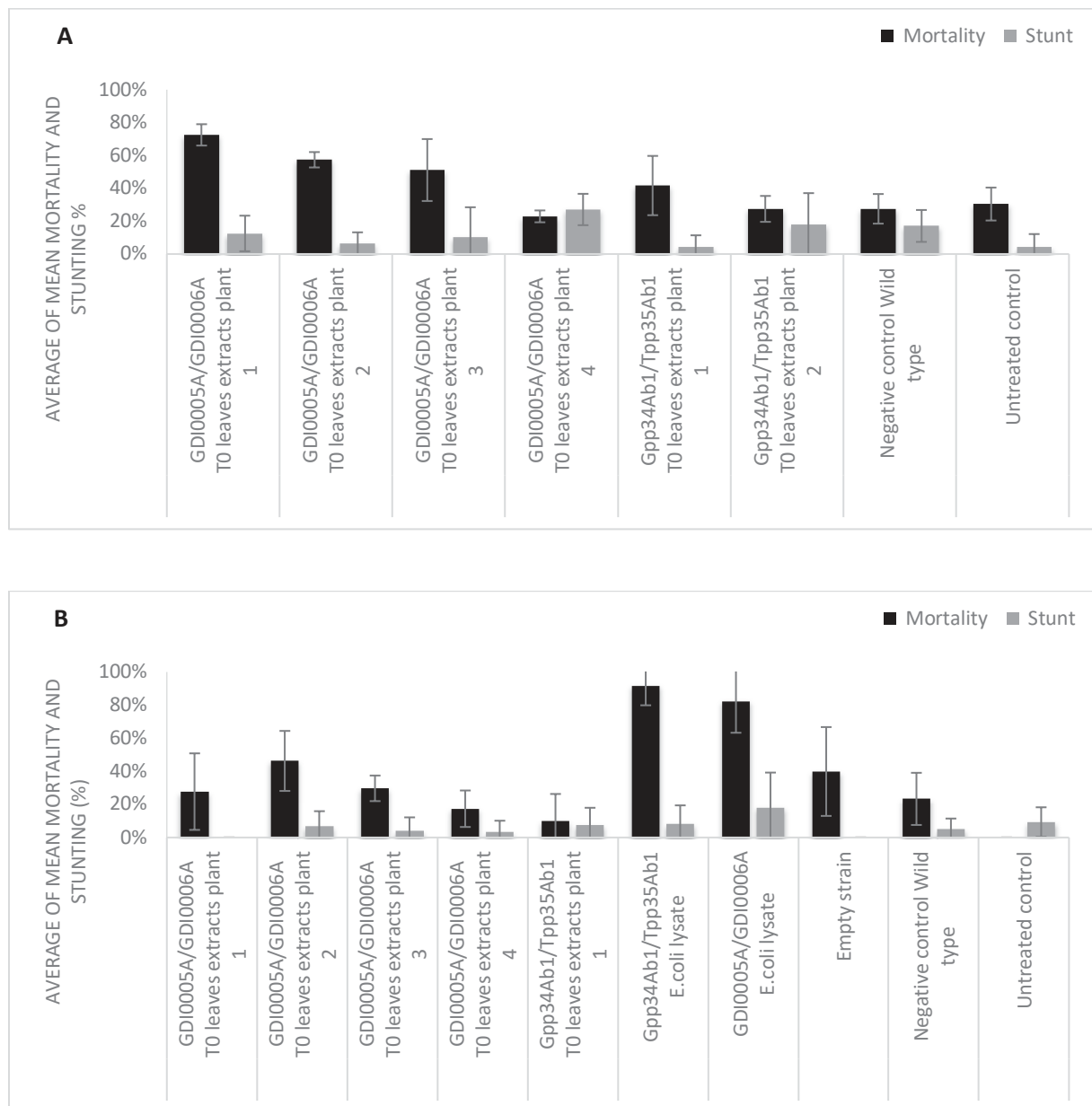


Figure 30: Evaluation of the activity of GDI0005A/ GDI0006A expressed in T0 maize plants against WCR neonates (USDA ARS non-diapause susceptible colony) 5 days after infestation using artificial diet overlay bioassays and conducted in two different labs: A (CABI-Hungary), B (Limagrains-France). Activity was evaluated by assessing the percentage of mortality (black boxes) and the percentage of stunting (grey boxes). Each WCR larvae was exposed to ~200 μ L of WCR artificial diet overlaid with 20 μ L of dialyzed total protein leaf extracts containing GDI0005A/GDI0006A. Negative control consisted of diet overlaid with total protein leaf extracts from the wild type plants. Positive controls consisted of diet overlaid with total protein leaf extracts from T0 Gpp34Ab1/Tpp35Ab1 plants, Gpp34Ab1/Tpp35Ab1 *E. coli* lysate and GDI0005A/GDI0006A mixed *E. coli* lysates. For each experiment, a total of 48 WCR neonate larvae were analyzed. Statistical analyses were conducted using Fisher exact test comparing each sample results (mortality and stunting) to the negative control. P-values of less than 0.05 were considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The error bars show the standard error of the mean (SEM).

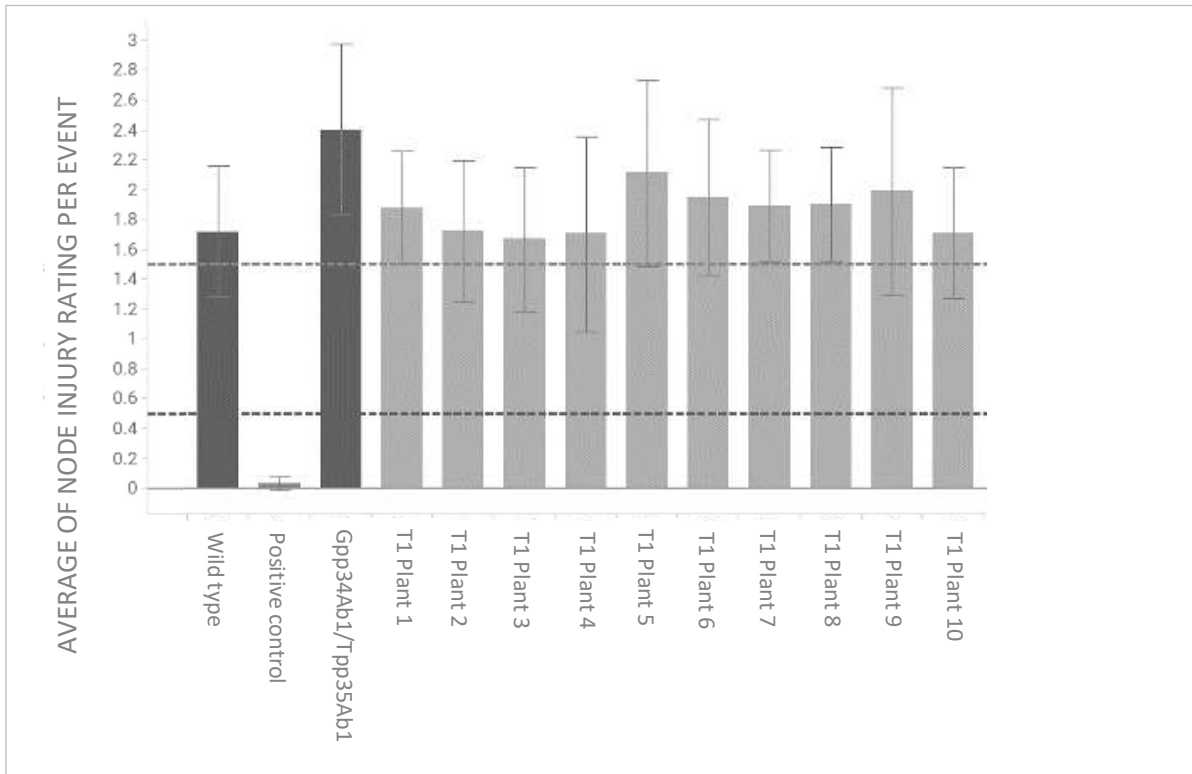


Figure 31: GDI0005A/GDI0006A T1 plants resistance evaluation against WCR neonates (USDA ARS non-diapause susceptible colony) using a two week root trainer assay test. Injury from corn rootworm larval feeding was visually assessed using the Iowa State 0–3 Node-Injury Scale (Oleson et al., 2005). The wild type was used as a negative control. Plants with injury score less than 0,5 are considered resistant. Susceptible plants score ranges between 1.5 and 3.

5 Discussion and perspectives

Due to its adaptability to pest management measures, the Western Corn Rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte) is one of the most economically important maize pests (Early et al., 2018; Adhikari et al., 2020; De Groote et al., 2020; FAO, 2020). Up to now, commercial pesticidal proteins, used either as biopesticides or in transgenic maize traits, are mostly derived from a unique bacteria *Bacillus thuringiensis* (Bt). The specificity of Bt pesticidal proteins, as well as their high efficacy in both biological and transgenic strategies, have contributed to their success. As could be expected, overuse of Bt proteins has led systematically to the emergence of resistant populations (Gassmann, 2021). Thus, the focus of recent research has been on investigating the microbial world to find new potential biological agents carrying insecticidal proteins that could be deployed in hybrid maize.

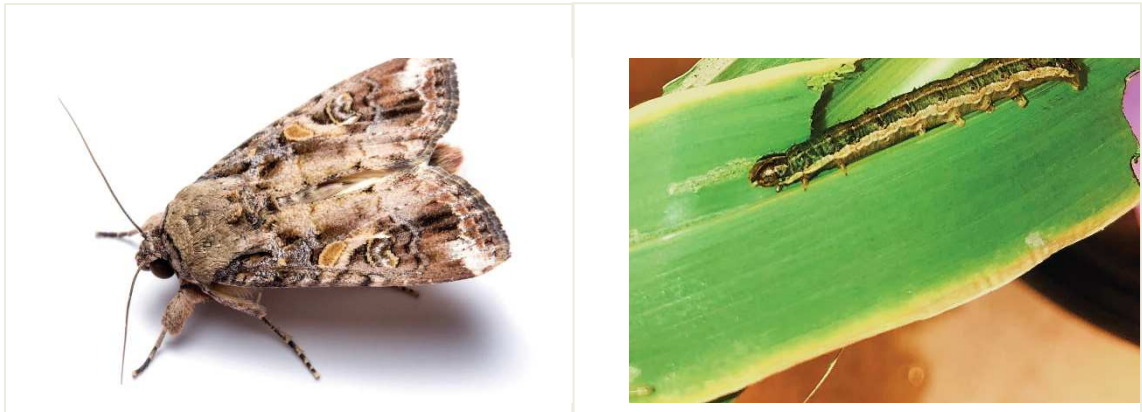
In this study, we describe the identification of a group of a two component pesticidal proteins from *Chryseobacterium* genus active against rootworms and having similarities to a large protein family first identified by (Gruver et al., 2016). Most of the active proteins claimed in this patent were found in gram negative bacteria such as *Pseudomonas*, *Burkholderia* and *Parococcus*. While tested against WCR neonate resistant strains to the commercially deployed proteins Cry3Bb1 and Gpp34Ab1/Tpp35Ab1, our proteins called GDI0005A and GDI0006A induced a significant weight loss on both larvae strains. This may suggest a new mode of action that could complement existing trait genes. Further competition experiments are needed to check if GDI0005A/ GDI0006A share binding sites with commercial proteins. Sequence analysis and motifs prediction have only revealed a presence of type II lipoprotein N-terminal signal peptide and a conserved cysteine (Cys) residue for GDI0006A and its similar sequences. After signal peptide cleavage by signal peptidase, proteins undergo additional posttranslational modification, such as the insertion of a fatty acid to the amino-terminal diacylglyceryl-Cys residue, to anchor the ‘mature proteins’ to the outer membrane of the original bacteria (Braun and Hantke, 1974; Nakayama et al., 2012; Sankaran and Wu, 1995). This could explain the purification difficulties seen along the process for these proteins. n-Dodecyl β -D-maltoside (DDM), a non-ionic gentle detergent known to enhance membrane proteins stability (Rouse et al., 2013) have improved GDI0006A solubilization and helped its purification. Despite the unexpected size after purification, the 40 kDa protein was active when tested with the purified GDI0005A against WCR. This could indicate that this protein represents the active form of GDI0006A. Further tests are needed to optimize the GDI0006A purification and ensure a better yield for LC50 studies to determine its level of activity when tested with GDI0005A. MALDI-

TOF analysis could be useful for further protein characterization. Despite its role in outer membrane stabilization and assembly, lipoproteins play a key role in host-pathogen interaction for immune response activation and virulence (Chimalapati et al., 2012; Machata et al., 2008). Many studies have been focusing on bacterial lipoproteins and their roles mainly for clinical research using human and animal pathogens (Elsner et al., 2002; Fletcher et al., 2004). No evidence of lipoprotein with insecticidal activities has yet been described.

The first trials to express GDI0006A *in planta* were not a success. Difficulties have been observed with both stable and transient expression. Expressing foreign proteins in a plant system remains challenging to ensure sufficient high-level expression for insect control. Codon optimization is required for better protein expression in plants (Watts et al., 2021). Since the codons were already engineered to bring it more into line with plant-preferred codon usage, we decided to target GDI0006A in different cellular organelles (chloroplast, apoplast, protein body, nucleus, peroxisome). Many researches have demonstrated that addressing the protein of interest in different cell compartments could enhance its accumulation in the plant (Dai et al., 2000; Conley et al., 2011; Shen et al., 2017). However, with GDI0006A this approach was unsuccessful. In addition, a phytotoxicity was observed when the protein was targeted in the nucleus or in the chloroplast. Unstable mRNA, non-efficient translation or rapid protein degradation are possible hypothesis to explain the low level of GDI0006A accumulation *in planta* (Watts et al., 2021). The low level of GDI0006A expression in both maize and *N. benthamiana* of GDI0006A could explain the absence of activity. Thus, overcoming the limitation linked to GDI0006A insufficient production level *in planta* remains critical. Otherwise, the aim of using it in association with GDI0005A for a biotech approach to control WCR larvae will be impossible to achieve.

We have shown that *Chryseobacterium* strains carrying GDI0005A and GDI0006A have an activity against WCR larvae. Additional components in *Chryseobacterium* could be involved in this insecticidal activity with or without the two proteins GDI0005A and GDI0006A. To demonstrate a correlation between *Chryseobacterium arthrospiraerae* activity and our insecticidal proteins, further investigation needs to be undertaken. The role of GDI0005A and GDI0006A could be investigated by knocking out GDI0005A or GDI0006A genes. In addition, coimmunoprecipitation and yeast two-hybrid experiments could be considered to explore the potential interaction between the two proteins. This may help us to optimize their expression in maize plants.

**CHAPTER 2: Assessment of the potential role
of the *Junonia coenia* densovirus (JcDV) to
enhance bacterial insecticidal proteins potency**



1 Introduction

Densoviruses are orally transmitted viruses, for which the midgut is the first gate to enter the organism. The work of Wang et al., (2013) has contributed to the understanding of the molecular mechanisms that allow JcDV, a densovirus that infects FAW, to cross the insect gut epithelium. In particular, they have demonstrated that the passage of this virus disrupts the permeability of the gut. Thus, it would be interesting to evaluate the potentiality of this densovirus to enhance the toxicity of gut binding insecticidal proteins, such as Bt insecticidal proteins.

As previously mentioned, JcDV genome encodes three non-structural proteins (NS1, NS2 and NS3) and four structural proteins, the capsid proteins (VP1, VP2, VP3 and VP4) (Bossin et al., 2003; Bruemmer et al., 2005). VP4 is the major capsid protein and constitutes the surface of the virus particle. VP4 proteins are able to self-assemble into VLPs when expressed in a recombinant baculovirus system (Bruemmer et al., 2005; Croizier et al., 2000). Besides, they are also capable to pass through the midgut and can be used to vectorize molecules. Indeed, it was shown that insecticidal proteins expressed as fusion proteins with VP4 were unable to produce VLPs but could be delivered from the midgut to the insect hemocoel (Kemmerer and Bonning, 2018; Bonning and Kemmerer, 2020). This recent discovery has paved the way for the study of possible interactions between VP4 and gut binding insecticidal proteins while expressed individually in plants.

VLP production has been widely explored especially for medical applications. VLPs can be obtained using many different expression systems (Nooraei et al., 2021) including bacteria (Huang et al., 2017), yeast (Saraswat et al., 2016), baculovirus/insect cells (Airenne et al., 2013), mammalian cells (Ren et al., 1997) and plants (Charland, 2012). The correct folding and assembly of VLP is complex and highly reliant on the structure of the viral protein, the expression system and the experimental conditions (pH, temperature...) (Le and Müller, 2021; Nooraei et al., 2021). Hence, the interest of evaluating the production of VLPs JcDV VP4 in a plant expression system since it is safer to use VLPs that do not contain the whole virus genome either for biocontrol or transgenic approach.

