

Dottorato in Scienze Biotecnologiche – XXIX ciclo

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TRANSEPITHELIAL DELIVERY OF MACROMOLECULAR BIOPESTICIDES

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Summary

The need to reduce the use of chemical pesticides in agriculture and the decrease in number of available molecules has promoted intense research efforts towards the identification of new bioinsecticides of natural origin and of appropriate delivery strategies. This has allowed the isolation and characterization of a wealth of bioactive molecules, mainly peptides and proteins derived from different sources such as bacteria, viruses, insect predators/parasitoids, arthropods and plants, which are natural antagonists of insects. Moreover, the study of the molecular mechanisms mediated by these novel bioinsecticides and the identification of their cognate receptors offer the opportunity to develop bioinspired strategies mimicking the negative effects on host insects by natural antagonists through the use of “RNAi mediated crop protection” technologies. The high specificity of these antagonistic associations and the targeted gene silencing action of properly tailored dsRNAs confer to these novel tools and strategies for pest control a high degree of selectivity, that make them ideal candidates for inclusion in sustainable IPM plans. However, specific delivery strategies have to be developed in order to allow these molecules to resist the harsh physicochemical environment of the insect gut and to overcome the lining epithelial layer.

The present work aims at providing new delivery strategies for proteinaceous and oligonucleotidic molecules to control phytophagous insect pests.

We focused, first, on the use of XXX as delivery vector of a neurotoxin derived from the salivary glands of the spider *Segestria florentina*, SFI2.6, and, then, screened specific XXX domains to assess their capacity to mediate transepithelial delivery of fused toxins. SFI2.6/XXX, SFI2.6/XXX domain1, SFI2.6/XXX domain2, SFI2.6/XXX domain3 fusion proteins have been produced in yeast. Recombinant SFI2.6/XXX and SFI2.6/XXX domain1 have been produced in suitable amounts for their use in feeding bioassays on the pea aphid *Acyrtosiphon pisum*. The high mortality obtained with these fusion proteins suggests that XXX and domain1 are extremely efficient carriers for the oral delivery of toxins active in the body cavity. Future studies including the other two XXX domains may further contribute to the development of novel delivery strategies.

To promote the feasibility of RNAi technology for pest control, we propose a new delivery strategy of dsRNAs, here used to target host immune genes, in order to induce an immunosuppressive status enhancing the impact of entomopathogens. Two immune genes of the noctuid moth *Spodoptera littoralis* have been considered (*SI 102* and *SI gasmin*), one of which (*SI gasmin*) has been isolated and characterized as part of this thesis work, showing its potent role as opsonizing factor mediating phagocytosis. Vectors for the expression in bacteria of *SI 102* and *SI gasmin* dsRNA have been designed and recombinant bacteria producing *SI 102* dsRNA, when orally delivered to *S. littoralis* larvae were able to specifically reproduce the high level of gene silencing observed with dsRNA obtained *in vitro*, along with the immunosuppressive phenotype induced.

In conclusion, the results obtained with both fusion proteins and dsRNA-expressing bacteria are very promising and contribute to inspire new ideas for the delivery of insecticidal molecules, in particular of macromolecules targeting haemocoelic receptors or silencing host immune genes, in order to enhance insect sensitivity to natural antagonists.

Riassunto

I metodi oggi disponibili per la protezione delle colture dagli insetti si basano essenzialmente sull'uso di insetticidi chimici di sintesi. L'impiego e, soprattutto, l'abuso di questi ultimi ha creato una serie problemi, come lo sviluppo di resistenza negli insetti, la contaminazione ambientale ed gli effetti indesiderati sugli artropodi utili, importanti fornitori di servizi ecologici quali gli antagonisti naturali e gli impollinatori. La riduzione dell'uso di questi prodotti è uno dei principali obiettivi da perseguire al fine di rendere sempre più sostenibile l'agricoltura sia dal punto di vista ecologico che economico. Privilegiare l'adozione di strategie biologico-integrate e di prodotti alternativi a quelli chimici di sintesi non è solo opportuno, ma anche imposto dalla direttiva europea sull'uso sostenibile dei pesticidi ("Sustainable use of pesticides and alternative non chemical methods for plant protection and pest management", 2009/128/EC).

Il controllo biologico (De Bach, 1964) ha un ruolo fondamentale nella lotta integrata agli insetti dannosi (Integrated Pest Management, IPM), che è basata sull'uso di tutti i possibili mezzi di lotta, nel rispetto di principi economici, ecologici e tossicologici (Viggiani, 1994). Nella sua accezione più stretta, il controllo biologico classico include l'uso di antagonisti naturali per il controllo degli insetti dannosi. Fra i principali antagonisti naturali degli insetti ci sono proprio gli insetti, predatori e parassitoidi, altri artropodi, nematodi, virus e microrganismi entomopatogeni. Molto spesso, formulati commerciali a base di questi ultimi e/o di loro tossine vengono denominati come bioinsetticidi. Talora, tale termine viene usato anche per indicare molecole o complessi di molecole naturali, spesso prodotte da antagonisti di insetti, che hanno attività insetticida. I bioinsetticidi rappresentano una valida alternativa ai composti chimici di sintesi soprattutto per la bassa tossicità nei confronti di organismi non-target e vertebrati, la ridotta persistenza nell'ambiente e la minore probabilità di insorgenza dei fenomeni di resistenza, grazie alla presenza di differenti componenti bioattive.

Negli ultimi anni molte attività di ricerca sono state finalizzate all'individuazione di nuove molecole bioinsetticide. Tra queste, la maggior parte è rappresentata da composti, principalmente peptidi e proteine, isolati da batteri, virus, predatori/parassitoidi degli insetti, artropodi e piante (Whetstone and Hammock, 2007). Accanto all'individuazione, è fondamentale la ricerca volta alla definizione di opportuni metodi di somministrazione orale di questi composti, che possono essere soggetti a degradazione enzimatica nel lume intestinale dell'insetto e, nel caso di target localizzati nell'emocele, essere incapaci di attraversare l'epitelio intestinale in forma attiva e quantità sufficiente (Bonning and Chougule, 2014).

Più recentemente, ha inoltre riscosso grande interesse nella comunità scientifica l'utilizzo dell'RNA di interferenza (RNAi) per il controllo degli insetti dannosi (RNAi mediated crop protection) (Gu and Knipple, 2013). L'RNAi, un meccanismo di regolazione dell'espressione genica mediata da piccoli RNA a doppio filamento, può essere sfruttato per il controllo degli insetti tramite la somministrazione orale di molecole di dsRNA aventi come bersaglio geni fondamentali per la sopravvivenza degli insetti dannosi e quindi causandone la morte. L'RNAi può inoltre essere utilizzato indirettamente, interferendo geni importanti nella risposta immunitaria degli insetti dannosi, al fine di esaltare su di essi l'impatto di antagonisti naturali come i microrganismi entomopatogeni (Chen *et al.*, 2015; Caccia *et al.*, 2016). L'identificazione di geni bersaglio specifici e, come nel caso di bioinsetticidi di natura

proteica, di opportune strategie di delivery del dsRNA sono requisiti necessari per l'impiego su larga scala di strategie di controllo degli insetti dannosi basate su RNAi. Questo progetto di tesi mira alla definizione di efficienti strategie di somministrazione di proteine insetticide di origine naturale e di molecole di dsRNA in grado di alterare la risposta immunitaria degli insetti dannosi e, quindi, di potenziare l'effetto di microrganismi entomopatogeni, in particolare di *Bacillus thuringiensis*.