In this chapter, we aim to study the potential synergy between JcDV and *Bacillus thuringiensis* Cry2Ab2 protein since both affect the FAW midgut permeability (Pardo-López et al., 2013; Wang et al., 2013b). This study will shed the light to potential of using the combination of entomopathogenic densoviruses with bacterial insecticidal proteins in a biocontrol program.

We also intend to express VP4 in a plant expression system and check its ability to self-assemble into VLPs that are able to enter and cross the midgut epithelium barrier similarly to JcDV. This proof of concept will explore the possible synergies between bacterial insecticidal proteins and VLPs through a transgenic approach.

2 Evaluation of the potential interaction between JcDV and Cry protein

2.1 Cry protein purification and activation

Cry2Ab2, known for its activity against FAW, was used to perform bioassays and *ex vivo* experiments with the Ussing chamber system. To conduct experiments, the Cry2Ab2 protoxin (71 kDa) was fused to a His-MBP tag (45 kDa) and expressed in an *E. coli* system. The purification was performed in the laboratory of Renaud Vincentelli. As previously discussed, Cry proteins initially produced as insoluble protoxins need to be solubilized and activated by the midgut enzymes. Thus, prior to their use for *ex vivo* experiments, purified Cry2Ab2 was activated using both trypsin and FAW midgut juice obtained as described in (Liu et al., 2020). In both cases we obtained a doublet band of ~55 kDa and ~45 kDa (Figure 32). To be in the same physiological conditions, we decided to activate Cry2Ab2 with the midgut juice for the following experiments.

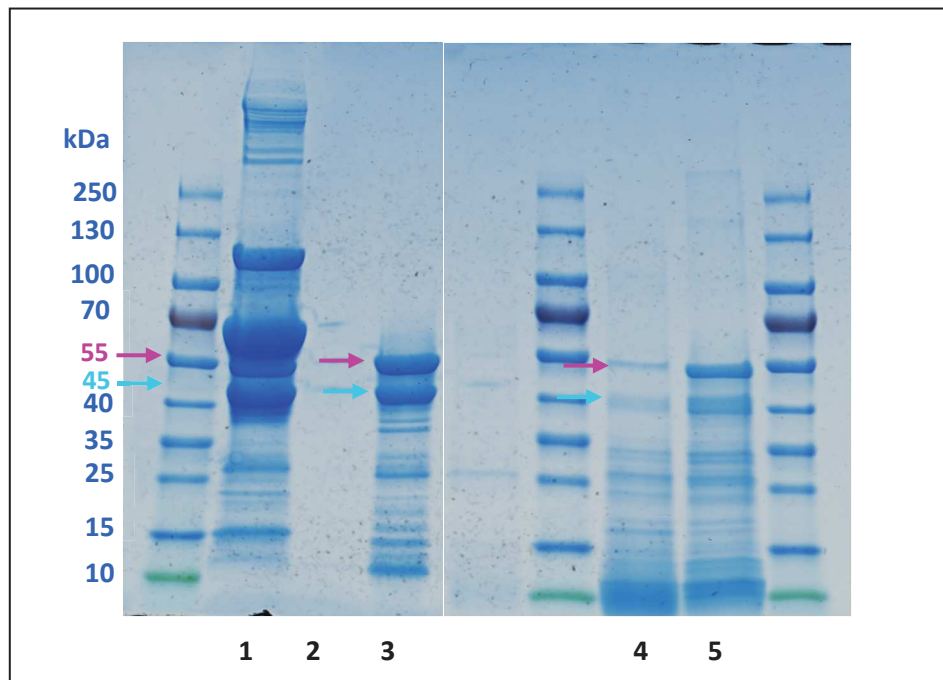


Figure 32: Cry2Ab2 activation comparison using trypsin and FAW midgut juice. 1: purified His-MBP_Cry2Ab2, 2: negative control, 3: purified His-MBP_Cry2Ab2 activated with trypsin, 4: purified His-MBP_Cry2Ab2 activated with FAW midgut juice 10x dilution, 5: purified His-MBP_Cry2Ab2 activated with FAW midgut juice 5x dilution. Blue arrows indicate the band corresponding to 45 kDa, Pink arrows correspond to the band with a molecular weight of 55 kDa

2.2 Lethal median dose (LD50) determination

Prior to performing bioassays with the mixtures of Cry2Ab2 and JcDV, LD₅₀ of both Cry2Ab2 and JcDV had to be determined. Sublethal doses were used for bioassays to compare the effects of JcDV and Cry2Ab2 against FAW neonates when tested separately and in mixture.

For LD₅₀ experiments, different doses of Cry2Ab2 and JcDV were tested individually against FAW neonates. According to the dose-response analysis, LD₅₀ were estimated as 793 ng/cm² for Cry2Ab2 and 3,72 10⁸ virus equivalent genome (veg) per cm² for JcDV (Figure 33).

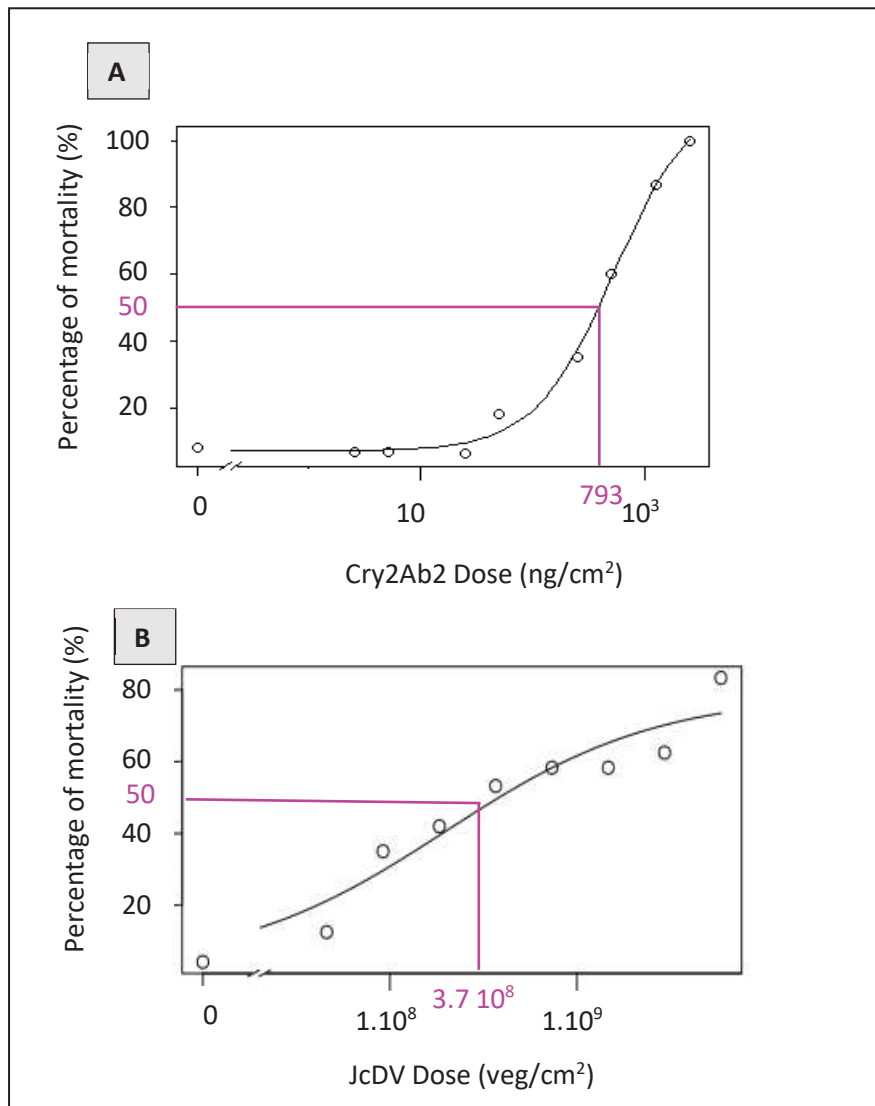


Figure 33: FAW neonates dose response curves when orally exposed to (A) Cry2Ab2 over 5 days and (B) JcDV over 8 days. Experiments were conducted using artificial diet overlay bioassay. Each FAW larvae was exposed to ~200 μ L of FAW artificial diet overlaid with 20 μ L of samples: Cry2Ab2 (Different doses) JcDV (different doses). For each experiment, a total of 48 FAW neonates were analyzed. Dose-response analysis were performed using R studio software version 4.1.2.

2.3 Effects of JcDV and Cry2Ab2 mixture on the FAW midgut permeability

Since both JcDV and Cry2Ab2 disturb separately the FAW midgut permeability, we evaluated their effects when combined. To do this, we tested the passage of small fluorescent dextrans (4 kDa-FITC Dextran) in the presence of Cry2Ab2, JcDV or the mixture of both (JcDV + Cry2Ab2) using the Ussing chamber. This apparatus consists of 2 compartments separating the midgut from the luminal and basal sides. Both compartments contain physiological solutions to keep the tissue alive (see material and methods section). Cry2Ab2 (0.78 mg/mL) and JcDV ($1.35 \cdot 10^9$ veg) with 0.2 mg/mL of the 4 kDa-FITC Dextran were added at the same time to the luminal compartment. When tested alone, JcDV and Cry allowed passage of dextrans through the midgut epithelium 30 min after start of experiments. By contrast, when the two were put together in the luminal compartment, dextrans were detected in the basal compartment only after 1 hour (Figure 34). These results suggest that the presence of both JcDV and Cry2Ab2 together tends to delay the permeabilization of the midgut. More replicates are needed to evaluate the significance of these results.

2.4 Cry2Ab2 effects on the passage of JcDV

We also studied the passage of JcDV through the intestine in the presence of Cry2Ab2 using Ussing chamber. The number of viruses was quantified by qPCR to estimate the viral flow in the basal compartment. After 1-hour incubation, the viral flow seems to be much higher ($3.6 \cdot 10^7$ veg/min/mm²) when JcDV was tested alone comparing to the mixture (JcDV + Cry2Ab2) where no virus seems to pass. JcDV flow seems to be slowly initiated ($8.8 \cdot 10^5$ veg/min/mm²) 2 hours after incubation with the mixture (JcDV + Cry2Ab2) while it appears to have stopped with JcDV only by 2 hours (Figure 35). These results suggest that the presence of Cry2Ab2 impedes and delays the passage of the virus through the midgut.

2.5 Effects of JcDV + Cry2Ab2 mixture on FAW larvae survival

Evaluation of the activity of JcDV + Cry2Ab2 mixture was performed using diet overlay bioassay. The JcDV LD₅₀ ($3.72 \cdot 10^8$ veg/cm²) was mixed with a lower sublethal dose of Cry2Ab2 (25.45 ng/cm²). Data from Figure 36 show that JcDV alone was significantly active against FAW neonates. The presence of Cry2Ab2 in the mixture seems to inhibit the FAW mortality induced by JcDV. These results confirmed the preliminary results from *ex vivo* experiments (Figures 34 and 35) suggesting an antagonism between JcDV and Cry2Ab2.

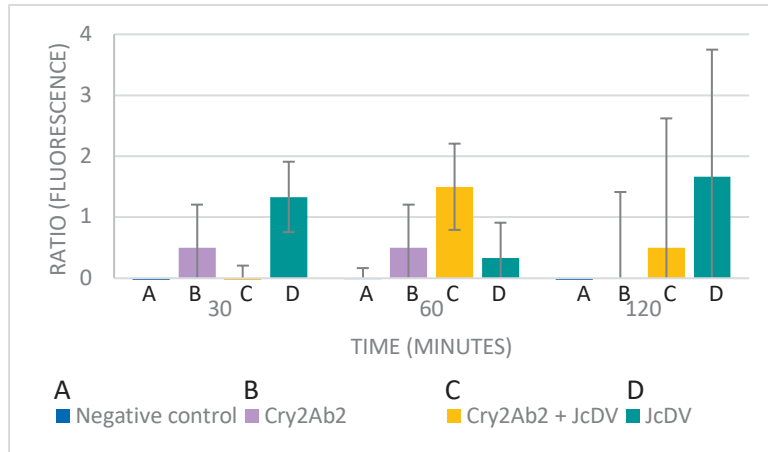


Figure 34: Evaluation of the midgut permeability in the presence of both JcDV and Cry2Ab2. 6 instar FAW midguts were incubated 2 hours with the samples containing (0.2 mg/mL) 4kDA-FITC Dextran: the yellow boxes with C letter are samples containing the mixture (0.78 mg/mL) of the active Cry2Ab2 (with the midgut juice) + (1.35 10^9 veg) JcDV, the green boxes with D letter contain (1.35 10^9 veg) JcDV alone + midgut juice, the purple boxes with B letter contain (0.78 mg/mL) of the active Cry2Ab2 alone with midgut juice. The negative control (midgut juice only) is represented by the blue boxes with A letter. Sampling was made at 30 min, 60 min and 120 min in both compartments. The fluorescence was measured at 520 nm. The results are shown as a ratio (Fluorescence of the basal compartment / total fluorescence (basal + luminal)). Two replicates were carried out, and the significance was determined using the Kruskal-Wallis test.

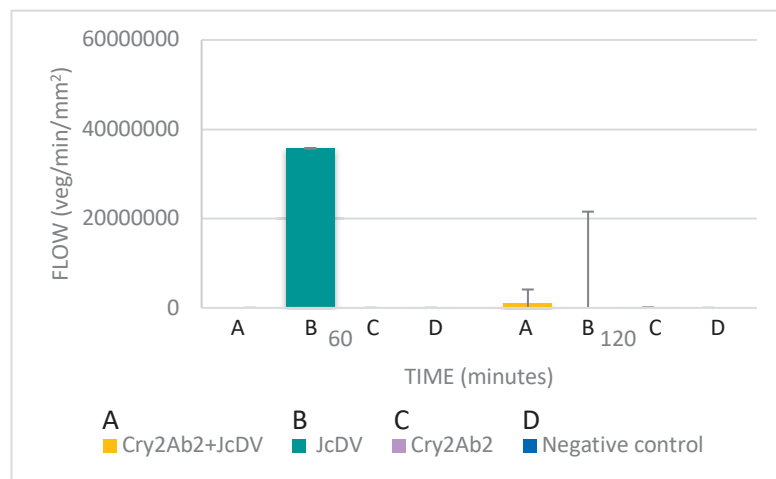


Figure 35: Evaluation of JcDV flow in the presence/ absence of Cry2Ab2. The virus number in the basal compartment was determined by qPCR. The flow was calculated based on the following formula: (virus number/(time*tissue area)). Two replicates were carried out, and the significance was determined using the Kruskal-Wallis test. The yellow boxes with C letter are samples containing the mixture (0.78 mg/mL) of the active Cry2Ab2 (with the midgut juice) + (1.35 10^9 veg) JcDV, the green boxes with D letter contain (1.35 10^9 veg) JcDV alone + midgut juice, the purple boxes with B letter contain (0.78 mg/mL) of the active Cry2Ab2 alone with midgut juice. The negative control (midgut juice only) is represented by the blue boxes with A letter.

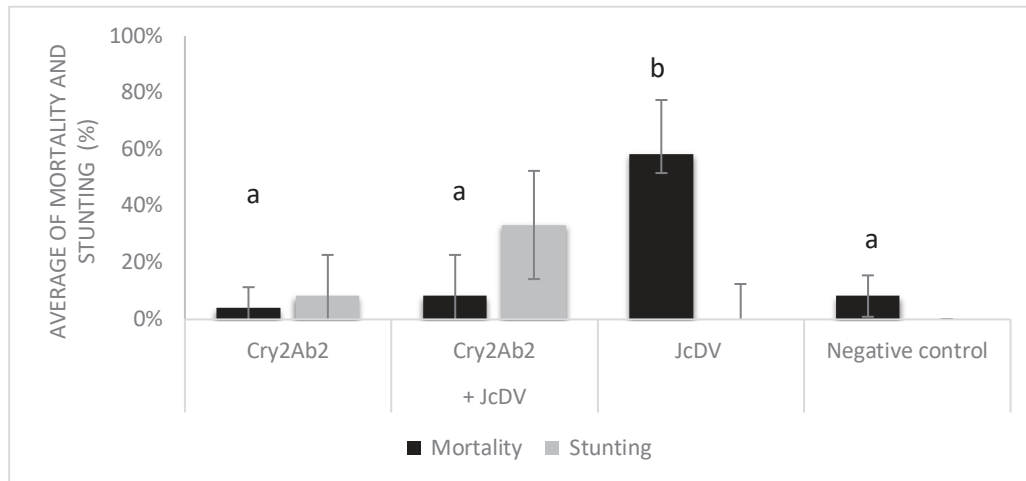


Figure 36: Evaluation of the activity of JcDV + Cry2Ab2 in mixture against FAW neonates 8 days after infestation using artificial diet overlay bioassays. Activity was evaluated by assessing the percentage of mortality (black boxes) and the percentage of stunting (grey boxes). Each FAW larvae was exposed to ~200 μL of FAW artificial diet overlaid with 20 μL of samples: Cry2Ab2 Cry2Ab2 (25.45 ng/cm^2), Cry2Ab2 (25.45 ng/cm^2) + JcDV LD50 ($1.23 \cdot 10^8$ veg), JcDV ($1.23 \cdot 10^8$ veg). The negative control consisted of diet overlaid PBS buffer. For each experiment, a total of 48 FAW neonates were analyzed. Statistical analyses were conducted using Tukey HSD test comparing both stunting and mortality in all samples. The error bars show the standard error of the mean (SEM).

3 Evaluation of VP4 expression *in planta*

3.1 Expression of VP4 in Tobacco transient expression system

The objective is to evaluate the possibility of expressing VP4 proteins and their capability to self-assemble into VLPs in a plant expression system. We have tested the agroinfiltration approach to transiently produce this protein in *Nicotiana benthamiana* (NB) leaves under the control of the CsVMV promoter as previously represented in (Figure 21). Three different constructs were generated: C1 (VP4 only), C2 (VP4 with His tag) and C3 (VP4 fused to eGFP) protein. Molecular constructions and transformation into *Agrobacterium* strains were done by the Limagrain's TEV laboratory headed by Laurent Beuf. By 7 days after infiltration of NB leaves with *Agrobacterium tumefaciens* carrying VP4 with the different constructions, total proteins were extracted, and samples were used to conduct Western blots, Ussing chamber and microscopy analysis. A 3 mm^2 leaf sections expressing VP4 were prefixed with paraformaldehyde for transmission electron microscopy. No signs of phytotoxicity were observed 7 days post-infiltration suggesting that VP4 is not toxic for NB.

3.1.1 VLP-VP4 detection in NB leaf extracts

Different experiments were conducted to verify the expression of VP4 in NB leaves and its self-assembly into VLP. The Western blot analysis using an anti-JcDV antibody in Figure

37 (A+B), showed that VP4 is well expressed at the correct size (around 47 kDa) with the 3 constructs in both replicates. The eGFP protein seems to be expressed at the correct size (26 kDa) in the SDS-page gel (Figure 37A).

The negative staining on C1 leaf extract and observation with transmission electron microscope (TEM) revealed the presence of pseudo-particles of around 25 nm (Figure 38 A&C) similar to those formed by VP4 when expressed in a baculovirus expression system (Figure 38B). These results demonstrated that VP4 could self-assemble in a plant expression system.

3.1.2 VLP-VP4 localization in NB leaf sections

VP4 was first immunolocalized in NB leaf thin sections (200 μm) using an anti-JcDV primary antibody and an Alexa fluorTM 488 secondary antibody. The confocal images revealed a high concentration of VP4 around structures similar to the chloroplasts as well as inside/around nucleus (Figure 39).

Ultra-thin NB leaf sections expressing VP4 (C1 construct) were observed with TEM after positive staining. Structures having a similar shape and size to VLP-VP4 (around 25 nm) were localized near the chloroplasts (Figure 40). An immunogold staining using an anti-JcDV antibody may be used to confirm that these structures are indeed VLP-VP4.

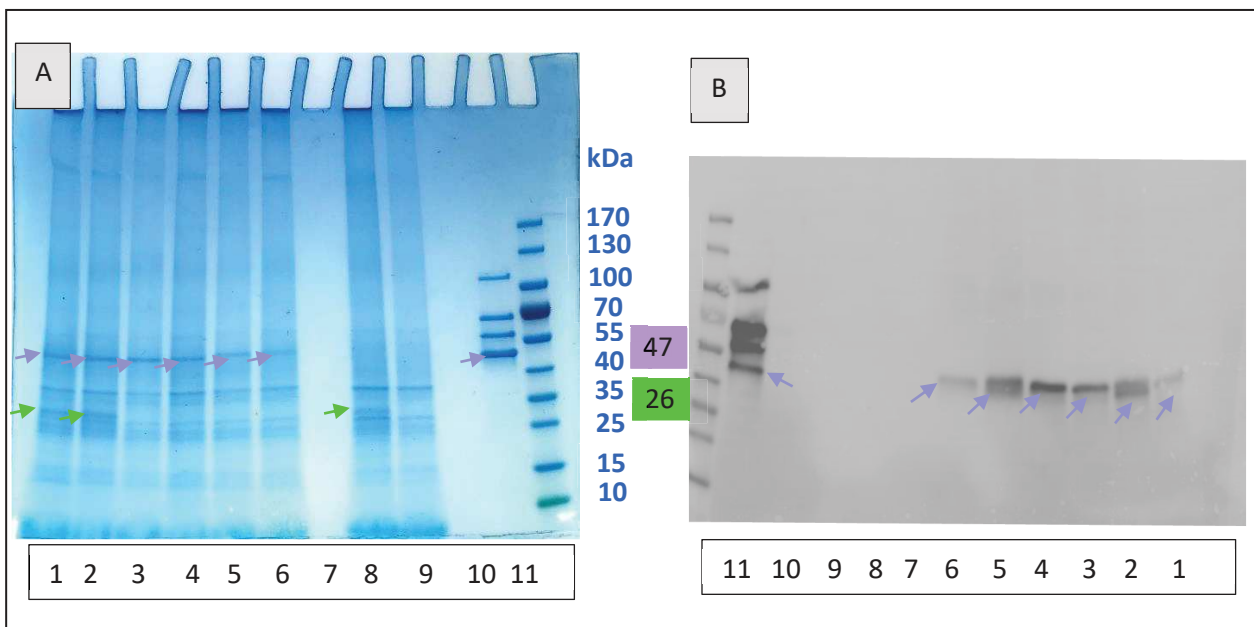


Figure 37: *Nicotiana benthamiana* leaf extracts expressing VP4 analysis using western blot with anti-JcDV antibody. Proteins were revealed by enhanced chemiluminescence (Millipore, Burlington, MA, USA) using a Chemidoc imager (Biorad). Lanes 1&2: C3 construct (VP4+eGFP) 2 biological replicates, lanes 3&4: C2 construct (His_VP4) 2 biological replicates, lanes 5&6: C1 construct (VP4 only) 2 biological replicates, lanes 7&10: PBS buffer, lane 8: eGFP, lane 9: P19 (gene silencing), lane 11: purified JcDV expressing the 4 capsid proteins (VP1,VP2, VP3, VP4).

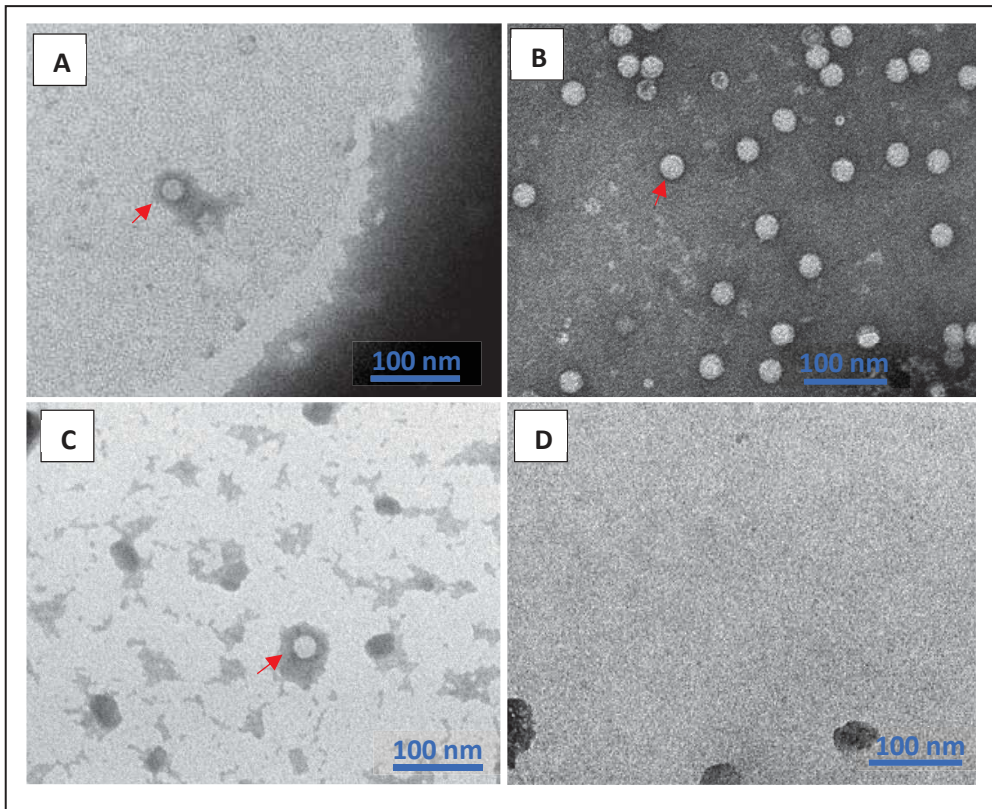


Figure 38: VP4 pseudo-particles formation in NB leaves (A&C) and in baculovirus system (B) observed with electron transmission microscopy after negative staining. D: negative control NB leaf extracts containing P19: gene silencing. A&C: NB leaf extracts containing, Red arrows for VLPs

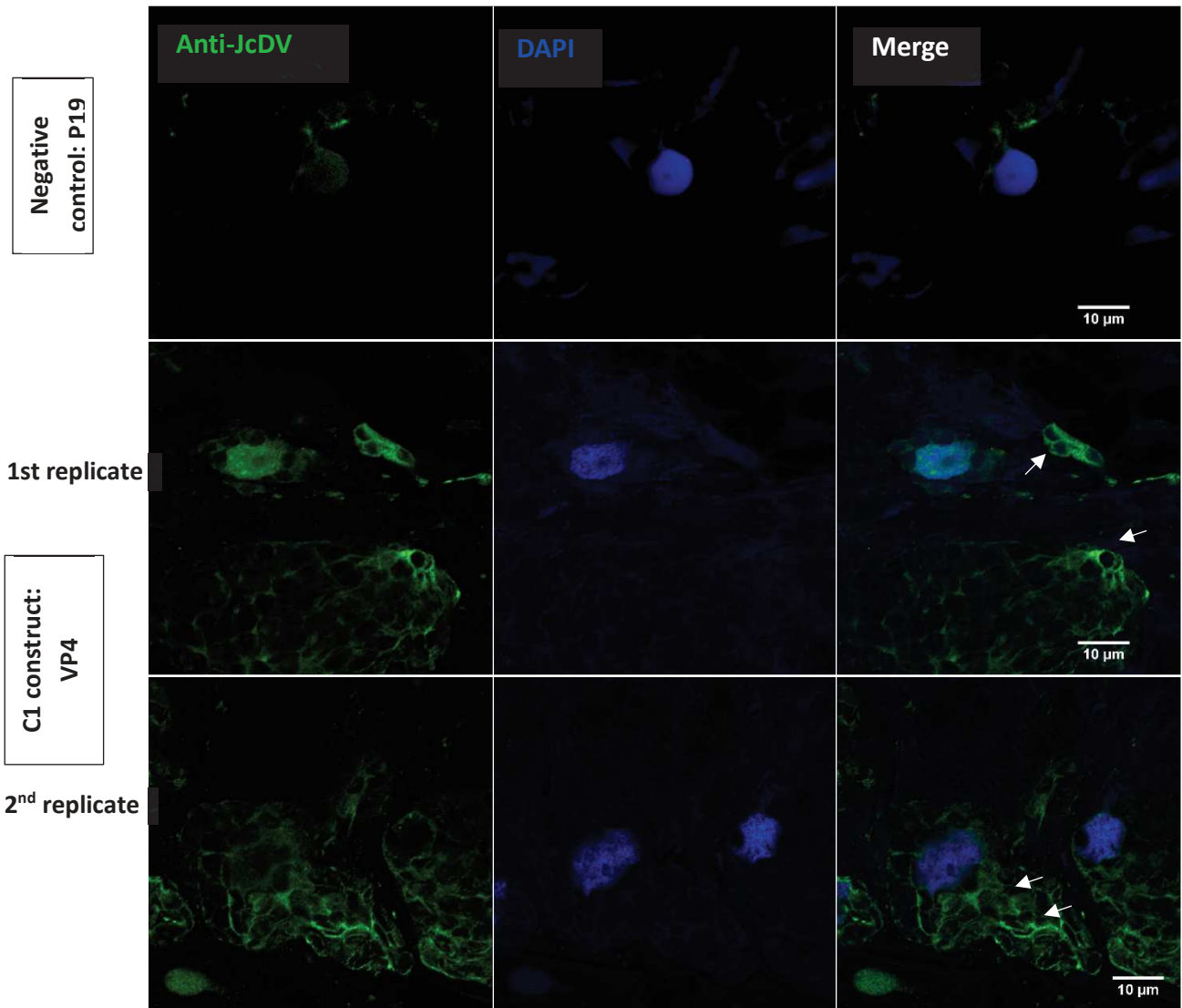


Figure 39: VP4 detection in NB leaf sections visualized with confocal microscopy after immunolabeling. DAPI is used to label cells nuclei. Alexa fluor 488 anti-rabbit secondary antibody was used to label JcDV. White arrows indicate the eventual position of Chloroplasts. Bars, 10 μ m.

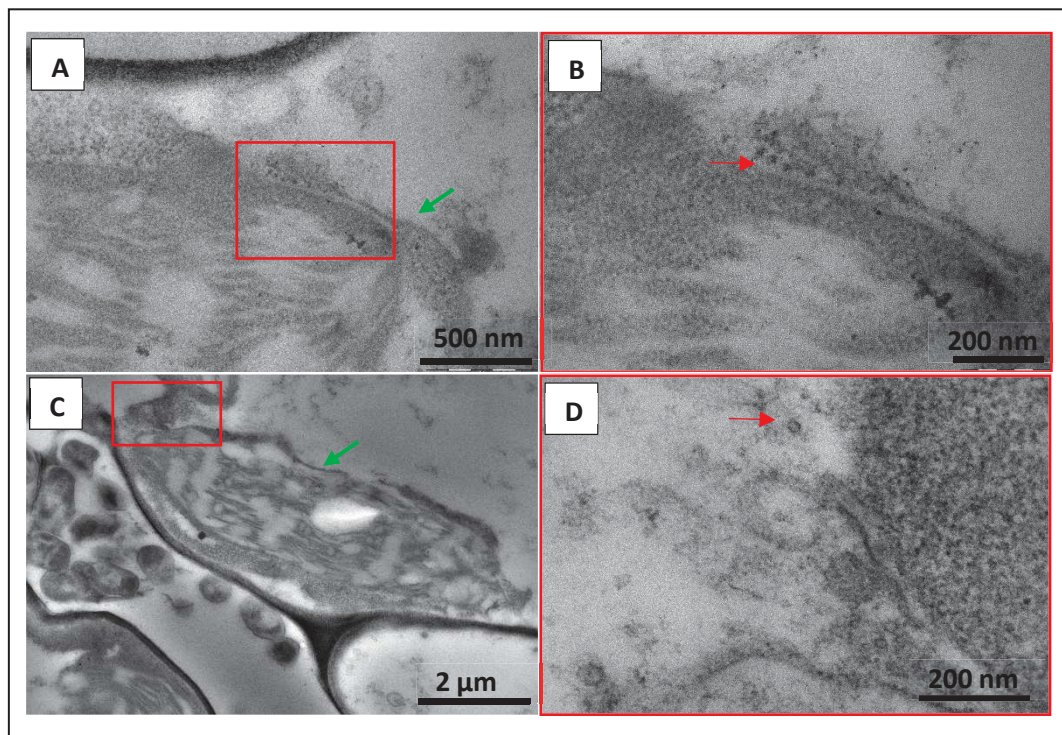


Figure 40: Ultrathin NB leaf sections observed with Electron transmission microscope after positive staining. Red arrows indicate particles with similar size as VLP, green arrows indicate chloroplast. B is a zoomed caption of the red rectangle in A. D is a zoomed caption of the red rectangle in C.

3.2 Evaluation of VP4 effects on FAW midgut larvae

3.2.1 *Ex vivo* experiment: Ussing chamber

As previously discussed, it has been demonstrated that VP4 can imitate JcDV and cross the intestinal epithelium by transcytosis when expressed in fusion with other proteins using a recombinant baculovirus (Kemmerer and Bonning, 2018). Besides, VLP-VP4 with the same expression system have been shown to enter *Lymatria dispar* cell line by endocytosis (Vendeville et al., 2009).