L'assorbimento di proteine *in vivo* nell'intestino di insetto è stato riportato da molti autori (Jeffers and Roe, 2008; Burand and Hunter, 2013) e gli studi *in vitro* dei meccanismi responsabili di questo processo nell'intestino di lepidottero hanno messo in evidenza che il processo coinvolto è la transitosi (XXX). In particolare, XXX viene internalizzata grazie ad un meccanismo di endocitosi (XXX) mediato dal legame della XXX con un recettore "XXX", omologo al recettore XXX espresso in molti epiteli assorbenti di mammifero (XXX).

Queste informazioni hanno stimolato l'idea di studiare l'abilità della XXX, o dei suoi tre domini, di fungere da molecola vettore per proteine bioinsetticide con target emocelico, in particolare per la neurotossina SFI2.6 derivante dal veleno del ragno *Segestria florentina*. Tale tossina, caratterizzata da motivi strutturali che la rendono estremamente resistente alla proteolisi e alla degradazione ambientale, altera la neurotrasmissione bloccando selettivamente i canali del calcio in insetto (Lipkin *et al.*, 2002).

Le sequenze codificanti la XXX e i suoi tre domini, ottenute dal database UniProt, sono state clonate nel vettore pGAPZ α , adatto per l'espressione di proteine ricombinanti in *Pichia pastoris*, che conteneva la sequenza codificante per la tossina SFI2.6. I costrutti per le proteine di fusione sono stati disegnati in modo da fondere all'estremità N-terminale una sequenza codificante un peptide segnale per la secrezione delle proteine ricombinanti nel mezzo di coltura del lievito, e all'estremità C-terminale una coda di istidine per la purificazione delle proteine ricombinanti mediante cromatografia di affinità. Le proteine sono state prima prodotte in coltura su piccola scala da singole colonie di lievito trasformate con i costrutti di fusione ed esperimenti di Western blot hanno evidenziato che le proteine sono state espresse. Un clone di lievito per ciascun costrutto è stato, quindi, utilizzato per la fermentazione e la conseguente espressione delle proteine ricombinanti su media scala per la valutazione dell'attività biologica su insetto. Le proteine sono state purificate mediante cromatografia di affinità e la loro concentrazione è stata misurata con il metodo di Bradford. La produzione ha avuto una resa modesta e le proteine di fusione contenenti XXX e dominio1 sono state prodotte in una quantità sufficiente per essere utilizzate in biosaggi sull'afide *Acyrtosiphon pisum*. La resa delle proteine di fusione contenenti i domini 2 e 3 è stata minore e non ha consentito l'effettuazione del biosaggio.

Per effettuare i biosaggi su afide SFI2.6/XXX e SFI2.6/XXX domain1 sono stati disciolti nella dieta artificiale a una concentrazione finale di 0.1 mg/ml. XXX 0.1 mg/ml e GNA 0.1 mg/ml sono state utilizzate come controlli negativi, mentre la sola acqua e la proteina SFI2.6/GNA sono state utilizzate come controlli positivi, in quanto la GNA si è già dimostrata un buon vettore su afide (Fitches *et al.*, 2004; 2012; Wakefield *et al.*, 2010; Nakasu *et al.*, 2016), sono state utilizzate come controlli positivi. L'esperimento è stato condotto su neanidi di terza età (30 per ogni trattamento). I risultati ottenuti mostrano che la sopravvivenza degli afidi a cui sono state somministrate le proteine di fusione SFI2.6/XXX e SFI2.6/XXX domain1 è significativamente ridotta rispetto ai controlli, a cui sono state somministrate la dieta, la XXX 0.1 mg/ml, o la GNA 0.1 mg/ml (Figura 1). Inoltre SFI2.6/XXX e

SFI2.6/domain1 mostrano una tossicità anche maggiore rispetto al controllo positivo rappresentato da SFI2.6/GNA (Figura 1).

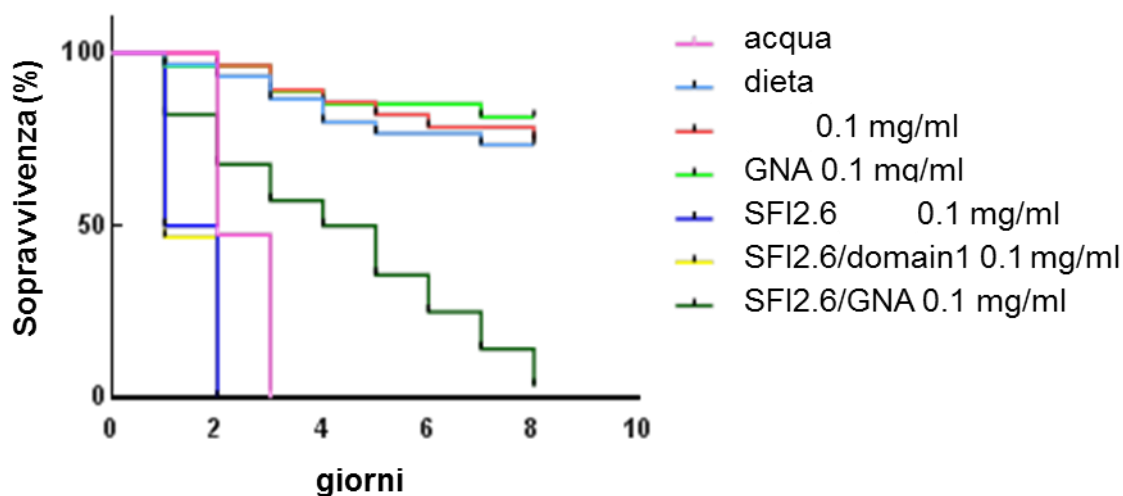


Figura 1. Curve di sopravvivenza Kaplan-Meier relative al biosaggio su dieta artificiale liquida, offerta in sacchetti di parafilm all'afide verde delle leguminose, *Acyrthosiphon pisum*.

In conclusione, i risultati ottenuti da biosaggi su afidi suggeriscono che sia la XXX che il suo dominio1 sono vettori estremamente efficienti per il trasporto transepiteliale della neurotossina SFI2.6. Sarà importante ottimizzare la produzione per ottenere maggiori rese e analizzare la tossicità orale nei confronti di *A. pisum* delle molecole di fusione contenenti il dominio 2 e 3, nonché eseguire biosaggi delle proteine di fusione su altri insetti fitofagi. I risultati potranno evidenziare eventuali differenze di tossicità e quindi consentire l'individuazione del miglior vettore tra le quattro proteine usate come carrier.

Un secondo aspetto studiato in questo lavoro di tesi è quello dell'individuazione di un efficiente vettore per le molecole di dsRNA, al fine di poter utilizzare il silenziamento genico su larga scala per il controllo degli insetti fitofagi in campo. In particolare, la ricerca è stata volta a sviluppare una strategia di delivery di molecole di dsRNA in grado di silenziare geni coinvolti nell'immunità dell'insetto fitofago scelto come modello, il lepidottero *Spodoptera littoralis*, e quindi di indurre una condizione di immunosoppressione per potenziare l'impatto di entomopatogeni.

Sono stati innanzitutto scelti due geni target da silenziare e coinvolti in diversi aspetti dell'immunità cellulare in *S. littoralis*. *Sl 102*, recentemente identificato e caratterizzato (Di Lelio *et al.*, 2014; Caccia *et al.*, 2016), è coinvolto nelle risposte di incapsulamento e nodulazione di organismi non-self e il suo silenziamento aumenta di circa 5 volte la tossicità di *B. thuringiensis* e delle sue tossine. *Sl gasmin* è stato identificato in *S. littoralis*, venendo caratterizzato a livello molecolare e funzionale per la prima volta in questo lavoro di tesi. *Sl gasmin*, omologo di *gasmin*, un gene coinvolto nella risposta immunitaria in larve di *S. exigua*, ha un ruolo chiave nella fagocitosi di patogeni.