Here we tested the capacity of VLP-VP4 expressed in NB leaves to pass through the midgut using a Ussing chamber. To check whether these pseudo-particles could cross the midgut epithelium, NB leaf extracts containing VLP-VP4 were incubated *ex vivo* with midgut from 6th instar FAW larvae using Ussing chamber for 2 hours. To measure the flow of VLPs from the luminal to basal compartments, total protein precipitation and immunoprecipitation using an anti-JcDV antibody were carried out on the samples from both compartments. Results were not conclusive. Non-specific bands at 70 kDa were detected in basal samples in both negative control (PBS extraction buffer) and VP4 leaf extracts with total protein precipitation (Figure 42 A). Immunoprecipitation blots revealed the presence of 2 bands of 50 and 25 kDa,

that probably correspond to the heavy and light immunoglobulin G (IgG) chains of anti-JcDV antibodies used in this experiment. VP4 seems to be detected only in samples from luminal compartment (lanes 9,10 & 11) (Figure 42 B). To suppress interference from the heavy IgG chain, another immunoprecipitation test was conducted with the same samples using anti-light chain secondary antibodies. Same results have been observed. Further optimization experiments are required to verify the ability of VLP-VP4 expressed in the plant system to cross the intestinal epithelium by transcytosis and whether this passage could permeabilize the gut like the JcDV viral particles.

We checked if VLP-VP4 were able to enter FAW midgut cells. After 2 hours *ex vivo* incubation with NB leaf extracts containing VLP-VP4 using Ussing chamber, the 6th instar FAW intestines were fixed using paraformaldehyde. Thin sections (200 μm) were observed with confocal microscopy after immunolabeling. Images showed a concentration of VP4 in the cytoplasm of epithelial cells (Figure 41). These results indicate that VP4 expressed in NB leaves can enter the intestinal epithelium.

3.2.2 Activity of purified VLP-VP4 in combination with Cry2Ab2: Preliminary results

To see whether the presence of VP4 could enhance the toxicity of Cry2Ab2, a sublethal dose of Cry2Ab2 (25.45 ng/cm²) was tested in mixture with VP4 (3.3 10^{10} veg/ cm²) purified from a recombinant baculovirus system expression (Croizier, 2000). The preliminary results did not show any significant effect of VP4 used either alone or in combination with Cry2Ab2. A non-significant trend to enhance the percentage of stunting has been observed with a mixture of VP4 and Cry2Ab2 (Figure 43). Further experiments using different concentration of VLP-VP4 produced in a baculovirus system and Cry2Ab2 are needed to check if a synergy between both molecules does exist. Once the synergy is confirmed, we can test the VLP-VP4 produced *in planta* in a mixture with Bt proteins.

As a main conclusion of this part, we confirmed the possibility to produce VLP-VP4 by expressing VP4 in plants.

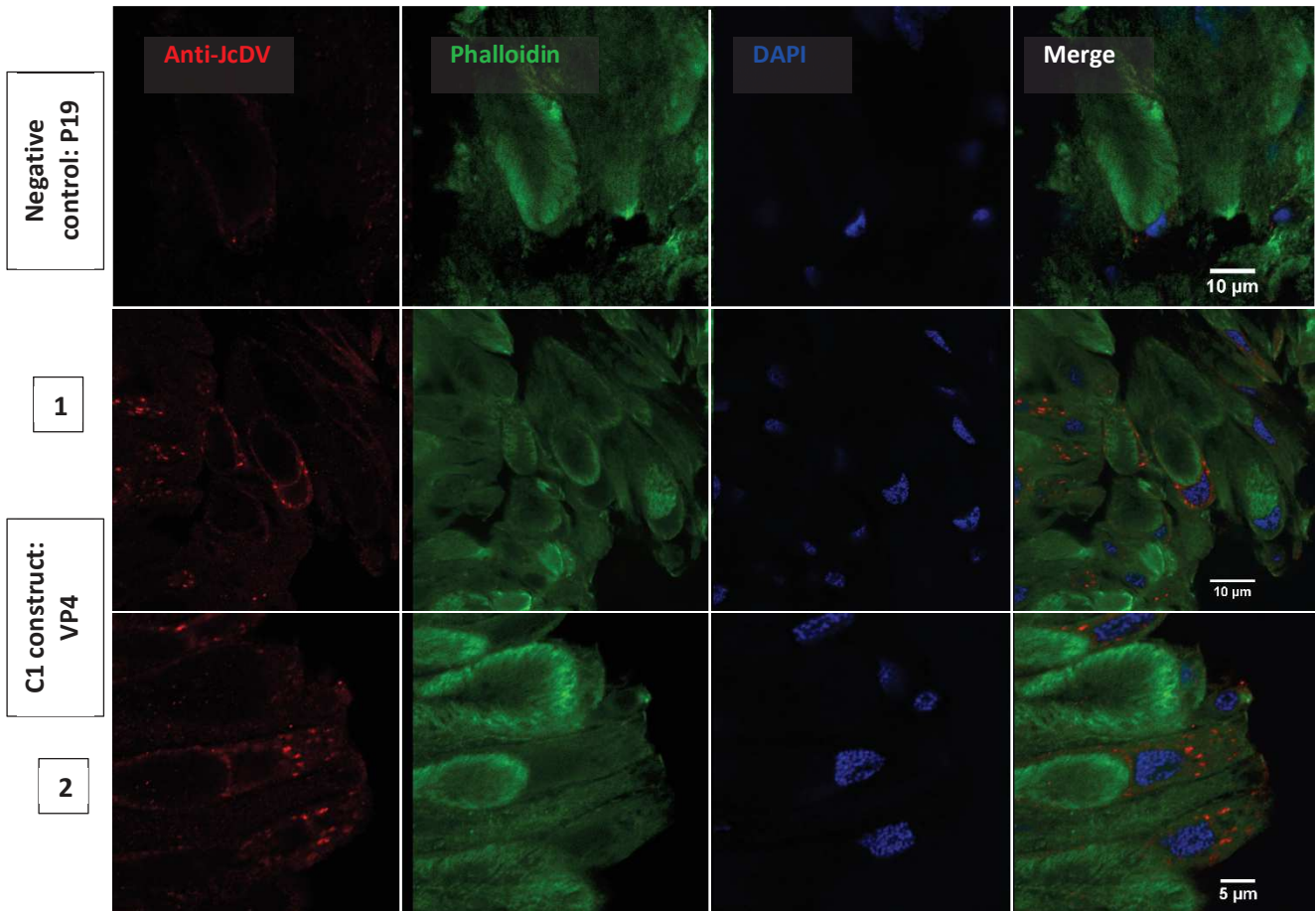


Figure 41: Evaluation of ability of VP4 expressed in NB leaves to cross FAW midgut. Thin sections were observed using confocal microscopy after immunolabeling of fixed FAW midgut thin section (200 μm) incubated for 2 hours in Ussing chamber with NB leaf extracts containing VP4 (C1 construct). DAPI was used to label cells nuclei, green fluorescent phalloidin was used to label actin filaments for epithelial cells and microvilli. 1&2 : two biological replicates

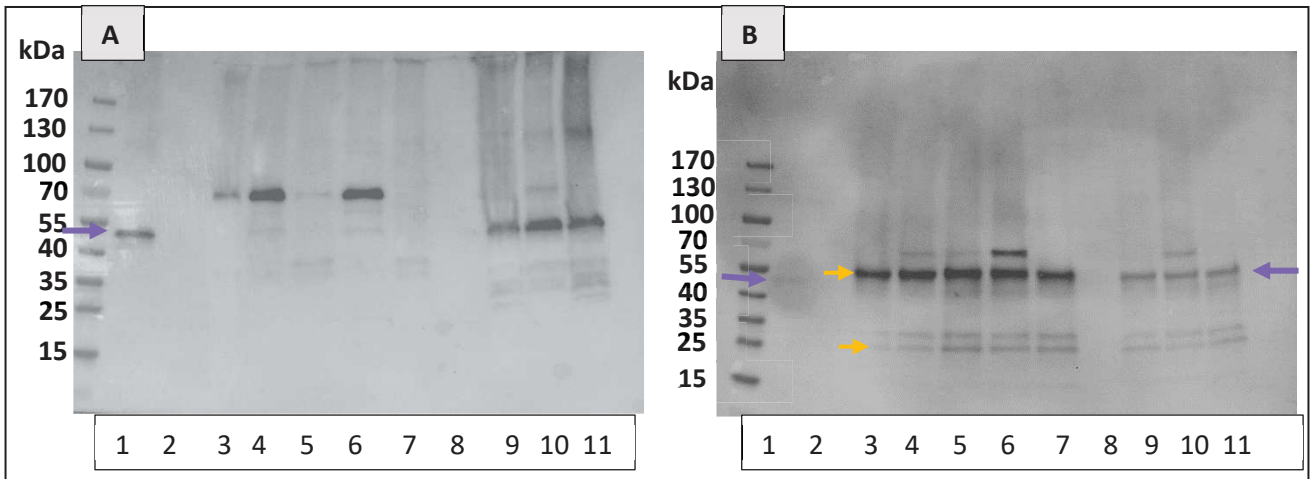


Figure 42: Total protein precipitation (A) and protein immunoprecipitation (B) analysis of NB leaf extracts expressing VP4 after 2 hours incubation in Ussing Chamber. 1: VP4 expressed in NB leaves (C1 construct), 2: PBS buffer, 3: basal compartment sample containing negative control (P19), 4: luminal compartment sample containing negative control (P19), 5&6&7: basal compartment samples containing VP4 expressed in NB leaves (C1 construct) (3 biological replicates), 8: PBS buffer, 9&10&11: luminal compartment samples containing VP4 expressed in NB leaves (C1 construct) (3 biological replicates). The purple arrows indicate the right molecular weight for VP4, the orange arrows indicate the molecular weight of the heavy and the light IgG chains

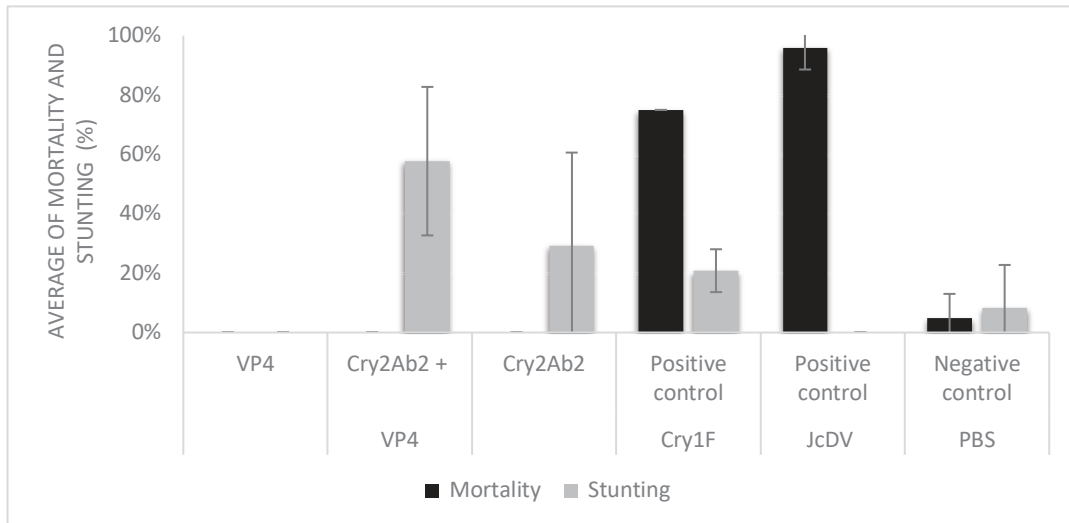


Figure 43: Evaluation of the activity of VP4 + Cry2Ab2 in mixture against FAW neonates 8 days after infestation using artificial diet overlay bioassays. Activity was evaluated by assessing the percentage of mortality (black boxes) and the percentage of stunting (grey boxes). Each FAW larvae was exposed to ~200 μ L of FAW artificial diet overlaid with 20 μ L of samples: Cry2Ab2 (25.45 ng/cm²), Cry2Ab2 (25.45 ng/cm²) + VP4 (10⁹ veg), VP4(10⁹ veg). The negative control consisted of diet overlaid PBS buffer. For each experiment, a total of 48 FAW neonate larvae were analyzed. Statistical analyses were conducted using Tukey HSD test comparing all samples together. The error bars show the standard error of the mean (SEM).

4 Discussion and perspectives

The fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith, 1797), originated from north America, is an invasive pest that threatens food security in many countries all over the world (Early et al., 2018; Adhikari et al., 2020; De Groot et al., 2020; FAO, 2020). Its exponential spread is pushing researchers to figure out new strategies/ molecules to control this insect. Bt insecticidal proteins, which are gut binding proteins, have been good alternatives to chemicals in controlling FAW for years (Brookes and Barfoot, 2020). To date, several Bt proteins are used in transgenic traits to control FAW including Cry1 proteins (Cry1F, Cry1Ac, Cry1Ab, Cry1A.105), Cry2 proteins (Cry2Ab2, Cry2Ae) and Vip proteins (Vip3A) (Huang, 2021). These proteins are known to destabilize midgut integrity by forming pores (Bravo et al., 2017; Syed et al., 2020; Gupta et al., 2021). The need to substitute or enhance the activity of the existing commercial Bt against FAW has increased with the appearance of field resistance to Cry1F and Cry1Ab with a potential cross resistance among all Cry1 proteins (Farias et al., 2014; Jakka et al., 2016a; Omoto et al., 2016; Flagel et al., 2018; Huang, 2021). Thus, there is an emerging interest to combine entomopathogens/molecules with different origins to delay resistance. JcDV, an orally transmitted densovirus, shows efficacy against FAW larvae under lab conditions (Mutuel et al., 2010; Wang et al., 2013b). Before reaching the target tissues for replication, JcDV crosses the midgut epithelium barrier. It has been demonstrated that this passage causes midgut disruption (Wang et al., 2013b). Since both Cry proteins and JcDV particles disrupt the FAW midgut, we were interested in studying the potential synergy between them. At first, we used an *ex vivo* approach to test the evolution of the 6th instar FAW midgut permeability over 2 hours incubation with a mixture of JcDV and Cry2Ab2 using Ussing chamber. The results revealed an important inhibition and delay of the transepithelial transport of JcDV and 4 kDa FITC-Dextran in the presence of Cry2Ab2 mixed with JcDV. These results have been confirmed by bioassay test using diet overlaid with a sublethal dose of Cry2Ab2 and the dose corresponding to the LD50 of JcDV against neonates. A significant decrease of mortality has been noticed with larvae fed on diet containing the mixture JcDV + Cry2Ab comparing to larvae exposed to only JcDV. This suggests an antagonism between Cry2Ab2 and JcDV. These results stand in parallel with the findings of (Liu et al., 2006) in which they observed an antagonism when *Helicoverpa armigera* (Ha) larvae were exposed at the same time to a mixture containing Cry1Ac and Ha nuclear polyhedrosis virus (HaNPV). An additive effect has been noticed when Ha was fed on diet containing Cry1Ac 2 days after being infected with HaNPV. It would be interesting to test this condition with Cry2Ab2 and JcDV in both

bioassay and *ex vivo* experiments to see if we find the same synergy. It would also be possible to test different doses for both to determine the best dose combination.

Competitive binding of Cry2Ab2 and JcDV to the same midgut receptors could explain this antagonistic interaction. It has been demonstrated in the PhD work of (Pigeyre, 2019) that JcDV interacts with lipid rafts in the FAW epithelial brush border. Moreover, proteomic analysis of proteins extracted from these membrane microdomain followed by Viral Overlay Protein Binding Assay revealed that JcDV interacts with some potential receptors including aminopeptidases N (APN) and alkaline phosphatases (ALP) (Pigeyre, 2019). APNs and ALPs are involved in the insecticidal activity of Bt proteins against lepidopteran larvae. In fact, two APNs (APN1 and APN6) were shown to be affected in case of the cabbage looper *Trichoplusia ni* resistance to Cry1Ac (Tiewsiri and Wang, 2011). APN2 has also been identified as a Cry2Ab binding protein in *Manduca sexta* (Onofre et al., 2017). Further demonstration of the role of these midgut proteins was provided by the observation that downregulation of APN4 and APL2 led to a decrease of Ha susceptibility to Cry2Aa. For FAW, little is known about Cry2Ab functional receptors. APN1 has been identified as a FAW functional receptor to Cry1Ca. The down-regulated expression of ALP has been reported to be involved in FAW resistance to Cry1F (Jakka et al., 2016a). APN and APL are not the only described toxin binding receptors. ABC transporters appeared to have a critical role in the mode of action of Bt insecticidal proteins (Heckel, 2021). For instance, resistance to Cry1F has been proved to be linked to a mutation in SfABCC2 gene (Banerjee et al., 2017). These results show that the mechanism of action of Cry1F may involve many receptors even though researchers have had more success with ABC transporters in studies of insect resistance to Bt proteins (Heckel, 2020).

Our findings (antagonism between JcDV and Cry2Ab2) suggest that JcDV and Cry2Ab2 may share the same binding sites. Since Cry2Ab2 and Cry1F seem to not have the same binding sites, possibly JcDV and Cry1F will also differ in their receptors. Therefore, it would be interesting to evaluate the potential synergy between JcDV and Cry1F. Further experiments are also needed to confirm the results obtained with the mixtures of Cry2Ab2 and JcDV. Competition binding assays with co-immunoprecipitation could be considered to test the interaction between both JcDV and Cry2Ab2. Knockout of ALP or APN would determine whether they are functional receptors for both JcDV and Cry2Ab2. This could be achieved by genome editing, since functional genomics has already been studied for FAW (Wu et al., 2018; Zhu et al., 2020).

In vivo, before reaching the midgut, molecules need to pass the peritrophic matrix (PM). This matrix provides a protection against pathogens and it is mainly composed of chitin and some highly glycosylated proteins. It has been demonstrated by Pigeyre et al., (2019) that JcDV is able to bind to some glycans in the PM and pass through it causing its disruption. Bt insecticidal proteins, as well, recognize and bind to PM glycoproteins by the lectin-like folds present in two of the three domains of most Cry proteins (Hayakawa et al., 2004; Valaitis and Podgwaite, 2013). Thus, it would be of big interest to study the effect of both Cry proteins and JcDV when combined together on the PM.

In the second part of this chapter, we were interested in expressing a JcDV capsid protein (VP4) *in planta* to check the feasibility of its use in a biotech approach. Results revealed a good level of VP4 expression in *N. benthamiana* leaves without any sign of phytotoxicity. Immunostaining of semi-thin leaf sections have shown a VP4 accumulation around nuclei and structures similar to chloroplasts. Immunogold experiments are needed to validate the exact localization of VP4 when expressed *in planta*. We have also demonstrated that VP4 could self-assemble to form VLPs. These VLPs were tested *ex vivo* using Ussing chamber. It has been shown that VLPs were able to enter the epithelial cells. Unfortunately, the passage of these VLPs across the epithelium barrier has not been confirmed yet. Some optimizations in protein immunoprecipitation technique are needed. To our knowledge, this study is the first proof of concept of JcDV VP4 expression evaluation in a plant system. Our aim was to use these VLPs as helper molecules for bacterial pesticidal proteins, that bind to receptors on intestinal cells, in order to create new transgenic plants that are more resistant to insects. The possible use of VP4 in combination of other insecticidal molecules has been demonstrated by (Bonning and Kemmerer, 2020). In fact, the goal of Bonning and Kemmerer's study was to use hemocoelic insecticidal proteins by ingestion via VP4 which will ensure the delivery from the FAW midgut to the target cells localized in the hemocoel. In this case, they have expressed fusion proteins in *E. coli*. In our study, we worked with insecticidal proteins that act by ingestion on the midgut cells. Thus, it will be interesting to further verify if VLPs made of VP4 expressed in plant

system would also be able to cross the midgut epithelium and affect the midgut permeability as does the virus JcDV. The second step would be to test the combined effect of both VLPs and bacterial insecticidal proteins. Since VP4 may have the same receptors as JcDV (which are still unknown), it would be preferable for the proof of concept to use another Bt protein that does not share the same binding sites as Cry2Ab2 to avoid the antagonism seen with JcDV. Cry1F and Vip3A could be good candidates to perform these experiments.

CONCLUSION

Insect pests are a major worldwide concern by threatening the production of staple crops such as maize. Considerable yield losses every year are mainly due to insect attacks in maize fields. Their high adaptability to pest management tactics makes their control more challenging. Coleopterans and lepidopterans are among the most important insect groups that cause serious damage to maize. Bt maize has been a good alternative for a long time until the appearance of resistant populations. The main challenge today remains to find new ecofriendly molecules and strategies to sustainably control these insects.

In this 3-year PhD program, my work focused on two maize pests, the Western Corn Rootworm *Diabrotica virgifera virgifera* (coleopteran) and the Fall Armyworm *Spodoptera frugiperda* (lepidopteran). For both insects, we looked for biotech and biocontrol solutions.

For biotech applications, we have discovered new pesticidal proteins active against WCR and present in different strains of *Chryseobacterium*. We demonstrated that the toxicity was dependent on two protein components as in the commercial binary proteins Cry34/Cry35 and that the mode of action of these proteins was different from the two existing commercial modes of action. Significant difficulties were encountered in purifying the second member of the binary protein due to its membrane-bound nature. However, I was able to achieve a partial purification which will require further optimization to conduct efficiency studies (LD50) of its activity against WCR with the second protein component. Preliminary studies to express active proteins in tobacco or maize were not successful. The potential use of this binary protein to generate transgenic maize resistant to WCR has therefore been put on hold pending further purification, activity, and expression data in the plant. A second approach for biotechnological applications was tested using a capsid protein (VP4) of an entomopathogenic FAW virus (JcDV). I first succeeded in expressing the VP4 protein in a plant system without any sign of phytotoxicity to the plant. I have demonstrated that this capsid protein can self-assemble into virus-like particles (VLPs) in planta. The next step will be to demonstrate that the VLPs produced in plants are able to cross the midgut like the whole JcDV virus and to test different methods for combining the VP4 protein with Bt toxins to evaluate the desired objective.

For biological approaches, we focused on the potential use of a densovirus JcDV and the Bt protein (Cry2Ab2) against FAW, both of which disturb midgut permeability. The two proteins were mixed for bioassay experiments to assess potential synergies. The results showed an unexpected antagonism confirmed by our *ex vivo* experiments using the Ussing chamber suggesting receptor binding competition or a physical interaction. As studies with other pests have shown synergy between a Cry protein and other enthomopathogenic viruses, further analysis are required. The most important result I have obtained in the field of biocontrol concerns the potential use of *Chryseobacterium* strains against WCR. Although the origin of the insecticidal activity observed with this bacterium and the correlation with the activity of our pesticidal proteins are still unknown, we have demonstrated their effectiveness *in vitro* against WCR larvae. The next steps will be to determine the method of producing an active ingredient from the bacterial strains that is compatible with the biocontrol regulations and with all safety concerns.

This thesis gave me the opportunity to do research in the field of crop protection from two distinct perspectives. This double vision has been present in all my approaches and encourages me to keep an open mind to imagine applications based on the experimental results.

Material and Methods

Insect rearing

Larvae of *Spodoptera frugiperda* were reared under controlled conditions (25 +/- 1°C, 65 to 70% relative humidity [RH], 16h light, 8h dark photoperiod) on a corn-based artificial diet.

WCR (EPPO code DIABVI) were mass-reared following the procedures of (George and Ortman, 1965; Singh and Moore, 1985; Branson et al., 1988). A non-diapause colony (USDA ARS, Bookings, USA) was used to infest artificial diet-based bioassay with neonates (<24h old). The population is considered susceptible to most insecticides or novel agents as it had not been exposed to any of those, and therefore resistance is considered unlikely (Magalhaes et al., 2007; Wright et al., 2000b)

Protein expression using *E. coli* BL21(DE3)

GDI0005A, GDI0006A and their homologous sequences were obtained from genome sequencing or Genebank and adapted to *E. coli* codon use and synthesized by GenScript Biotech (Leiden, Netherlands). The corresponding DNA fragments were cloned using Electra Protocol () into pD441-NH vector (ATUM, Newark, Californiamm, USA) under the control of isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible T5 promoter. Cloning was performed in the *E. coli* BL21DE3 strain suitable for protein overexpression. All the plasmids were verified by sequencing. The recombinant *E. coli* BL21 (DE3) strains were inoculated into 2 mL Luria–Bertani (LB) medium with 50 μ g/mL Kanamycin. After overnight cultivation at 37 °C, 1 % (v/v) of *E. coli* seed cultures were inoculated into auto inducible expression medium (Overnight Express Instant TB Medium - Novagen - 5 easy ref 71491-4) with 50 μ g/mL Kanamycin and cultivated at 30°C overnight under agitation at 200 rpm in an Infors HT Multitron Pro incubator (Infors AG, Bottmingen, Switzerland). Cultures were centrifuged for 20 min at 4000 rpm. Cell pellets were either resuspended in 50mM Tris, 150 mM NaCl pH 7,5 with or without 0,1% CHAPS/DDM and sonicated in an ice bath for 3 min using 500W sonicator probe (Fisherbrand FB505AEUK-220) or disrupted using BugBuster Protein Extraction Reagent (Merck Millipore, Billerica MA, USA) supplemented with 10 mg/mL of lysozyme and EDTA-free blend of protease inhibitors (Complete EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany).

Protein expression using *E. coli* Lemo21(DE3)

GDI0006A sequence was obtained from genome sequencing or Genebank and adapted to *E. coli* codon use and synthesized by GenScript Biotech (Leiden, Netherlands). The corresponding DNA fragments were cloned using Electra Protocol

(https://www.atum.bio/catalog/reagents/electra#__electra-overview) into pD441-NH vector (ATUM, Newark, California, USA) under the control of isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible T5 promoter. Cloning was performed in *E. coli* Lemo21 (DE3) strain suitable for membrane protein overexpression and regulated by L-rhamnose. Transformation was checked by PCR. The recombinant *E. coli* BL21 (DE3) strain was inoculated into 2 mL Luria–Bertani (LB) medium with 50 μ g/mL Kanamycin to produce a starter culture. After overnight cultivation at 37 °C, 3% (v/v) of *E. coli* seed cultures were inoculated into 10 mL LB medium with 50 μ g/mL Kanamycin and 2000 μ M of L-rhamnose and incubated at 37 °C. Upon reaching an optical density (OD₆₀₀) of 0.6–0.8 at 600 nm, protein production was induced by adding 400 μ M IPTG and re-incubated overnight at 30°C under agitation at 200 rpm in an Infors HT Multitron Pro incubator (Infors AG, Bottmingen, Switzerland). Cultures were centrifuged for 20 min at 4000 rpm. Cell pellets were resuspended in a sonication buffer (50 mM Tris, 150 mM NaCl pH 7.5) and sonicated on an ice bath for 3 min using 500W sonicator probe (Fisherbrand FB505AEUK-220).

Protein expression analysis

Proteins from cells lysates samples or plant extracts were separated by SDS-PAGE on a 4–15% Mini Protean TGX Precast stain free SDS-PAGE gel (BioRad). Proteins were directly visualized using a ChemiDoc (Chemidoc™ MP Imaging System, Bio-Rad) without further treatment, or visualized after Coomassie blue staining. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane and immunoblotted using specific antibodies. After blocking the membrane with a 3% Bovine Serum Albumin (BSA) solution, the membranes were incubated with the specific antibodies for 2 hours.

For GDI0005A/GDI0006A experiments we used anti-GDI0005A anti- α rabbit polyclonal antibody (1:5000; Genscript), anti-GDI0176A (GDI0006A homolog) anti-rabbit polyclonal antibody and the anti-His-6 mouse monoclonal antibody (1:1000; no. ab18184; Abcam). After washing with 1x Tris-buffered saline (TBS) containing 0.02% of Tween 20, the membranes were incubated 1 hour with alkaline-phosphatase conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:5000; no 65-6122; ThermoFisher) for GDI0005A/GDI0006A anti-bodies or rabbit anti-mouse immunoglobulin G (IgG) secondary antibody (1:5000; 31329; Invitrogen) for anti-His-6. The antibodies were detected using a Novex™ Alkaline Phosphatase Chromogenic Substrate (BCIP/NBT) (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions

For VLP-VP4 experiments, the rabbit or the mouse anti-JcDV capsid antibodies (1:1000; Eurogentec (Pigeyre et al., 2019)) were used. After washing with 1x Tris-buffered saline (TBS) containing 0.02% of Tween 20, the membranes were incubated 1 hour with the anti-rabbit secondary antibody HRP-conjugated (1:3000; Biorad, Hercules, CA, USA) or the anti-mouse light chain secondary antibody (Sigma-Aldrich). The antibodies revealed by enhanced chemiluminescence (Millipore, Burlington, MA, USA) using a Chemidoc imager (Biorad).

Protein purification

Lysate was clarified by a 10 min 12000 rpm centrifugation and 0.22 µm filtration. The supernatant was incubated with 10 mM imidazole and loaded onto a 5 mL HisTrap HP Ni-NTA chelating Sepharose column (GE Healthcare) preequilibrated in the lysis buffer (50mM Tris, 150 mM NaCl, pH 7.5 with or without 0,1% CHAPS/DDM) using the ÄKTA Purifier FLPC purification system (GE Healthcare) equipped with Unicorn 6.5 software. After washing with the washing buffer (50mM Tris, 150 mM NaCl, 20 mM imidazole, pH 7.5 with or without 0,1% CHAPS/DDM), the protein was eluted with the elution buffer (50mM Tris, 150 mM NaCl, 500 mM imidazole, pH 7.5 with or without 0,1% CHAPS/DDM). Peak fractions were pooled and further purified by gel filtration using HiPrep 26/10 Desalting column (Dutscher) equilibrated with 50 mM Tris pH 7.5, 150 mM NaCl with or without 0,1% CHAPS/DDM. Peak fractions were pooled and concentrated using an Amicon Ultra-15 filter (Millipore, MWC, 10000).

JcDV purification

Spodoptera frugiperda or *Spodoptera littoralis* L3 caterpillars, previously infected with JcDV virus, were ground in 1X TE buffer (1 mM Tris pH7.5; 0.1 mM EDTA pH 7.3) containing 2% ascorbic acid. The ground material was clarified at 10 000 g at 4°C for 10 min, then the viral pellet was filtered through 0.22 µm filter. The semi-purified JcDV virus was loaded on a density gradient of 15 to 55% of OptiPrep™ (Sigma) and ultracentrifuged 16 h at 100 000g at 8°C. The virus band in the 45% Optiprep layer was harvested and then dialyzed for 48 h using 1X PBS (Pigeyre et al., 2019). The quality of the virus stock was checked by negative staining and electron microscopy. The viral concentration was estimated at $3.62 \cdot 10^{10}$ virus equivalent genomes (veg) / µl by quantitative PCR (qPCR) as described in (Wang et al., 2013b). The viral infectious titer was determined in cell culture on permissive insect cells IPLB-Ld-652 (Goodwin et al., 1978) by immunofluorescence as described in (Li et al., 1996).

Protein expression in planta

- Transient expression: *Nicotiana benthamiana* Agroinfiltration

GDI0005A/ GDI0006A and their homologs, GDI0006A with different target signals and VP4 with or without His-tag/ GFP fusions sequences were codon-optimized for plants expression and synthesized by GenScript Biotech (Leiden, Netherlands). Constructs were then introduced into *Agrobacterium tumefaciens* strain LBA4404 (pSB1) under the control of CsVMV promoter and with neomycin phosphotransferase (NPTII) as a selectable marker gene for kanamycin resistance. Transformation and gene insertion were checked by real time-PCR using specific primers synthesized by GenScript Biotech (Leiden, Netherlands). Recombinant *A. tumefaciens* from one isolated colony were grown overnight at 28 °C in LB medium supplemented with 5 µg/mL tetracycline, 20 µg/mL kanamycin and 10 µg/mL rifampicin under agitation at 200 rpm. A glycerol stock was prepared by adding 1500 µL of the overnight culture to 500 µL of 50% glycerol in a 2 mL cryovial tube and preserved at -70°C after gently mixing. For agroinfiltration experiments, *A. tumefaciens* recombinant strains expressing individually P19 (a gene silencing suppressor from Artichoke Mottled Crinckle virus (Garabagi et al., 2012) and the genes of interest were then cultured and isolated on separate LB agar petri plates containing the adequate antibiotics. Single colony from each *A. tumefaciens* strain was inoculated, using a sterilized toothpick, into 10 mL of YEP broth medium, 100 µL of 0.6 M CaCl₂ with 20 µg/mL kanamycin for overnight culture at 28°C under agitation at 200 rpm. The overnight cultures were brought to an (OD₆₀₀) of (0.3-0.4) and re-incubated in the same medium at 28 °C for 1 hour until they reach an (OD₆₀₀) of 0.5. Cell pellets were washed with induction solution (50 mM MES; 2 mM NaH₂PO₄; 0,5% glucose; 100 µM Acetosyringone; pH 5.6 adjusted with NaOH) and then resuspended in the same solution. Recombinant *A. tumefaciens* carrying the genes of interest were mixed with *A. tumefaciens* expressing P19 and incubated for 1 hour at room temperature before co-infiltration. The mix was then slowly infiltrated on the underside of 3-4 weeks old *Nicotiana benthamiana* plants leaves using 1 mL syringe without needle. The infiltrated area was gently labeled with a black pencil. Seven days after infiltration, tissues were collected and used for protein extraction or confocal microscopy. Tissues infiltrated with only P19 were used as negative control whereas tissues with the mix of P19 and ZsGreen (gene coding for green fluorescence protein) were used to check the agroinfiltration efficiency.