Inizialmente è stato identificato un cDNA parziale nella library di EST (Expressed Sequence Tags) di *S. littoralis* che mostrava l'87% di identità di sequenza con il gene *gasmin* di *S. exigua*. E' stata quindi individuata la ORF (open reading frame) utilizzando il tool <https://www.ncbi.nlm.nih.gov/orffinder>, che poi è stata amplificata mediante PCR. Il cDNA codifica per una proteina predetta (*Sl gasmin*) di 346

amminoacidi (aa), con un putativo peptide segnale di 21 aa, che mostra un'identità di sequenza rispettivamente di 94% con *gasmin* di *S. littoralis*. Il profilo di espressione di *Sl gasmin* in differenti tessuti di larve di *S. littoralis* mostra i massimi livelli di trascrizione negli emociti e un incremento di trascritto in seguito a stimolazione immunitaria mediante iniezione di batteri Gram positivi (*Staphylococcus aureus*), Gram negativi (*Escherichia coli*) e lieviti (*Saccharomyces cerevisiae*). I risultati ottenuti suggeriscono quindi un ruolo chiave nella risposta immunitaria e per studiarlo nel dettaglio è stato utilizzato l'RNAi. I cambiamenti fenotipici associati al silenziamento di *Sl gasmin* hanno messo in evidenza il suo coinvolgimento nella fagocitosi di batteri sia Gram positivi (*S. aureus*) che Gram negativi (*E. coli*). La presenza di un putativo peptide segnale nella proteina codificata da *Sl gasmin* e la mancata fagocitosi associata al silenziamento hanno suggerito che *Sl gasmin* potesse avere un ruolo nell'ambiente extracellulare, probabilmente legandosi al patogeno per facilitarne il riconoscimento da parte degli emociti e quindi mediarne la fagocitosi. Per provare questa ipotesi è stato ideato un esperimento di recupero del fenotipo esponendo gli emociti ottenuti da larve silenziate (e quindi incapaci di fagocitare) al plasma derivante dai controlli. Questo ha permesso di ripristinare la funzione di fagocitosi di batteri *in vitro*. Al contrario, gli emociti ottenuti dai controlli hanno perso la loro attività fagocitaria quando risospesi nel plasma ottenuto da larve silenziate. Dunque *Sl gasmin*, una volta secreta dagli emociti, funge da agente opsonizzante nell'emolinfa ed è essenziale per mediare la fagocitosi.

Il silenziamento di tale gene aumenta, inoltre, la mortalità di larve di *S. littoralis* trattate con la tossina Cry1Ca di *B. thuringiensis*. Questo risultato dimostra il ruolo chiave che questo gene svolge *in vivo* nella risposta immunitaria dell'insetto.

Identificati i specifici geni bersaglio è stata quindi sviluppata una strategia di delivery del dsRNA in grado di silenziarli. La somministrazione orale è certamente il metodo più pratico e una tecnica molto promettente per realizzarla è rappresentata dall'espressione di molecole di dsRNA in batteri. Oltre all'abbattimento dei costi di produzione rispetto alla sintesi del dsRNA *in vitro*, le molecole di dsRNA risultano essere molto più stabili poiché meno esposte alla degradazione ambientale e nel lume intestinale dell'insetto, grazie alla protezione rappresentata dall'involucro batterico (Whyard *et al.*, 2009). Sono stati quindi costruiti vettori ricombinanti contenenti frammenti dei geni *Sl 102* e *Sl gasmin* (il gene *GFP*, assente in insetto, è stato usato come controllo) per l'espressione *in vivo* di molecole di dsRNA. I costrutti sono stati clonati utilizzando una strategia innovativa, basata sull'utilizzo del vettore *L4440*, che è stato convertito in un vettore Gateway. La tecnologia Gateway è un metodo di clonaggio universale basato sulla ricombinazione sito-specifica (Landy *et al.*, 1989). Questo sistema è risultato molto efficiente, permettendo un clonaggio veloce ed efficiente di entrambi i geni, *Sl 102* e *Sl gasmin*, e, più in generale, potrà risultare molto utile per il clonaggio di qualsiasi costrutto codificanti dsRNA da esprimere in batterio.

Il ceppo batterico di *E. coli* HT115 è stato trasformato con il vettore ricombinante contenente il frammento di *Sl 102* o *GFP* per la produzione di dsRNA. I batteri ricombinanti esprimenti *Sl 102* dsRNA e *GFP* dsRNA (batteri controllo) sono stati sottoposti a trattamento di sonicazione, al fine di ucciderli disgregandoli, facilitando, così, il rilascio nell'intestino delle molecole di dsRNA e riducendo anche i rischi associati alla loro immissione nell'ambiente. Sono poi stati condotti biosaggi su larve di *S. littoralis* somministrando oralmente i batteri ricombinanti sonificati tramite siringa o tramite applicazione su dieta artificiale. I livelli di trascritto del gene *Sl 102* negli emociti delle larve trattate con batteri esprimenti dsRNA *Sl 102* sono risultati

fortemente ridotti, con entrambe le modalità di somministrazione, rispetto alle larve controllo (trattate con batteri esprimenti dsRNA *GFP*) (Figura 2A). Inoltre, il silenziamento ottenuto mediante somministrazione orale è associato ad una ridotta capacità degli emociti di incapsulare sferette cromatografiche iniettate nelle larve trattate (Figura 2B), indicando chiaramente l'induzione di un fenotipo immunosoppresso.

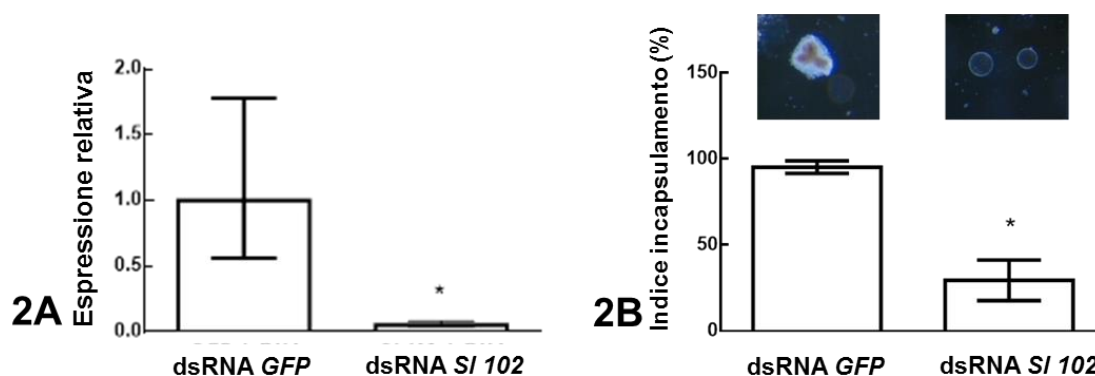


Figura 2. (A) Espressione relativa del gene *SI 102* negli emociti delle larve trattate oralmente con batteri esprimenti dsRNA *SI 102* o dsRNA *GFP* (controlli) e (B) rispettivi indici di incapsulamento di sferette cromatografiche da parte degli emociti.

In conclusione, la strategia di delivery orale del dsRNA *SI 102*, mediante l'utilizzo di batteri trasformati, è in grado di innescare una inibizione specifica dell'espressione del gene target e della conseguente immunosoppressione, riproducendo i risultati ottenuti mediante silenziamento con molecole di dsRNA prodotte *in vitro* (Di Lelio *et al.* 2014). Sarà interessante co-somministrare i batteri trasformati e *B. thuringiensis* (o le tossine da esso prodotte) per verificare l'utilizzabilità di questa tecnologia in campo, attraverso lo sviluppo di adeguate formulazioni.

Questo lavoro di tesi fornisce un contributo per lo sviluppo di nuove strategie di delivery orale sia di macromolecole proteiche con target emocelico, sia di molecole di dsRNA per il silenziamento di geni che svolgono ruoli chiave del sistema immunitario dell'insetto, al fine di potenziare l'azione di contenimento degli antagonisti naturali. Sebbene nel lavoro presentato siano state acquisite nuove interessanti informazioni relative alle possibili strategie di delivery di molecole ad attività insetticida ed i risultati ottenuti sono molto promettenti, ulteriori sforzi di sperimentazione dovranno essere messi in atto al fine di sviluppare le conoscenze utili per la formulazione di prodotti da immettere sul mercato.

INTRODUCTION

1.1 Bioinsecticides and Integrated Pest Management (IPM)

The use and, above all, the abuse of synthetic chemical pesticides highlighted serious issues, such as, for example, the resistance developed by target insects, environmental contamination, alteration of the main ecosystem services, reduction of natural enemies and pollinators populations. Reducing the use of these products is one of the main objectives to be pursued in order to make the agriculture both ecologically and economically more sustainable. The adoption of alternative products other than chemical synthetic insecticides is not only appropriate, but also imposed by the European Directive on the sustainable use of pesticides ("Sustainable use of pesticides and alternative non-chemical methods for plant protection and pest management" 2009/128/EC). For this purpose, a particularly important role is played by biological control, based on the use of parasites, predators and pathogens (De Bach, 1964), that, starting from the second half of the last century, has been increasingly adopted for plant protection against phytophagous insects (Pennacchio, 2014). This control strategy has successfully been improved for more than a century and still represents one of the key-aspects in the integrated pest management (IPM: the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that limit the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment) (Bale *et al.*, 2008).

Bioinsecticides are naturally occurring substances that control pests and have considerable importance in IPM, where they are used in combination with other tools including chemical pesticides as part of bio-intensive IPM.

Bioinsecticides are ideal candidates for insect control, since they show:

- low toxicity for non-target organisms and vertebrates (specificity of action);
- low pollution problems due to limited dose of use and fast degradation rates;
- low risk of pest resistance due to the occurrence of different bioactive components.

At present, bioinsecticides of different origin are commercially available. Among those derived from plants, we have extracts of neem seeds, commonly used in organic farming. Neem is an Indian plant (*Azadirachta indica*) whose seeds contain many limonoids, including the azadirachtin A, a potent antifeedant for many insects, that has a severe impact on neuroendocrine balance and development (Maia and Moore, 2011). A good level of insecticide activity is exerted by mixtures of essential oils. These are compounds obtained from aromatic plants, which show a complex mechanism of action (neurotoxic action, sexual confusion and inhibition of cytochromes, which are important enzymes in the detoxification of xenobiotics in insects) (Maia and Moore, 2011). These are just a few examples worth mentioning. Other bioinsecticides are metabolites produced by microorganisms. A brilliant example is offered by products based on mixtures of A and D spinosyns, metabolites produced by *Saccharopolyspora spinosa* during the fermentation process, which show selective affinity with nicotinic acetylcholine receptors of insects (Kirst, 2010).

Many commercially available bioinsecticides are formulated based on entomopathogenic microorganisms. Among them, the bacterium *Bacillus thuringiensis* provides the best example of a very effective natural antagonist used in widely used in spraying formulations and as a source of toxin genes expressed in transgenic plants (Romeis *et al.*, 2006), which encode proteins produced as parasporal inclusions during the sporulation process. These insecticidal toxins are extremely selective against insects and, then, ideal candidates for sustainable IPM plans (Bale *et al.*, 2008). The most widely used are the δ -endotoxins, which belong to the two large multigene families *cry* and *cyt* (Palma *et al.*, 2014). The Cry toxins are active against Lepidopteran larvae, Coleoptera, Hymenoptera and Diptera. The Cyt proteins are produced by *Bt* strains active against Diptera. In addition to these toxic proteins in parasporal crystals, some *Bt* strains also produce insecticidal proteins during the vegetative stage (Vegetative Insecticidal Proteins, VIP). Genes encoding Cry toxins have been used, since 1996, for the production of insect-resistant plants (James, 2005; Roh *et al.*, 2007). *Bt* plants are currently used in many countries and their use has led to a significant reduction in chemical pesticides applications (Toenniessen *et al.*, 2003; Brookes and Barfoot, 2005), without any scientific evidence of negative impact on non-target organisms and environment (Rukarwa *et al.*, 2014).

Entomopathogenic fungi and viruses, important natural regulators of insect populations, are also included in commercial products for insect control. Their specificity against insects is, in general, quite high, even though a certain degree of variation in the host range can be observed. The most widely used fungi belong to the Ascomycetes group (including the genera *Beauveria* and *Metarhizium*) (Wang and Wang, 2017), while, regarding viruses, Baculoviruses are successfully used for controlling lepidopteran larvae (Rohrmann, 2013). The Baculoviruses received a considerable attention also because they are particularly amenable for genetic manipulations aiming at enhancing their speed in killing the target insect or to broaden their host range (Kroemer *et al.*, 2015). In spite of the great potential of this biotechnological approach, there are still strong concerns that prevent the uptake of this technology and the use of modified microorganisms in crop protection.

1.2 Sources of new bioinsecticides and bio-inspired control strategies

Plant and microorganisms are clear examples of natural sources of molecules that regulate insect antagonistic interactions and thus can have insecticide activity. This basic concept of mimicking the natural killing mechanisms can be extended to the interaction between insects and other arthropods, i.e. insect natural antagonists, and underlies the potential of using them beyond the organism level, not only as biocontrol agents, but also as a source of molecules and genes that may have a relevant role in crop protection.

Among the wealth of insect natural enemies, predators and parasitoids are by far the most abundant and used as biocontrol agents (Bale *et al.*, 2008). However, recent studies do also uncover their interesting potential as source of molecules and genes to be used for the development of new biotechnologies for insect pest control (Pennacchio *et al.*, 2012). The molecules involved in the regulation of host physiology and its killing by parasitic wasps (Pennacchio and Strand, 2006) are ideal candidates for the development of new biological insecticides, in theory highly

selective, given the high stenophagy level exhibited by these beneficial insects. The identification of the genes regulating the molecular interactions between insect hosts and their natural antagonists offers the opportunity to identify new virulence factors and their cognate target receptors in the host, that can be used to develop innovative biotechnologies for insect control (Pennacchio *et al.*, 2012). These biotechnologies, based on molecules and genes that are produced and delivered by natural enemies, could fall into an expanded definition of biological control, which includes not only the use of the organisms, but also of genes and molecules derived from them.

The beneficial arthropods are active insect antagonists, and their strategy of host attack is very often based on the use of secretions involved in the capture/colonization and subsequent physiological/biochemical redirection to enhance the nutritional suitability of the victim and maximize the developmental success of their own progeny (Vinson *et al.*, 2001; Pennacchio and Strand, 2006; Pennacchio *et al.*, 2012). The most exhaustive information on molecules causing prey paralysis has been obtained for spiders and scorpions, because of their medical importance. These studies allowed the identification and molecular characterization of powerful neurotoxins, which are able to block in a very selective way different kinds of voltage-gated ion channels in arthropods (Zlotkin, 2005; Maggio *et al.*, 2005; Gordon *et al.*, 2007; Gurevitz *et al.*, 2007; Nicholson, 2007; Rohou *et al.*, 2007). The research work on these neurotoxins has attracted increasing attention in the agrochemical industry, due to the possibility that structure-activity studies may allow the development of new families of synthetic insecticides, starting from natural molecules, generated by long co-evolutionary processes.