- Stable expression: *Zea mays* Transgenesis

Maize transformation was mediated by *A. tumefaciens*. Immature embryos of maize inbred A188 were cocultured with recombinant *A. tumefaciens* strain LBA4404 (pSB1)

transformed with a binary vector containing genes coding for GDI0005A and GDI0006A each one driven by the Cassava Vein Mosaic Virus (CsVMV), ZsGreen (green fluorescence protein), AFPAT conferring resistance to glufosinate (herbicide) and NPTIII for Kanamycin resistance. After 7 days, inoculated embryos were transferred into selection medium amended with glufosinate and antibiotics. Through 8 weeks of selection, embryogenic callus clones that proliferated on selection medium were transferred on a regeneration medium with phytohormones. After 28 days, regenerated plantlets in normal morphology were transferred to pots and grown to maturity in greenhouse. T0 plants genotyping was performed by quantitative RT-PCR (qPCR). Positive qPCR plants with best gene expression were self-pollinized to produce T1 generation. Seeds segregation was verified by GFP fluorescence visualized under a fluorescence stereomicroscope (Leica MZ16F) using a GFP2 (Leica, Bannockburn, IL) filter.

Total protein extraction

Around 30 mg of plant tissues (agroinfiltrated *N. benthamiana* leaves or transgenic maize leaves and roots) were ground to a fine powder in nitrogen using micro-pestles in Eppendorf tubes. One mL of Phosphate Buffer Saline (PBS) pH 7.4 containing 0.2 % Tween 20 was added to each tube. After homogenizing, the extracted solution was centrifuged (12000 rpm, 10 min, 4 °C) and the supernatant was used for protein expression analysis, bioassays and with Transmission Electron Microscopy (TEM) analysis. For bioassays, agroinfiltrated *N. benthamiana* extracts were dialyzed against 1X PBS pH 7.4 with 0.2 % Tween 20 using a Slide-A-Lyser G2 dialysis cassette with a 2K Membrane Molecular Weight Cutoff (thermos scientific).

Ussing chamber experiments

Midguts were isolated from 6th instar larvae (2 days after molting) and inserted into the Ussing chamber (EM-CSYS-2; physiologic instruments) (Figure 44). The basal (hemolymphatic) and the luminal compartments were filled respectively with 700 μ L of HEMO (5 mM CaCl₂, 24 mM MgSO₄, 20 mM K gluconate, 190 mM sucrose, and 5 mM Tris adjusted to pH 7 with HCl) and LUME (5 mM CAPS [3-(cyclohexylamino)- 1-propanesulfonic acid] adjusted to pH 10 with tetramethylammonium hydroxide (TMAOH)) solutions. Both compartments were supplied with a constant flow of oxygen. After a few minutes for equilibration, 300 μ L of the tested sample (*N. benthamiana* extracts expressing VP4, the activated Cry proteins or the mix of activated Cry proteins with JcDV) was added to the luminal compartment. To maintain balance in both compartments, 300 μ L of HEMO

solution was simultaneously introduced to the basal compartment. Experiments were performed at room temperature for 2 hours. To test the permeability of FAW midgut, 0.3 $\mu\text{g/ml}$ Fluorescein isothiocyanate–dextran (FD4 3000-5000 MW; Sigma-Aldrich) was added to the luminal solution. The Ussing chamber was then protected from light to ensure the fluorescence stability. At the end of the experiment, solutions from the luminal and basal compartments were collected and compared by western blot using an anti-JcDV antibody (see above), qPCR as described in (Wang et al., 2013b) or fluorescence measures (495 nm excitation and 521 nm emission; Tecan). Three midguts were assayed for each sample and conserved for confocal microscopy. Statistical analyses were performed with a nonparametric Kruskal Wallis test.

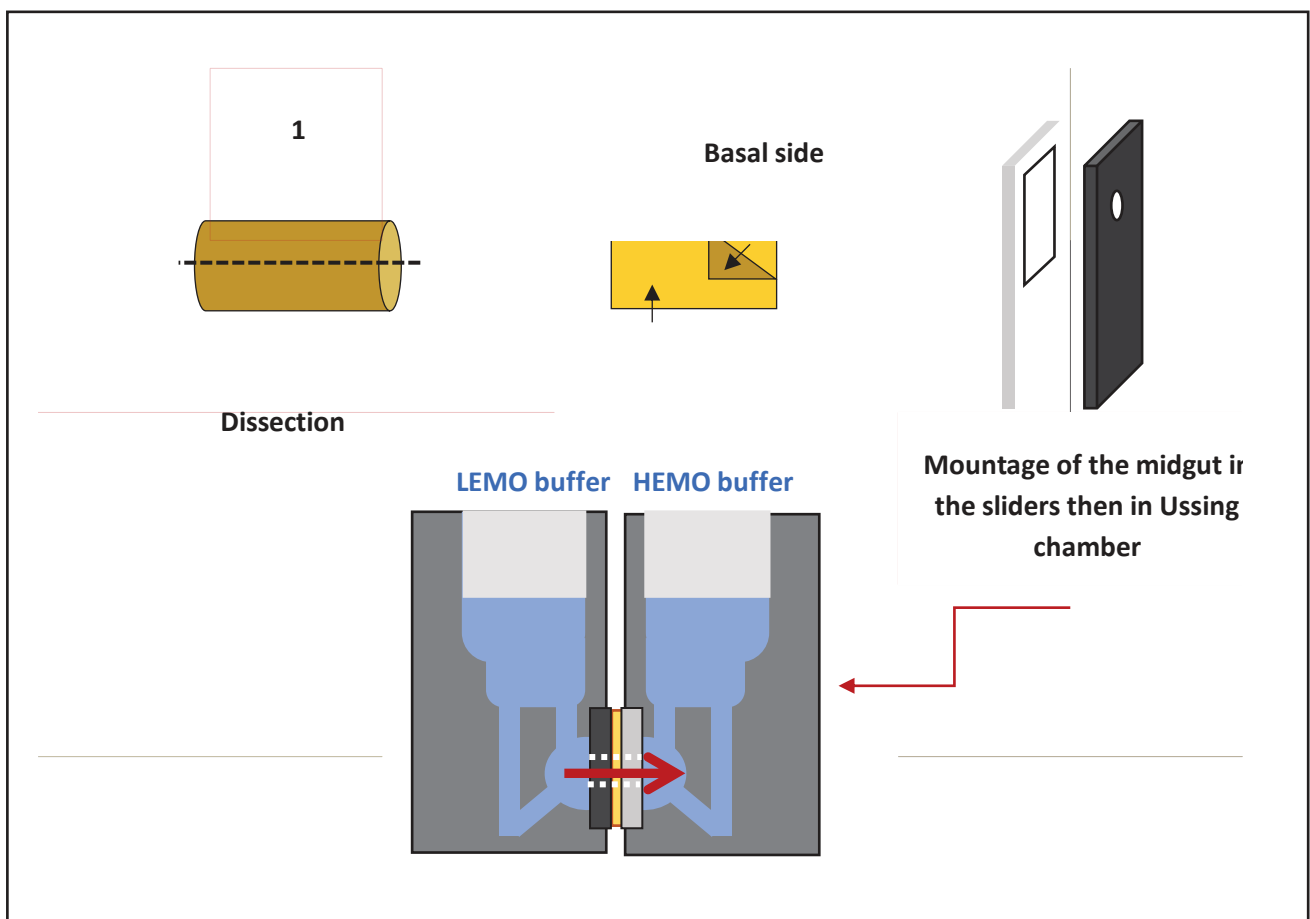


Figure 44: Schematic representation of Ussing chamber experiment

Cry2Ab2 activation

The 71 kDa Cry2Ab2 fused to the 45 kDa His-MBP tag was expressed in *E. coli* BL21 (DE3) as described above at Limgrain's lab. The purification was conducted at the laboratory of Renaud Vincentelli (AFMB, Marseille, France). Prior to its use in the Ussing chamber, the purified Cry2Ab2 protoxin was activated using 2 methods. A bovine trypsin was used (Sigma-Aldrich; T1426) at 1:20 trypsin: protein ratio (w/w), incubated at 30 °C for 2 hours and stopped by adding 1mM Benzamidine (Sigma-Aldrich; 12072). The other proteolytic activation method was the use of midgut juice enzyme complex as described by (Liu et al., 2020).

Total protein precipitation

VLP-VP4 samples, from both luminal and basal compartments, were collected 2 hours after being exposed to FAW midguts using Ussing chamber. To detect the passage of VLP-VP4 through the epithelial barrier, proteins were precipitated using sodium deoxycholate (2%) and 100% trichloroacetic acid. Samples were vortexed and centrifuged at 13000 rpm for 15 min at 4 °C. Pellets were washed with cold acetone, dried at room temperature for 20 min, and solubilized by vortexing in 2X SB denaturing buffer (4% SDS, 20% glycerol, 10% β -mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH 7). Samples were then boiled 5 min at 95°C and conserved at -20°C for Western blot analysis.

Protein immunoprecipitation

Immunoprecipitation was conducted using the same samples previously used for total protein precipitation. 150 μ L of each sample were incubated overnight with 10 μ L of the rabbit anti-JcDV capsid antibodies (Eurogentec (Pigeyre et al., 2019)). Samples were then incubated for 2 hours with 40 μ L of 50% protein-A-sepharose beads (GE-Healthcare) under shaking. Sepharose beads were then washed twice with the NET buffer (150 mM NaCl, 5mM EDTA, NP40 2% and 50mM Tris-HCl, pH 7.8) and once with the WASH buffer (500 mM LiCl, 1% β -mercaptoethanol and 100mM Tris-HCl, pH 7.8). After a short centrifugation (5 min at 13000 rpm), Sepharose beads were solubilized by gentle shaking in 10 μ L 5X SB denaturing buffer (4% SDS, 20% glycerol, 10% β -mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH 7) and processed (boiled at 95°C for 5 min) for SDS-PAGE and western blot.

Tissues fixation for immunofluorescence labeling and confocal microscopy

Three mm² sections of agroinfiltrated *N. benthamiana* leaves or entire VLP-VP4 treated FAW midguts were fixed for 2 hours under agitation with 4% paraformaldehyde in 1X PBS at pH 7.2 at 4°C. The tissues were then washed 3 times for 10 min with 1X PBS

and kept at 4°C for further use. Prior to the immunolabeling step, tissues were permeabilized with 1X PBS, containing 0.05% of Tween 20 and 0.1 M of glycine for 15 min. Tissues were then immersed in 12-well plates containing warm 8% and 4% agarose for *N. benthamiana* leaves and FAW midgut tissues, respectively. After polymerization, 200 µm sections were cut using a Leica vibratome and incubated for 1 hour in a blocking solution (5% BSA and 0.1% Triton in 1X PBS,) at room temperature under gentle shaking. Sections were then incubated overnight with the primary rabbit anti-JcDV capsid antibody (1/1000; Eurogentec), washed 3 times for 10 min in 1X PBS with 0.05% of Tween 20 and then incubated for 1 hour at room temperature with the secondary anti-rabbit IgG antibody (1/500; Alexa Fluor™ 488; Invitrogen). Phalloidin (1/300; Sigma-Aldrich) may be added to label actin cytoskeleton of the midgut epithelial cells. After repeated washing, sections were mounted using Vectashield mounting medium containing DAPI (Vectorlabs) for confocal microscopy observations.

Images were acquired using a 63×/1.4 Plan-Apochromat Oil PH3 objective on a confocal Zeiss LSM 880 microscope, equipped with suitable filters for the dyes, and a FastAiryscan detector to improve image resolution (MRI platform, Montpellier). We used ZEN black software to operate the microscope. Images were processed with the Image J software.

Electron microscopy

- Negative staining

Negative staining was performed with the *N. benthamiana* extracts containing the VP4 to check whether VP4 can form an empty capsid *in planta*. Samples were observed by transmission electron microscopy after negative staining using a carbon-coated copper TEM grids (Carbon film, 400 mesh copper; Euromedex) and 2% phosphotungstic acid (PTA). The grid was brought into contact with a drop of the sample for 3 minutes, then washed with a drop of 2% PTA and left in contact for one minute with 2% PTA. The grid was then dried before being imaged with a transmission electron microscope JEOL 1200 EX II, operating at 100 kV (MEA platform, University of Montpellier).

- Positive staining

The 3 mm² *N. benthamiana* leaf sections expressing VP4 were prefixed with 4% paraformaldehyde in 1X PBS for 2 hours at room temperature. The sections were washed 3 times for 10 min in 1X PBS before being incubating in 2% osmium tetroxide for 1 h. Following washing with distilled water, sections were dehydrated in increasing concentrations of alcohol (50%, 70%, 100%) (10 min, 3 times each bath). They were impregnated with Epon resin,

embedded in silicone molds and left overnight for alcohol evaporation, then polymerized 48 h at 60 °C. Ultra-thin sections (100 nm) were cut using an ultramicrotome fitted with a diamond knife. They were mounted on carbon-coated copper TEM grids (Carbon film, 400 mesh copper; Euromedex) and subsequently stained with UranylLess for 2 min, followed by Lead acetate for 3 min. The grids were then dried before being observed with a transmission electron microscope JEOL 1200 EX II, operating at 100 kV (MEA platform, University of Montpellier).

Insect bioassays

- Fall armyworm bioassay

FAW bioassays were conducted in 96-well plates by adding 20 µL of purified Cry proteins or purified JcDV/VP4 or the mix of purified Cry proteins with JcDV/VP4 to 200 µL diet (General Purpose Lepidoptera Diet Frontier Scientific F9772 diet). The diet was dispensed into a 96-well plate using an 8-channel pipette (Finni pipette Multistepper 4540500 Thermo Fisher, Marietta, GA), delivering 200 µl into each well and allowed to cool. Then, 20 µl of the samples was dispensed (Socorex Acura 826 XS 20-200 µl, Ecublens, Switzerland) onto each well of the diet plate as directed in the experimental plan. The wells were dried in a laminar flow hood for approximately 1 hour or until all inoculated wells were dried. FAW neonates were obtained from the INRAE DGIMI insectarium platform. Eight wells per replicate and 3 replicates per treatment were infested with a single larva for each experiment providing 24 datapoints per experiment for each sample. After infesting each plate, 2 holes were poked into the sealing film using a 000-insect pin and plates were placed in the growth chamber (Conviron A1000, Manitoba, Canada) at 25 °C, 50% RH, 0:24 L:D for 5 days. Insecticidal activity was scored as dead, stunted (survived with no growth), no activity (growth similar to negative controls) after 5 days. Stunting was qualitatively assessed as an indicator for sublethal effects in comparison to the size and form of larvae in the negative controls. After scoring, the experimental plates were placed in a -80°C freezer (Thermo Fisher Scientific TSE 400A, Marietta, GA) overnight. The next day the plates were removed and thawed to room temperature and the total weights (A&D GR-200 Analytical Balance, Columbia, MD) for each sample replicate were collected and then divided by the number of individuals to calculate mean larval weight per 8-well replicate. Statistical analyses were determined using Fisher exact test comparing each sample results (mortality and stunting) to the negative control. Then, for the weights analysis, Kruskal-Wallis test was applied. P-values of less than 0.05 were considered significant.

- **Lethal median dose LD50**

Lethal median dose studies were conducted using diet overlay bioassay as described above. Different doses of Cry2Ab2 and JcDV were tested. Dose response curves were obtained with log-logistic model using the drc package of R studio (Ritz et al., 2015) software version (4.1.2).

- **Western corn rootworm bioassay**

WCR bioassays on susceptible strains were conducted in 96-well plates by adding 20 μ L of cleared *E. coli* lysate or purified proteins to each well. Each treatment was applied to 8 wells of each plate per bioassay. Each treatment-dose combination was tested in at least two biological replicates. Order of treatments were shifted every other plate to avoid edge effects. Plates were dried for 1 to 1.5 h, and then cooled for 1 h in a fridge at 3 °C to 5 °C. One neonate larva was placed per well using a fine artist brush. A fast-moving, healthy-looking larva was chosen, and lifted from the end of abdomen with the brush, moved towards a well surface, and allowed to crawl off the brush onto the diet. Larvae were not placed in treatment column order but rectangular to avoid systemic errors. After every 12th larva, the brush was cleaned in 70% ethanol followed by sterile tap water. The filled plate was closed with an optically clear adhesive qPCR seal sheet (#AB-1170, Thermo Scientific, USA or #BS3017000, Bioleader, USA) allowing data assessments without opening the plate. Four to five holes were made with flamed 00-insect pins into the seal per well to allow aeration. The plates were incubated at 24 \pm 2 °C and 50 to 70% relative humidity (RH) in the dark in a ventilated incubator (Friocell 22, MMM Medcenter, Munich, Germany) for 5 days. Bacterial lysate containing Gpp34Ab1/Tpp35Ab1 (<https://www.rcsb.org/>) was used as a positive control. Bugbuster buffer, Tris-NaCl buffer and DDM buffer were used as negative controls. Insecticidal activity was scored as dead, stunted (survived with no growth), no activity (growth equal to controls) after 3 and 5 days. Statistical analyses were determined using Fisher exact test comparing each sample result to the corresponding negative control. Results were considered positive if larval mortality and stunting were statistically higher than in the negative control and recorded at 3- and 5-days post treatment. Stunting was qualitatively assessed as an indicator for sublethal effects in comparison to the size and form of larvae in the negative controls. Feeding was assessed through observing food remains, frass, and diet in the larval gut to assure that diet and a treatment had been ingested.