The high biodiversity of insect parasitoids and their need to solve specific issues posed by the colonization of different ecological niches promoted the development of numerous virulence strategies in different phylogenetic lineages (Pennacchio and Strand, 2006). The consequent host physiological alterations, triggered by factors of parasitic origin injected by the ovipositing wasp females, such as venom, symbiotic viruses and ovarian secretions, are of key importance for the success of parasitism, as they are essential to finely modulate the recipient host microenvironment. This unique capacity to inject a complex mixture of host regulation factors is conferred by the presence in parasitic Hymenoptera of the ovipositor, which is one of the most important morphological adaptations that has significantly promoted the intense adaptive radiation process and evolutionary success of these wasps (Quicke, 1997).

The astonishing biodiversity of parasitic wasps offers an unparalleled opportunity to isolate a wealth of new bioinsecticide molecules, which may range from aggressive neurotoxins typically occurring in the more basal lineages of idiobiont parasitoids (i.e. wasps that paralyze and suppress their hosts at the oviposition by using potent venom blends), to host regulation factors by koinobiont species (i.e wasps with their larval stages developing into living hosts) disrupting essential physiological functions, such as immunity and neuroendocrine balance, and reproduction (Pennacchio and Strand, 2006; Pennacchio *et al.*, 2012). The growing information on the molecular mechanism of action of these parasitoid-derived molecules and on their receptors in the insect hosts offers not only the opportunity to use them as new bioinsecticides, but also to define novel insect control strategies mimicking their effect. Indeed, current molecular technologies, such as RNA interference (RNAi) and genome editing (Pennacchio *et al.*, 2012; Perkin *et al.*, 2016) can allow to reproduce the host alterations by parasitic wasps by hitting the cognate receptors of their virulence factors. A brilliant proof of concept corroborating the high potential of this approach has been recently provided by Caccia *et al.* (2016), who demonstrated a 5-6 fold

enhancement of *Bt* efficacy by silencing an immune gene of the host, which is targeted by the immunosuppressive strategy of a parasitic wasp (Falabella *et al.*, 2012; Di Lelio *et al.*, 2014).

One of the major issues to address for profitably using the great molecular biodiversity of insect natural enemies is how to deliver their virulence factors in order that they can reach the receptors, located in the body cavity, in an undegraded form, following oral administration. The problem to solve is to survive the harsh environment of the gut and to overcome its epithelial wall.

1.3 Delivery of bioinsecticides

There is a strong dichotomy between bioactive molecules whose receptors are exposed in the gut lumen and those that have to reach the receptors placed within or behind the gut epithelium. Once orally administered, the bioinsecticides reach the intestinal lumen where they find the intestinal barrier, consisting of the peritrophic membrane (PM) and the intestinal epithelium. The PM is an extracellular thin sheet lining the midgut lumen, largely made of chitin and proteins, which protects the midgut columnar epithelium and plays an important role in digestive physiology (Terra, 2001). PM represents the main barrier to the transit of pathogenic and potentially toxic macromolecules (Lehane, 1997). The intestinal epithelium has the double role to produce the digestive enzymes (Terra and Ferreira, 1994) and to absorb the nutrients (Giordana *et al.*, 1998). The main cells involved in these basic functions are the columnar cells. They are polarized cells presenting the apical membrane, towards the intestinal lumen, folded to form a brush border. In the larval stage of moths, close to this cell type there are also the goblet cells, which confer functional peculiar characteristics to the intestinal epithelium (Giordana *et al.*, 1998). The goblet cells determine the extrusion of K^+ ions, which by accumulating in the insect midgut lumen promote the absorption of amino acids against gradient. The K^+ extrusion is linked to HCO_3^- and OH^- fluxes which lead to extreme luminal alkalisation, typical of the lepidopteran midgut (Giordana *et al.*, 1998). The efficacy of orally administered insecticidal molecules targeting haemocoelic receptors is strictly related to their ability to cross the intestinal barrier, so it is of substantial importance to understand the mechanisms of macromolecule absorption in the gut, in order to develop strategies to enhance their rate of uptake and transit to the haemocoel.

Thus, in addition to the identification of bioinsecticidal molecules, a fundamental issue is the development of appropriate delivery strategies that allow the orally administered molecules to reach unaltered their haemocoelic targets. For this purpose, many research efforts focused on the identification of stabilizers, able to decrease or avoid degradation in the insect gut lumen, and carriers, able to facilitate the entrance of bioinsecticidal molecules into the cells and the transepithelial transport (Pennacchio *et al.*, 2012). As far as the stabilizers, excellent results have been obtained through the PEGylation (covalent bond between the polyethyleneglycol and the bioinsecticide molecule). In fact this technology has been proved to be capable of protecting proteinaceous insecticidal molecules from degradation, increasing their oral toxicity (Jeffers *et al.*, 2012; Jeffers *et al.*, 2014). Microencapsulation, mostly used to protect the bioinsecticides from environmental degradation, has also been successfully applied to increase the stability of the bioactive proteinaceous and nucleotidic molecules within the insects intestinal lumen (Richards *et al.*, 2014; Lin *et al.*, 2016). Regarding the carrier molecules, one of the

most significant examples is represented by lectins, derived from plants, and in particular GNA (*Galanthus nivalis* agglutinin). GNA, in fact, is able to cross unaltered the intestinal barrier of numerous insects, but also to act as a vector for proteinaceous molecules with haemocoelic targets, such as neuropeptides and neurotoxins produced by arthropods, as spiders and scorpions (Fitches *et al.*, 2002; Fitches *et al.*, 2004). Other carriers of interest are represented by cell penetrating peptides (CPP), a group of peptides rich in arginine and lysine able to cross the cell plasma membranes both alone or conjugated with macromolecules with low cellular permeability. These peptides have been demonstrated able to efficiently transport proteins across the plasma membranes of intestinal cells and to increase protein absorption across the intestinal epithelium in *Bombyx mori* larvae (Cermenati *et al.*, 2011). CPP are also able to enhance the entry of nucleic acids into insect cells (Chen *et al.*, 2012). Insect pathogenic microorganisms can be also used as delivery systems.

To achieve transepithelial delivery of macromolecules, viruses and microorganisms can be also considered as vectors. The insect pathogenic viruses, in particular belonging to *Baculoviridae* family, have received considerable attention since their easy genome manipulation allows the introduction of exogenous genes encoding toxins targeting haemocoelic receptors (Kamita *et al.*, 2005; Inceoglu *et al.*, 2006). Similarly, entomopathogenic fungi can be manipulated to express bioinsecticidal toxins and allow their penetration through the cuticle, one of the main obstacles for the direct application of molecules with insecticidal action (Wang and Leger, 2007). Nucleic acids, such as dsRNAs, may be carried by bacteria which confer protection and stability to the molecules, thereby preventing the degradation in the intestinal lumen (Kim *et al.*, 2015). These are just a few examples of microorganism-mediated delivery, which exploit the capacity they have to overcome host barriers preventing the penetration of bioactive molecules into the body cavity of target insects.

We have briefly outlined above the major research areas in the development of new bioinsecticides which require special attention by the scientific community in order to fill the many gaps that prevent a profitable exploitation of the natural biodiversity of insect natural antagonists. This work aims at contributing new ideas in this context by considering both new oral delivery strategies of macromolecules targeting haemocoelic receptors or the silencing of host immune genes, in order to enhance its sensitivity to natural antagonists.

2 FUSION PROTEINS FOR THE ORAL DELIVERY OF INSECTICIDAL MOLECULES

2.1 Introduction

2.1.1 Protein absorption in insect gut

In the last decade, the study of peptide and protein absorption by the insect gut has received extensive attention because of the considerable impact this information may have on the development of new delivery strategies for insecticide macromolecules targeting haemocoelic receptors (Fitches *et al.*, 2001; Jeffers and Roe, 2008).

The intestinal epithelium in animals represents a barrier which allows a selective transit of molecules between the gut lumen and the internal compartments. Small organic molecules and ions can cross the intestinal barrier either via paracellular or transcellular pathway. The paracellular route is modulated by regulating cell junction permeability, while the transcellular pathway of ions and small solutes by the asymmetric distribution of membrane carriers and channels at the opposite poles of the cell membrane. Macromolecules can also be transported across the gut by transcytosis, a complex progression of intracellular events that exploits the membrane traffic involved in internalisation and secretion at the apical and basal poles of the cell (Mostov *et al.*, 2000). Proteins internalized via receptor-dependent or -independent endocytosis in clathrin-coated or clathrin-free vesicles are targeted to the endosome. The reduction in pH to 5.5-6 within the endosome results in conformational changes in some receptors causing the release of the ligand. From here, ligands and receptors may be translocated to the apical membrane (retroendocytosis), the basolateral membrane (transcytosis), or stay attached to receptors to continue along the endolysosomal pathway (Bonning and Chougule, 2014).

Transcytosis has been widely studied in diverse mammalian epithelia (Apodaca, 2001; Tuma and Hubbard, 2003). Following the first suggestion by Palade (1953), on the possible occurrence of transcytosis, several subsequent studies have addressed the functional bases of this transport process. It is now clear that transcytosis widely occurs in epithelial tissues (Brown and Stow, 1996; Mostov *et al.*, 2000; Apodaca, 2001), but it is not restricted to polarised cell types (Tuma and Hubbard, 2003). The molecular mechanism of transcytosis has been thoroughly studied in mammals, both *in vivo* (Sai *et al.*, 1998; Ziv and Bendayan, 2000) and *in vitro* (Heyman *et al.*, 1982; Kiliaan *et al.*, 1998), or, more frequently, by using model epithelial monolayers (Gekle *et al.*, 1995; Gekle *et al.*, 1997; Ellinger *et al.*, 2001). The cytoskeleton plays a key-role in this absorption process (Apodaca, 2001), which is energy-dependent and regulated by internal signals.

During the years many efforts have been devoted to the study of the transport of peptides and proteins across the insect gut, such as as proctolin (Bavoso *et al.*, 1995), Aea-TMOF (Borovsky and Mahmood, 1995), Neb-TMOF (Zhu *et al.*, 2001), small proteins from cobra and scorpion venom (Primor *et al.*, 1980; Fishman *et al.*, 1984; Zlotkin *et al.*, 1992), large proteins, like albumin (Nogge, 1970), horseradish peroxidase (Fishman and Zlotkin, 1984; Modespacher *et al.*, 1986), *Galanthus nivalis* agglutinin (GNA) (Powell *et al.*, 1998; Fitches *et al.*, 2001), green fluorescent protein (Habibi *et al.*, 2002) or immunoglobulins (Allingham *et al.*, 1992; Ben-Yakir and

Shochat, 1996). They demonstrated *in vivo* that those molecules are able to cross the intestinal barrier, but the mechanism involved in the process was not clarified. A few years later, the mechanism of intestinal absorption of several proteins have been studied in larval Lepidoptera. In XXX *et al.*, XXX they show interesting data on protein translocation across the isolated midgut of XXX perfused *in vitro*. They demonstrated for the first time, under *in vitro* controlled experimental conditions, that unchanged XXX is transported through the insect midgut by transcytosis. A further study clarified that the mechanism involved in XXX internalization is clathrin-mediated endocytosis and a XXX receptor is responsible for XXX recognition by XXX intestinal cells (XXX *et al.*, XXX). The uptake by endocytosis of macromolecules starts with the formation of vesicles derived from the invagination and pinching off of plasma membrane portions. This phenomenon is a mixture of multiple mechanisms that fall into two wide categories (Conner and Schmid, 2003): phagocytosis (i.e., the uptake of large particles) and pinocytosis (i.e., the uptake of fluid and solutes). Although phagocytosis occurs in specialized cells, pinocytosis is common to all cells and occurs by four different ways: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin-and caveolae-independent endocytosis (Conner and Schmid, 2003). The best-studied cell uptake mechanism is the clathrin-mediated endocytosis, which ensures an efficient internalization of diluted solutes by high-affinity receptors concentrated in pits coated on their cytosolic side by clathrin and other associated proteins. In mammalian absorptive epithelia XXX, a multiligand receptor plays a fundamental role in clathrin-mediated endocytosis of different ligand. XXX is a 600-kDa transmembrane protein belonging to the LDL receptor family, acting in mammalian polarized epithelia as a receptor for hormones, vitamin binding proteins, enzymes, enzyme inhibitors, and proteins like albumin and lactoferrin (Moestrup and Verroust, 2001; Christensen and Birn, 2002). These coated pits invaginate and pinch off to form the endocytic vesicles that are delivered to early endosomes, from which ligands and receptors will be addressed to their proper destination. Numerous regulatory proteins are involved in this process (Conner and Schmid, 2003), and the cytoskeleton is implicated both in the formation of the endocytic vesicles and in their release from the plasma membrane (Murray and Wolkoff, 2003; Smythe and Ayscough, 2006). Receptor-mediated endocytosis is distinctly a more efficient mechanism of transport. The K_m value for XXX, calculated in the kinetics experiments performed with XXX columnar cells in culture, is in the same range of the apparent affinity constants for XXX endocytosis in mammalian absorptive cells (Gekle, 1995; 1996; 1998). Clathrin-mediated endocytosis is also involved in XXX internalization in *S. littoralis* midgut cells, although the nature of the receptor involved in the recognition of XXX in this insect is still unknown (XXX *et al.*, XXX).

However, the transcellular pathway is not the only mechanism involved in the protein absorption. In fact the permeation mechanisms to rich the haemolymphatic for some proteins, such as horseradish peroxidase (HRP), involve both transcytosis and paracellular pathway (XXX *et al.*, XXX). HRP enzyme was detected in the junctions between adjacent cells. The presence of HRP is evident not only in the apical part of the junction, but also further down in the space between adjacent cells (XXX *et al.*, XXX).

The ability of proteins and peptides to cross the insect midgut epithelium suggests that insecticide macromolecules targeting haemocoelic receptors can be orally delivered. This finding may allow the exploitation of the highly specific molecules that control physiological processes in insects or that regulate pathologies induced by

natural antagonists, in order to develop innovative insect control strategies. For example, the titre alteration of hormones or biologically active peptides (Whetstone and Hammock, 2007), which could be carried out by their direct administration and/or by an alteration of their metabolism, may surely damage insect survival and reproduction or reduce their capacity to face environmental challenges. To this purpose, the use of insecticidal macromolecules produced by insect antagonists to regulate and damage host physiology and reproduction appears promising (Binnington and Baule, 1993; Beckage and Gelman, 2004; Pennacchio *et al.*, 2012). The oral delivery of insecticide proteins and peptides targeting haemocoelic receptors requires both a thorough understanding of their degradative pathway in the midgut lumen and of their capacity to cross the gut epithelium unaltered and still biologically active.

Among the numerous pests, lepidopteran larvae are probably one of the most economically important phytophagous group. In consideration of the possibility to control them by using potent natural toxins from insect predators, it is essential to identify effective molecules and to define suitable delivery strategies to make them reach haemocoelic targets. This latter aspect appears particularly amenable for this type of studies, thanks to the set of information available on the intestinal physiology underpinning the absorption of intact macromolecules.