Root trainer assay

GDI0005A/GDI0006A T1 maize plants resistance was evaluated against WCR neonates (USDA ARS non-diapause susceptible colony) using a two week root trainer assay test as described in (Dönitz et al., 2015). T1 maize plants expressing GDI0005A and GDI0006A were planted into root trainer pots containing Metro Mix soil. V3 to V4 plants were infested with up to 150 WCR eggs suspended in agar solution. Two weeks post-infestation, the plant roots were washed and injury from WCR larval feeding was visually assessed using the Iowa State 0–3 Node-Injury Scale (Oleson et al., 2005). The wild type was used as a negative control. Plants with injury scores less than 0.5 were considered resistant. Susceptible plants score ranged between 1.5 and 3.0.

Rapport détaillé (en français)

Problématique et objectifs

Le maïs (*Zea Mays*) est la troisième culture la plus importante dans le monde après le riz et le blé (Kyenpia et al., 2009). Depuis 1996, la protection du maïs aux Etats-Unis et en Amérique du Sud (Brésil, Argentine) contre les insectes ravageurs repose essentiellement sur l'utilisation de plantes exprimant des entomotoxines ayant comme origine *Bacillus thuringiensis* (Schnepf et al., 1998 ; Adang et al., 2014). Cependant, l'utilisation massive de ces toxines, comme pour toute pression de sélection qui devient constante, a conduit à l'émergence de souches d'insectes résistants (Onstad, 2008 ; Tabashnik, 2008). D'où la nécessité pour les sociétés de semences de chercher d'autres molécules ayant des modes d'action différents pour créer de nouvelles variétés résistantes. C'est dans cette perspective que mon travail de thèse s'est inscrit, avec l'objectif d'identifier de nouvelles protéines entomotoxiques contre 2 des principaux ravageurs du maïs : la chrysomèle des racines du maïs (*Diabrotica virgifera virgifera*) (WCR) et la légionnaire d'automne (*Spodoptera frugiperda*).

Cette thèse s'inscrit dans un partenariat public-privé entre l'entreprise semencière Limagrain (basée à Chappes-France) et l'Institut National de la Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAe) DGIMI de Montpellier. Elle est co-financée par le ministère de l'Enseignement supérieur et de la Recherche et Limagrain au travers d'une bourse CIFRE. Limagrain a développé un programme de recherche en biotechnologie sur la lutte contre les insectes, en partenariat avec la société allemande KWS, en créant la joint-venture Genective. L'objectif principal de l'entreprise est de développer de nouvelles générations de maïs transgénique à forte efficacité contre les insectes. Elle vise les marchés américains et en particulier le marché nord-américain pour lequel les deux sociétés ont un semencier commun (AgReliant). Le projet de thèse est lié à un projet appelé DIETETIC (2016-2020) porté par Limagrain, et associant un autre partenaire privé (PROTEUS), deux laboratoires publics du CEA (Genoscope et LEMIRE) et un laboratoire d'une organisation internationale, CABI, situé en Hongrie. Ce projet, financé par la SEMAE (Organisation Interprofessionnelle des Semences et des Plantes, anciennement GNIS), avait pour objectif d'identifier des protéines insecticides contre la chrysomèle des racines du maïs (WCR), un ravageur apparu en France en 2002 et causant des dégâts croissants ces dernières années. Le deuxième ravageur ciblé dans la thèse est la chenille légionnaire d'automne. Les travaux concernant ces insectes ont été réalisés dans le cadre d'une collaboration entre Limagrain et l'équipe DIDI (Dynamiques des Interactions entre Densovirus et Insectes) qui fait partie de l'unité mixte de recherche DGIMI entre l'INRAe

et l'Université de Montpellier (<https://www6.montpellier.inrae.fr/dgimi/>). Pour ce ravageur, nous avons étudié l'interaction potentielle entre un virus entomopathogène et des protéines insecticides bactériennes. L'objectif était d'évaluer si la combinaison des deux pourrait améliorer la pathogénicité des protéines insecticides d'origine microbienne et par la suite, utiliser le virus pour renforcer l'efficacité de nos protéines candidates. Ainsi, l'objectif principal de cette thèse est d'explorer le monde microbien pour identifier de nouvelles bactéries/composés actifs contre les ravageurs du maïs, et distincts de ceux actuellement utilisés soit en biocontrôle, soit dans le maïs transgénique.

Dans un premier temps, nous avons recherché des protéines insecticides candidates ayant une activité contre *D. virgifera virgifera*. Ces protéines candidates ont été recherchées parmi celles dérivées de bactéries autres que *Bacillus* spp. Ces protéines devaient avoir un mode d'action différent de celui des protéines actuellement disponibles sur le marché. Dans cette partie, nous nous sommes concentrés sur la caractérisation de quelques protéines insecticides qui ont montré une activité contre les populations de WCR sensibles mais également aux populations résistantes aux toxines actuellement utilisées (Cry3 et Gpp34Ab1/Tpp35Ab1), et donc ayant potentiellement un nouveau mode d'action.

Dans un deuxième temps, nous avons effectué des tests préliminaires en utilisant des protéines Bt commerciales pour évaluer si le densovirus *Junonia coenia* densovirus (JcDV), connu pour infecter les larves de *S. frugiperda*, pourrait améliorer la pathogénicité des protéines insecticides. Cette partie a consisté à étudier l'interaction entre les toxines Bt commerciales et soit le virus JcDV, soit la protéine majoritaire de la capsid (VP4). Cette dernière est capable de s'auto-assembler en pseudo-particules et de véhiculer des peptides insecticides quand ils sont exprimés en fusion dans *Escherichia coli* (Bonning and Kemmerer, 2020). Dans cette étude, nous avons également exprimé la protéine VP4 *in planta* pour vérifier sa potentialité de former des pseudo particules actives. Ici, l'objectif est de les utiliser pour augmenter la pathogénicité des protéines insecticides bactériennes dans une approche biotechnologique pour créer de nouvelles variétés de maïs avec une grande efficacité contre FAW.

Chapitre 1 : Identification et caractérisation de protéines insecticides contre WCR

1. Stratégie

L'identification de nouvelles protéines « candidates » ayant une activité contre les lépidoptères et coléoptères se fait *in silico* par la recherche de séquences homologues à des

toxines connues à partir de génomes bactériens. Dans le cadre du projet DIETETIC, 2500 génomes bactériens ont été séquencés dans cette optique et analysés afin d'identifier des séquences ayant une homologie avec des toxines connues. Les protéines sont ensuite produites sous une forme recombinante dans *E. coli*. Les lysats sont testés sur des larves du premier stade (L1) des différents insectes cibles (souche sensible) en utilisant la technique de bio-essai décrite par (Graser et Walters, 2016). La mortalité des larves est évaluée après 3 et 5 jours. Ces tests de criblage de routine se font entre Biogemma et notre partenaire du CABI pour la chrysomèle des racines du maïs. Des tests ont également été faits sur d'autres ravageurs du maïs : chez Biogemma pour la pyrale du maïs (*Ostrinia nubilalis*) et chez Genective pour un ensemble d'autres espèces de l'ordre des lépidoptères (la noctuelle du maïs (*Helicoverpa zea*), l'armigère (*Helicoverpa armigera*), la légionnaire d'automne (*Spodoptera frugiperda*), la pyrale du Sud-Ouest (*Diatraea grandiosella*)). Si une toxine candidate montre une activité contre une souche sensible d'une espèce donnée, elle est ensuite testée contre des souches résistantes à des toxines commerciales pour déterminer si le mode d'action est similaire ou différent. La société Genective, a un accès à de nombreuses souches résistantes disponibles des différents insectes du maïs, entres autres, les souches de *D. virgifera virgifera* résistantes à Cry3Bb1 (Vaughn et al., 2005) et le couple Cry34Ab et Cry35Ab (Ellis et al., 2002). Si le niveau de toxicité est suffisant en particulier sur les souches résistantes aux toxines commerciales, la toxine candidate est classée comme « Protéine Active » *in vitro*. Pour tester son activité *in planta*, la toxine peut être exprimée soit de façon transitoire chez *Nicotiana benthamiana* par agro-infiltration, soit de façon stable chez le maïs par transgénèse. L'activité des extraits de protéines totales est testée en utilisant la même technique de bio-essai décrite précédemment. Une fois l'activité toxique validée sur extraits de plantes, l'activité entomotoxique est évaluée sur des plantes de maïs transformées en serre dans un premier temps, puis en champ dans un deuxième temps. Ces essais sont réalisés aux Etats Unis par Genective. Ils varient selon l'insecte cible : test sur les racines pour *D. virgifera virgifera* et tests sur feuilles pour les lépidoptères. Les dommages sur la plante sont évalués 1 à 2 semaines après infestation.

2. Identification et caractérisation d'une nouvelle famille de protéines insecticides contre WCR

2.1 Une nouvelle toxine binaire isolée de *Chryseobacterium arthrosphaerae* contrôle *Diabrotica virgifera virgifera* via un mode d'action différent de celui des toxines commerciales existantes (article)

En se basant sur la stratégie décrite ci-dessus, nous avons identifié une nouvelle famille de protéines pesticides binaires actives contre les larves de WCR. Ces protéines ont toutes été trouvées dans plusieurs souches d'espèces de *Chryseobacterium*. Nous avons concentré notre étude sur un couple de protéines appelé GDI0005A/GDI0006A trouvé dans le même opéron du génome de *Chryseobacterium arthrosphaerae*. Elles ont été choisies pour leurs faibles similitudes avec une classe de protéines binaires insecticides (PIP) décrites dans un brevet de Pioneer (Gruver et al., 2016). L'activité consiste en une mortalité significative et des effets subléthaux sur des larves de WCR sensibles. Lorsqu'il a été testé sur des souches de WCR résistantes aux protéines insecticides provenant de *Bacillus thuringiensis* disponibles dans le commerce (Cry3Bb1 et Gpp34Ab1/Tpp35Ab1), une perte de poids significative a été observée. Ceci suggère que GDI0005A/GDI0006A pourrait avoir un récepteur différent de celui des toxines Cry, impliquant un nouveau mode d'action.

Les protéines GDI0005A et GDI0006A ont été isolées à partir de la souche *Chryseobacterium arthrosphaerae* ZSTG2175. Cette souche provient d'une collection de notre collaborateur CEA LEMIRE (Cadarache) isolée du désert du Sahara. Dans les collections publiques, nous avons identifié une autre souche de *C. arthrosphaerae* (DSM25208) dont le génome était très proche. Un test PCR à partir de cultures liquides a confirmé la présence des gènes codant pour GDI0005A et GDI0006A dans les deux souches. Le séquençage des produits PCR a aussi validé que les deux souches portent des séquences identiques à celles de GDI0005A/ GDI0006A. Dans le but de les utiliser en biocontrôle, les deux souches ont été cultivées avec un milieu d'auto-induction riche. Deux répliques biologiques indépendants ont été faits. Les deux expériences ont révélé que les échantillons des souches ZSTG2175 et DSM25208 provoquaient une mortalité et un retard de croissance chez les larves de chrysomèle au bout de 5 jours. L'activité de la souche DSM25208 était moins faible que celle observée avec la souche ZSTG2175. Ces résultats ouvrent de larges perspectives pour des applications de biocontrôle. Cependant, d'autres études seront nécessaires pour démontrer une corrélation entre l'activité des souches ZSTG2175/ DSM25208 et les protéines insecticides GDI0005A/GDI0006A.

L'ensemble de ces données a été utilisé pour la rédaction d'un article par Jabeur *et al* intitulé « A novel binary pesticidal protein from *Chryseobacterium arthrosphaerae* controls *Diabrotica virgifera virgifera* via a different mode of action to existing commercial pesticidal proteins » soumis au journal PLOS ONE le 04/10/21.

3. Evaluation du niveau d'efficacité de GDI0005A/ GDI0006A :

Pour caractériser le niveau de toxicité de GDI0005A/GDI0006A, la concentration létale médiane (LC50) doit être déterminée. Par conséquent, des protéines GDI0005A et GDI0006A purifiées sont requises pour effectuer ces bio-essais. Alors que la protéine GDI0005A a été purifiée avec succès, des difficultés ont été rencontrées pour exprimer et purifier la protéine GDI0006A. Une analyse de la séquence de la protéine GDI0006A et de ses homologues a permis de détecter un peptide signal de type II situé à l'extrémité N-terminale avec un résidu cystéine (Cys) conservé. Selon les outils de prédiction, GDI0006A est probablement une lipoprotéine ancrée dans la membrane externe. Cela pourrait expliquer les difficultés observées tout au long du processus de purification de ces protéines. Des travaux ont été entrepris pour améliorer la solubilité et l'expression de la protéine GDI0006A. Le challenge a été de choisir un détergent qui préserve l'activité et la structure de la protéine. Le n-dodécyl β -D-maltoside (DDM), un détergent doux non ionique, connu pour améliorer la stabilité des protéines membranaires (Rouse et al., 2013), a facilité l'extraction et la purification de GDI0006A. La fraction finale purifiée ayant une taille de 40 kDa (inférieure à la taille attendue 52kDa) était active lorsqu'elle a été testée avec GDI0005A purifiée contre les larves de chrysomèle. Ces résultats montrent que la fraction active de GDI0006A est une protéine faisant 40 kDa. Une analyse par MALDITOF serait nécessaire pour vérifier la séquence de cette fraction.

4. Evaluation de l'expression et de l'activité de GDI0005A/GDI0006A *in planta* :

Dans le but d'étudier l'expression et l'activité de GDI0005A/GDI0006A *in planta*, nous avons utilisé 2 approches : l'agro-infiltration pour produire transitoirement ces protéines dans des feuilles de *Nicotiana benthamiana* (NB) et la transgénèse pour les exprimer de façon stable chez le maïs. GDI0005A/GDI0006A ont été exprimés sous le contrôle du promoteur viral constitutif fort du virus de la mosaïque de la veine du manioc (CsVMV) pour exprimer fortement les protéines (Oyelakin et al., 2015). Les constructions moléculaires et la transformation dans *Agrobacterium tumefaciens* (AT) ont été réalisées par le laboratoire TEV de Limagrain dirigé par Laurent Beuf. 7 jours après l'infiltration des feuilles de NB avec AT portant les gènes d'intérêt, les protéines totales ont été extraites et les échantillons ont été utilisés pour réaliser des analyses par western blots et des bio-essais contre les larves de WCR. Les résultats montrent que GDI0005A était fortement exprimé à la bonne taille (environ 66 kDa). En revanche, les premiers essais d'expression de GDI0006A *in planta* n'ont pas été un succès. Des difficultés ont été observées aussi bien avec l'expression stable que transitoire. L'expression de protéines bactériennes dans un système végétal reste un défi pour assurer un niveau

d'expression suffisant pour contrôler les insectes. L'optimisation des codons est nécessaire pour une meilleure expression des protéines dans les plantes (Watts et al., 2021). Puisque les codons avaient déjà été modifiés pour les rendre plus conformes à l'utilisation en plante, nous avons décidé d'adresser GDI0006A dans différents organites cellulaires (chloroplaste, apoplaste, corps protéique, noyau, peroxyosome). De nombreuses recherches ont démontré que le ciblage de la protéine d'intérêt dans différents compartiments cellulaires peut améliorer son accumulation dans la plante (Conley et al., 2011; Dai et al., 2000; Shen et al., 2017). Cependant, avec GDI0006A, cette approche s'est avérée infructueuse. De plus, une phytotoxicité a été observée lorsque la protéine a été adressée dans le noyau ou dans le chloroplaste. Un ARNm instable, une traduction non efficace ou une dégradation rapide de la protéine sont des hypothèses possibles pour expliquer le faible niveau d'accumulation de GDI0006A dans les plantes (Watts et al., 2021). Le faible niveau d'expression de GDI0006A dans le maïs et dans NB pourrait expliquer l'absence d'activité du couple GDI0005/ GDI0006A. D'où la nécessité de surmonter le problème lié à la production insuffisante de GDI0006A *in planta* pour pouvoir atteindre l'objectif d'utiliser GDI0006A en association avec GDI0005A dans une approche biotechnologique pour lutter contre les larves de la chrysomèle du maïs.