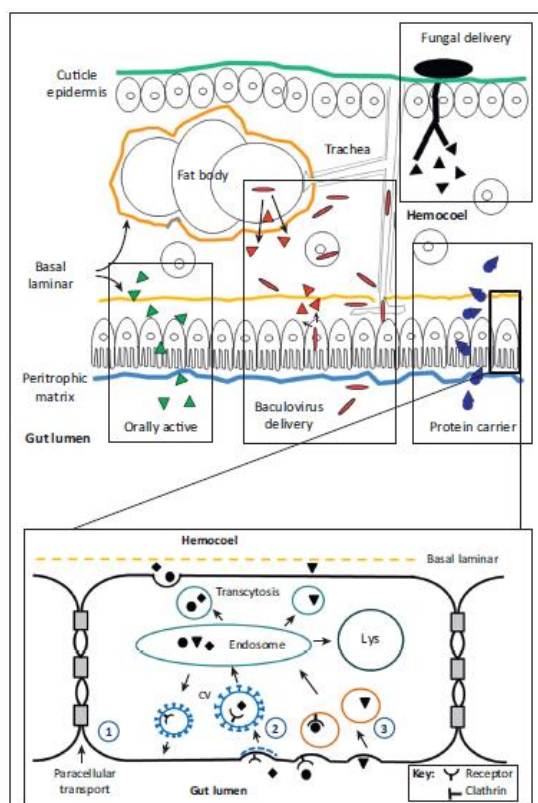


Figure 1. Delivery of intrahemocoelic toxins for management of insect (Bonning and Chougule, 2014).

2.1.2 Spider venoms: a promising source of novel bioinsecticides

Arachnid venoms, which are complex peptidic libraries, have received particular attention (King and Hardy, 2013). Based on the number of species and number of toxins present in the venom of those examined, there are an estimated 0.5-1.5 million arachnid-derived insecticidal peptides (Windley *et al.*, 2012), and at least 10 million

bioactive spider-venom peptides (King *et al.*, 2008). Of the 800 peptides in the ArachnoServer 2.0 Database, 136 are insecticidal with 38 being insect selective, 34 nonselective, and 64 of unknown phyletic selectivity (Windley *et al.*, 2012).

Spiders belong to the order Araneae, one of the largest groups in the arachnids, which mainly feed on insects and other arthropods.

Spiders have, during their evolution, developed a complex pre-optimized combinatorial peptide library of neurotoxins, enzymes, antimicrobial and cytolytic peptides in their venom glands to diversify their toxin pool (Sollod *et al.*, 2005). Given that many spiders rely upon their venom to immobilize or kill their prey, it is not unexpected that they have a wide variety of insect-selective toxins contributing to the development of bioinsecticides (King, 2007; Nicholson, 2007). Spider venoms contain a large number of bioactive molecules and toxins. They fall into various chemical groups such as polyamine-like toxins, that interfere with glutamic acid receptors and block neuromuscular transmission (Grishin *et al.*, 1986; Kawai *et al.*, 1991), low molecular weight proteins or peptides that affect neuronal or membrane ion channels and receptors through pre- or post-synaptic actions, and high molecular weight neurotoxins that interact with specific pre-synaptic receptors (Lipkin *et al.*, 2002). Insecticidal toxins synthesised by spiders typically cause paralysis due to disruption of the activity of neuromuscular junctions. These toxins are usually cysteine-rich polypeptides with 55 to 60 amino acid residues. Some of the venomous species that have been studied extensively include *Agelenopsis aperta*, *Atrax robustus*, *Hololena curta*, *Phoneutria nigriventer*, *Plectreurys tristis* and *Segestria florentina* (Stapleton *et al.*, 1990; Diniz *et al.*, 1993; Quistad and Skinner, 1994; Reily *et al.*, 1995; Newcomb *et al.*, 1995; Pallaghy *et al.*, 1997).

Interestingly, a large number of these phyla-specific toxins target voltage-gated ion channels, such as voltage-gated sodium and calcium channels (Na_v and Ca_v respectively), to promptly modify ion channel gating and kinetics.

2.1.2.1 Toxins targeting Na_v channels

The Na_v channel mediates the increase in sodium conductance during the rapid depolarization phase of the action potential. Therefore, this channel represents a key structural element controlling cellular excitability in biological systems. Na_v channels are transmembrane proteins which provide the current pathway for fast depolarization of excitable cells to prime action potential generation (Hodgkin and Huxley, 1952). Their structure principally includes a single pore forming ~2000-residue glycoprotein α -subunit in eukaryotic Na_v channels (Catterall, 2001). The α -subunit is formed of four homologous domains (I–IV) connected by cytoplasmic linkers (Catterall, 2000; Morgan *et al.*, 2000; Yu and Catterall, 2003). Each of these domains has six putative transmembrane segments (S1–S6). The four domains fold together in a clockwise orientation, where domains I and IV are brought in proximity, to form the outer pore atrium and the selectivity filter. This is created by the S5–S6 linker loops from each domain forming re-entrant pore loops that dip into the transmembrane area of the protein (Catterall, 2000; Li *et al.*, 2001). The S4 segments, which are the most conserved segments, have positively charged amino acids (Arg or Lys) at intervals of three residues and transport gating charges outward thus acting as voltage sensors to prime voltage-dependent activation by moving outward as a result of changes in the electric field (Stuhmer *et al.*, 1989; Chen *et al.*, 1996; Yang *et al.*, 1996; Chanda and Bezannila, 2002; Cestè le *et al.*, 2006). Na_v channel inactivation is mediated by a short intracellular loop connecting domains III and IV, containing the hydrophobic amino acid residues motif (IFM) (West *et al.*, 1992). The α -subunit is also associated with one or two smaller auxiliary subunits

($\beta 1$, $\beta 2$, $\beta 3$ and/or $\beta 4$) of about 30 kDa that are required for normal kinetics and voltage-dependence of gating but are not required for ion flux, ionic selectivity and pharmacological modulation (Schreibmayer *et al.*, 1994; Isom *et al.*, 1995; Yu *et al.*, 2003). The Na_V channel is a target for numerous drugs, neurotoxins and insecticides. These bind to at least seven neurotoxin binding sites and either disrupt conductance or modulate Na_V channel gating. Hainantoxin-I appears to target site-1 to block Na_V channel conductance (Li *et al.*, 2004). Magi 2 and Tx4(6-1) slow Na_V channel inactivation via an interaction with site-3 (Corzo *et al.*, 2003). The δ -palutoxins, and most likely μ -agatoxins and curtatoxins, target site-4 (Corzo *et al.*, 2000). In addition, several other spider neurotoxins, such as δ -atractoxins, are known to target both insect and vertebrate Na_V channels (Nicholson *et al.*, 2004) most likely as a result of the conserved structures within domains of voltage-gated ion channels across phyla.

2.1.2.2 Toxins targeting Ca_V channels

Ca_V channels form membrane pores that open as a result of membrane depolarization to allow the influx of extracellular calcium ions. They are responsible of a wide range of critical intracellular processes, including muscle contraction, hormone and neurotransmitter release, neurotransmission, and regulation of enzymatic activities and patterns of gene expression (Catterall, 2000).

Ca_V channels are divided into two wide superfamilies based on their voltage-dependence of activation: low-voltage-activated (LVA) Ca_V channels are activated by small membrane depolarizations and show rapid voltage-dependent inactivation, whereas high-voltage-activated (HVA) Ca_V channels are only activated by larger depolarizations and inactivate more slowly.

The quaternary structure of HVA Ca_V channels is more complex than that of voltage-gated sodium and potassium (K_V) channels and typically comprises 4-5 subunits: (i) a pore-forming $\alpha 1$ subunit of ~170-250 kDa; (ii) an extracellular $\alpha 2$ subunit; (iii) a transmembrane δ subunit that is covalently linked to $\alpha 2$ via a disulfide bond to form an ~170kDa $\alpha 2$ - δ complex (De Jongh *et al.*, 1990); (iv) an intracellular 50–78 kDa β subunit; (v) in some cases, a transmembrane γ subunit of 25-36 kDa (Catterall, 2000; Kang and Campbell, 2003; Bourinet and Zamponi, 2005). In contrast, LVA Ca_V channels primarily consist of just the pore-forming $\alpha 1$ subunit, with little evidence of regulation by additional subunits (Perez-Reyes, 2003; Bourinet and Zamponi, 2005; Catterall *et al.*, 2005).