Chapitre 2 : Évaluation du rôle potentiel du densovirus *Junonia coenia* (JcDV) dans l'amélioration de l'activité des protéines insecticides d'origine bactériennes

1. Interaction protéines virales/toxines :

Le but de cette étude est de tester si des protéines virales du densovirus *Junonia coenia* densovirus JcDV qui infecte les larves de *Spodoptera frugiperda* (Mutuel et al., 2010) pourraient améliorer la toxicité des toxines candidates. Pour se faire, la toxicité d'un mélange de toxine et de protéines virales sera testée sur des larves de *S. frugiperda* par la technique de bio-essai. Pour évaluer le passage des toxines candidates au travers de la membrane en présence des protéines virales, la méthode de la chambre de Ussing sera utilisée. JcDV est composé de trois protéines non structurales (NS1, NS2 et NS3) et de quatre protéines structurales, les protéines de capsid (VP1, VP2, VP3 et VP4) (Bossin et al., 2003; Bruemmer et al., 2005). Il a été démontré que VP4, la protéine majoritaire de la capsid, s'auto-assemble spontanément lorsqu'elle est exprimée seule en utilisant un système baculovirus (Bruemmer et al., 2005; Croizier et al., 2000). De plus, cette pseudo-particule (également connue sous le nom de *virus-like particle* VLP) est capable de traverser l'épithélium intestinal et de transporter divers peptides insecticides lorsqu'ils sont exprimés sous forme de protéine de fusion (Bonning and Kemmerer, 2020; Kemmerer and Bonning, 2018). Cette propriété de VP4 ouvre la voie à l'étude

des interactions possibles entre VP4 et les protéines insecticides d'origine bactérienne lorsqu'elles sont exprimées dans les plantes. Dans cette optique, la protéine VP4 sera exprimée de façon transitoire chez *Nicotiana benthamiana* par agro-infiltration. Les extraits de protéines totales à partir des zones infiltrées avec des *Agrobacterium tumefaciens* contenant le gène qui code pour VP4, seront utilisées pour vérifier le niveau d'expression (par western blot) et la formation des pseudo-particules (par coloration négative en microscopie à transmission électronique). Ces mêmes extraits seront testés en chambre d'Ussing pour évaluer le passage des VP4 à travers l'intestin des larves de FAW. Des coupes fines sur des morceaux de feuilles agro-infiltrées seront réalisées pour vérifier l'expression des VP4s dans les tissus des plantes (par immunomarquage avec une visualisation en microscopie confocale) ainsi que leur localisation dans les cellules (par coloration positive en microscopie à transmission électronique).

1.1 Etude de l'interaction entre JcDV et une toxine Bt commerciale Cry2Ab2 :

Une dose sub létale de Cry2Ab2 et la dose correspondant à la LC50 du JcDV ont été mélangées et testées contre les larves de *S. frugiperda*. Lorsque JcDV et Cr2Ab2 sont combinées, on observe une diminution de la mortalité des larves due au virus. L'effet du mélange de virus et de Cry2ab2 sur la barrière intestinale a été testé *ex vivo* en chambre d'Ussing. Le passage de molécules de Dextran-FITC (4kDa) du côté luminal vers le compartiment basal a été évalué après 2 heures d'incubation avec l'intestin. Les résultats ont montré qu'il y a plus de passage de molécules de Dextran quand le virus est incubé seul avec l'intestin par comparaison au mélange virus + Cry2ab2 confirmant les bio-essais sur les larves. De plus, les données de PCR quantitative indiquent un passage significativement plus élevé du virus en absence de la toxine Cry2Ab2. Ces résultats montreraient qu'il existe potentiellement un antagonisme entre la toxine Cry2Ab2 et le JcDV qui pourrait être lié à une compétition pour les récepteurs intestinaux. D'après la littérature, l'aminopeptidase et l'alkaline phosphatase sont 2 récepteurs actifs pour les protéines Bt de type Cry (Gómez et al., 2018; Jakka et al., 2016, 2016; Onofre et al., 2017). L'interaction de JcDV avec ces 2 récepteurs a déjà été démontrée dans les travaux de thèse de (Pigeyre, 2019). Il est donc possible qu'il y ait une compétition potentielle pour ces 2 récepteurs quand la protéines Cry2Ab2 et JcDV sont mélangés et testés ensembles.

2. Expression de la protéine VP4 *in planta* :

La séquence nucléique codant pour la protéine VP4 a été mise sous le contrôle d'un promoteur de plantes et inséré dans un vecteur binaire puis intégré dans une souche d'*Agrobacterium tumefaciens* pour réaliser des expressions transitoires dans des feuilles de tabac. Aucun signe de phytotoxicité n'a été observée sur les feuilles de *Nicotiana bentamiana* après infiltration de la souche *Agrobacterium* contenant le gène qui code pour VP4. Le niveau d'expression de la VP4 a été vérifié sur un extrait de feuilles agro-infiltrées, par western blot en utilisant un anticorps anti-VP de JcDV. Ces extraits de feuilles agro-infiltrées ont également été observés au microscope électronique à transmission après coloration négative. Les résultats ont montré que la protéine VP4 est capable de s'auto-assembler en plante pour former des pseudo-particules de diamètre attendu (Gosselin-Grenet et al., 2015) de 25 nm de environ. Un immunomarquage de la VP4 sur des coupes fines des feuilles agro-infiltrées a révélé une localisation de la VP4 autour des chloroplastes et des noyaux. Dans certaines coupes, un marquage nucléaire a été mise en évidence. L'effet de l'extrait de protéines totales des feuilles de NB contenant la VP4 sur la barrière intestinale de *Spodoptera frugiperda* a été évalué *ex vivo* en chambre d'Ussing. Après 2 h d'incubation, l'intestin a été fixé en utilisant du paraformaldéhyde. L'immunomarquage de la VP4 sur des coupes fines de l'intestin a montré une concentration de la VP4 dans le cytoplasme des cellules intestinales, notamment au niveau des microvillosités. Trois répliques ont été effectués. Ces résultats indiquent que les VLP constituées de VP4 (VLP-VP4) sont capables d'entrer dans l'épithélium intestinal. D'autres expériences seront nécessaires pour savoir si les VLP-VP4 sont capables de traverser l'épithélium intestinal par transcytose comme le font les particules virales de JcDV, et si l'entrée et/ou la transcytose entraîne une perméabilisation intestinale comme le fait JcDV.

Conclusion générale

Les insectes présentent une menace constante pour les plantes cultivées. Ceci est notamment vrai pour le maïs qui subit chaque année des pertes de rendement importantes dues principalement aux attaques d'insectes. La grande adaptabilité des insectes aux stratégies de lutte rend leur contrôle plus difficile. Les coléoptères et les lépidoptères sont parmi les groupes d'insectes les plus importants qui causent de sérieux dégâts au maïs. Le maïs Bt a été une bonne alternative jusqu'à l'apparition de populations résistantes. Le principal défi aujourd'hui reste celui de trouver de nouvelles molécules et stratégies respectueuses de l'environnement pour lutter durablement contre ces insectes.

Dans le cadre de ce programme de thèse, mon travail s'est concentré sur deux principaux ravageurs du maïs, la chrysomèle des racines du maïs *Diabrotica virgifera virgifera* (coléoptère) et la chenille légionnaire d'automne *Spodoptera frugiperda* (lépidoptère). Pour ces deux insectes, nous avons recherché des alternatives aux molécules existantes qui soient utilisables soit pour développer de nouvelles variétés de maïs résistantes (solutions biotechnologiques) soit directement en lutte biologique.

Pour les applications biotechnologiques, nous avons découvert de nouvelles protéines pesticides actives contre le WCR et présentes dans différentes souches de *Chryseobacterium*. Nous avons démontré que la toxicité dépendait de deux composants protéiques comme pour les protéines binaires commerciales Cry34/Cry35 et que le mode d'action de ces protéines était différent des modes d'action commerciaux existants. D'importantes difficultés ont été rencontrées pour purifier le second membre de la protéine binaire en raison de sa nature membranaire. Cependant, j'ai pu réaliser une purification partielle mais qui nécessitera une optimisation supplémentaire avant de pouvoir mener des études d'efficacité (LC50) de la protéine binaire contre le WCR. Les études préliminaires visant à exprimer les protéines actives dans NB ou dans le maïs ont échoué. L'utilisation potentielle de cette protéine binaire pour générer du maïs transgénique résistant à WCR a donc été mise en attente en attendant d'autres données de purification, d'activité et d'expression dans la plante. Une deuxième approche pour des applications biotechnologiques dirigée contre l'autre ravageur du maïs *S. frugiperda* a été testée en utilisant une protéine de capsid (VP4) d'un virus entomopathogène de la FAW (JcDV). J'ai d'abord réussi à exprimer la protéine VP4 dans un système végétal sans aucun signe de phytotoxicité pour la plante. J'ai démontré que cette protéine de capsid peut s'auto-assembler en particules de type viral (VLP) in planta. La prochaine étape consistera à démontrer

que les VLP produites dans les plantes sont capables de traverser l'intestin moyen comme le virus JcDV entier et à tester différentes méthodes de combinaison de la protéine VP4 avec des toxines Bt pour évaluer leur complémentarité afin d'atteindre l'objectif recherché (i.e. accroître la toxicité des toxines bactériennes).

Dans une perspective d'utilisation en lutte biologique, nous avons évalué le potentiel de la combinaison du densovirus JcDV et de la protéine Bt (Cry2Ab2) contre la FAW. Les deux sont connues pour leur action sur la perméabilisation de l'intestin de FAW. Les deux protéines ont été mélangées pour des expériences de bio-essai afin d'évaluer les synergies potentielles. Les résultats ont montré un antagonisme inattendu confirmé par nos expériences *ex vivo* utilisant la chambre d'Ussing, suggérant une compétition pour les mêmes récepteurs ou une interaction physique. Le résultat le plus important que j'ai obtenu dans l'approche de biocontrôle concerne l'utilisation potentielle de souches de *Chryseobacterium* contre la chrysomèle du maïs. Bien que l'origine de l'activité insecticide observée avec cette bactérie et la corrélation avec l'activité de nos protéines pesticides soient encore inconnues, nous avons démontré leur efficacité *in vitro* contre les larves de WCR. Les prochaines étapes seront de déterminer la méthode de production de la molécule active à partir des souches bactériennes qui soit compatible avec la réglementation du biocontrôle et sans risque de sécurité.

Cette thèse m'a donné l'opportunité de faire des recherches dans le domaine de la protection des cultures selon deux perspectives distinctes biotechnologiques et biologiques. Cette double vision a été présente dans toutes mes approches.

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Abstract: Due to their great adaptability to management strategies, insect pests are a constant threat to crops. Maize is the third most important staple crop in the world after wheat and rice. The western corn rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte, 1868) and the fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith, 1797) are among the most serious pests of maize and are responsible for massive yield losses every year. Bt maize expressing insecticidal proteins from *Bacillus thuringiensis* has long been a good alternative to chemicals. However, their overuse has led to the development of resistant populations. Hence, the need to find new compounds and strategies for sustainable control of these insects. In this PhD project, we aimed to develop biotech and biocontrol solutions for both WCR and FAW. In the first part, I was particularly interested in the study of binary insecticidal proteins named GDI0005A/GDI0006A identified by screening bacterial genomes and test against WCR larvae. These proteins were identified in *Chryseobacterium arthrosphaerae* genome. Heterologous expression of the two components was performed in *E. coli* system. One of the two components (GDI0006A) was a membrane lipoprotein and resulted in low amounts successfully purified. Nonetheless, the combination of the two proteins was active against WCR including WCR resistant to the currently marketed Cry3Bb1 and Gpp34Ab1/Tpp35Ab1 proteins. These results suggest that the new binary toxin may have different binding sites and thus a distinct mode of action compared to commercial toxins. The binary proteins were then expressed successfully in plant expression systems. However, the expression at low level of GDI0006A lead to no significant activity in planta. Finally, we tested the bacteria *C. arthrosphaerae* and show that it had activity in vitro against WCR, making of it a potential BCA against WCR.

The second part was devoted to studying the possible synergy between *Junonia coenia* densovirus (JcDV) and a Bt insecticidal protein Cry2Ab2 for biocontrol applications. Both are known to infect FAW and disturb the midgut permeability. However, when tested together either with *ex vivo* or *in vivo* approaches, we have noticed an antagonism suggesting a competition for the same midgut receptors. We also checked the feasibility of using VP4, the major capsid protein of JcDV in a biotech approach to enhance the activity of other gut binding insecticidal proteins. The preliminary results have shown a good expression of VP4 in *Nicotiana benthamiana* leaves without any sign of phytotoxicity. We demonstrated the ability of VP4 to self-assemble into virus-like particles (VLPs) in plant. When tested *ex vivo*, VLP-VP4 were able to penetrate the FAW intestinal cells. This proof of concept will be useful to explore the possible synergies with gut-binding insecticidal proteins to create new generations of transgenic maize resistant to FAW.

Keywords: *Diabrotica virgifera virgifera*, *Spodoptera frugiperda*, maize, insecticidal protein, *Junonia coenia* densovirus, insect midgut, synergy, biocontrol, resistance, transgenesis, virus-like particles

Résumé : Du fait de leur grande adaptabilité aux méthodes de lutte, les insectes demeurent une menace constante pour les plantes cultivées. Le maïs (*Zea mays* L.) est la troisième culture la plus importante dans le monde après le riz et le blé. La chrysomèle des racines du maïs (WCR) *Diabrotica virgifera virgifera* (LeConte, 1868) et la chenille légionnaire d'automne (FAW) *Spodoptera frugiperda* (J.E. Smith, 1797) sont parmi les ravageurs les plus importants du maïs et sont responsables de fortes pertes de rendement chaque année. Le maïs Bt exprimant des protéines insecticides provenant de *Bacillus thuringiensis* a été pendant longtemps une bonne alternative aux pesticides. Cependant, leur utilisation massive a conduit à l'apparition de populations de ravageurs résistantes, d'où la nécessité de trouver de nouvelles molécules et stratégies biotechnologiques ou de contrôle biologique pour gérer durablement ces insectes. Mes travaux de thèse s'inscrivent dans cet objectif et visent à trouver de nouvelles solutions contre WCR et FAW. Dans la première partie, je me suis particulièrement intéressée à une protéine binaire appelée GDI0005A/GDI0006A identifiée dans un criblage contre des larves de WCR. Ces protéines ont été isolées de la bactérie *Chryseobacterium arthrosphaerae* et ont montré une activité insecticide, lorsqu'elles sont exprimées sous forme de protéines recombinantes, contre des populations de WCR sensibles et résistantes aux protéines commerciales Cry3Bb1 et Gpp34Ab1/Tpp35Ab1. Ces résultats suggèrent que ces nouvelles protéines insecticides pourraient avoir des modes d'action différents de ceux des protéines Bt actuellement déployées et donc un nouveau mode d'action. Le deuxième membre de la protéine binaire (GDI0006A) est une lipoprotéine membranaire que j'ai purifiée partiellement. Cette propriété a été un facteur limitant pour évaluer le niveau d'activité de la protéine GDI0005A/GDI0006A. Nous avons essayé d'exprimer la protéine binaire en plante en utilisant deux systèmes d'expression : transitoire et stable. Les deux ont montré une faible accumulation de GDI0006A entraînant une perte de l'activité par rapport aux essais au labo avec des lysats bactériens. Nous avons également démontré que des cultures de la bactérie d'origine *C. arthrosphaerae* avait une activité contre WCR ce qui en fait un agent de biocontrôle potentiel contre les chrysomèles.

La deuxième partie a été consacrée à l'étude de l'éventuelle synergie entre *Junonia coenia* densovirus (JcDV) et une protéine insecticide Bt Cry2Ab2 dans une perspective de les combiner pour des applications de biocontrôle. Les deux sont connus pour infecter FAW et perturber la perméabilité intestinale de ce ravageur. Cependant, lorsqu'ils ont été testés ensemble avec des approches *ex vivo* et *in vivo*, nous avons plutôt remarqué un antagonisme suggérant une compétition avec les mêmes récepteurs intestinaux. Nous avons aussi vérifié la faisabilité de l'utilisation de VP4, la protéine majoritaire de la capsid de JcDV, dans une approche biotechnologique pour améliorer l'activité d'autres protéines insecticides qui ciblent l'intestin. Les résultats préliminaires ont montré une expression importante de VP4 dans les feuilles de *Nicotiana benthamiana* sans aucun signe de phytotoxicité. Nous avons aussi mis en évidence la capacité de VP4 à s'auto-assembler en pseudo-particules virales (VLP) dans la plante. Ces VLP-VP4 ont été capables de pénétrer dans les cellules intestinales de FAW lorsqu'elles ont été testées *ex vivo*. Cette preuve de concept sera utile pour explorer les synergies possibles avec des protéines insecticides qui affectent l'intestin dans le but de créer de nouvelles variétés de maïs transgéniques résistants à FAW.

Mots clés : *Diabrotica virgifera virgifera*, *Spodoptera frugiperda*, maïs, protéine insecticide, *Junonia coenia* densovirus, intestin de l'insecte, synergie, biocontrôle, résistance, transgenèse, pseudo-particule virale