Most, if not all, spider venoms contain peptide toxins that strongly modify the activity of Ca_V channels in both the peripheral and central nervous system of insects. Some toxins, such as PLTX-II, could become the defining pharmacology for specific subtypes of insect Ca_V channels. It was shown almost 20 years ago that PLTX-II blocks presynaptic Ca_V currents in *Drosophila* nerve terminals (Branton *et al.*, 1987; Leung *et al.*, 1989), but only recently has it been shown that the toxin most likely targets the Dmca1A Ca_V channel (Kuromi *et al.*, 2004). Some are phylogenetically indiscriminate peptides, such as ω -Aga-IVA, with high affinity for both insect and vertebrate channels and acting as a pore blocker (Chong *et al.*, 2006).

In addition to provide tools for ion channel characterization, spider toxins that specifically target insect Ca_V channels might prove to be valuable for the development of novel insecticides.

2.1.2.3 *Segestria florentina* venomous toxins

The venom purified from *S. florentina* glands contains about 25 polypeptide components (Sagdiev *et al.*, 1987). The crude venom extract has been demonstrated to inhibit neurons action potential, which elicits paralysis in cockroaches after intra-

peritoneal injection (Lipkin *et al.*, 2002). In contrast to reported effects of other invertebrate toxins, this crude extract has no haemolytic or proteolytic activity on the insect neuromuscular system.

S. florentina crude venom has one insecticidal toxin and two vertebrate specific neurotoxins (Sf-1 and Sf-2) (Sagdiev *et al.*, 1987). Moreover, the calcium channel antagonist SNX325, has been isolated from the venom (Newcomb *et al.*, 1995).

A few years ago, Lipkin *et al.* (2002) performed purification, structural and cloning analysis of the insecticidal toxins obtained from *S. florentina* salivary glands (showed in Figure 2). Low molecular weight polypeptides (<12 kDa) are responsible for the insecticidal activity of the venom. On the basis of N-terminal amino acid sequences a family of eight genes encoding highly homologues polypeptides (SFI1-SFI8) was revealed. All deduced polypeptides consist of 46 amino acids residues. Comparison of primary structures of SFI1-SFI8 with other spider toxins suggests that this family might share structural and functional relationships with other small spider neurotoxins, several of which are known to be highly selective agonists/antagonists of different Ca_v channels (Lipkin *et al.*, 2002). Lipkin *et al.* (2002) suggests that if the toxins of SFI family of possess a similar mode of action, they have the potential to be used as selective molecules to block Ca_v channels and to act like biopesticides.

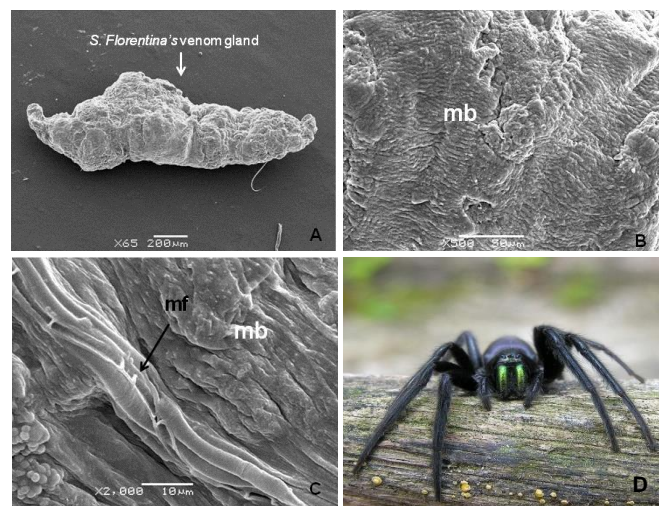


Figure 2. A) The venom gland of *S. florentina* is shaped like a bulb and covered by a bulky muscle layer B) Distinctive muscle bundles (mb) at higher magnification C) The nerve fibers (nf) among the muscle bundles (Benli *et al.*, 2013). D) *Segrestria florentina* spider image (www.wikipedia.org).

2.1.3 Fusion proteins containing neurotoxins

Some of the spider toxins have high phyletic specificity, and high potency mediated through novel mode of action, which makes them promising molecules to develop novel effective bioinsecticides. The strong and selective mode of action of spider neurotoxins would make them ideal candidates to be used in environmentally compatible pest management technologies, if suitable delivery systems could be devised. Whetstone and Hammock (2007) note, “considering the total number of species that produce insect-specific toxins and the variety of toxins within each venom type, the potential for the development and application of novel biopesticides from these sources appears virtually limitless, however, only if provided with suitable delivery systems”. Therefore, the problem with protein-based technologies is not in finding insecticidal proteins, but finding a suitable delivery method for these proteins. In fact with few exceptions, neurotoxins are not orally active because they are evolutionary meant to be injected.

The use of insecticidal fusion proteins containing a toxic peptide or protein fused to a “carrier” is an example of strategy to overcome this limitation. Protein based biopesticides represent environmentally friendly approaches to pest control, as they are biodegradable and combine efficacy with specificity. In addition to naturally occurring protein biopesticides like *B. thuringiensis* toxins, recombinant proteins with insecticidal activity can be produced using biotechnological methods.

2.1.4 Potential carrier proteins: proteins that move from the insect gut into the haemocoel

Creating fusion proteins by combining two or more proteins has recently become a widespread technique among researchers. The components of fusion proteins act together to produce effects that are not observed when they act independently. Fusion proteins could therefore be useful in addressing issues relating to protein delivery and, in particular, of proteinaceous bioinsecticides.

The carrier molecule confers oral activity to the insecticide which would normally be toxic when injected into the insect, since it directs the transport of the fusion molecule across the insect gut, as so allows the toxin to reach its site of action (Fitches *et al.*, 2002; 2004; 2012; Pyati *et al.*, 2014). Fusion proteins, thanks to the presence of a carrier, acquire good stability, so they cannot be degraded in the environment or digested by gut enzymes of pests, and high toxicity, with activity towards pests comparable to the toxic proteins themselves.

As stated before, numerous papers describe the movement of a diverse range of proteins from the insect gut into the haemocoel in a broad range of arthropods (Jeffers and Roe, 2008; Burand and Hunter, 2013) and it has been unequivocally demonstrated that orally delivered proteins are able to cross the intestinal barrier *in vivo* and to reach the haemocoel undegraded both in blood-feeding insects (Nogge, 1970; Fishman and Zlotkin, 1984; Modespacher *et al.*, 1986; Allingham *et al.*, 1992) and in phytophagous insects (Ben-Yakir and Shochat, 1996; Powell *et al.*, 1998; Fitches *et al.*, 2001; Habibi *et al.*, 2002; Jeffers *et al.*, 2005; Kurahashi *et al.*, 2005). Among the plethora of proteins that have been proven to cross the insect gut there are XXX, immunoglobulins (IgG), teratocyte-secreted protein (TSP)14, GNA, horseradish peroxidase. Some of the proteins that transcytose across the gut epithelium of insects (IgG, XXX and horseradish peroxidase), also transcytose across mammalian epithelial cells. Increasing experimental evidence indicates that ingested numerous proteins can in part reach the haemocoel undegraded, but the information on the mechanisms involved in protein transport across the insect gut is very limited, in spite of the implications that this may have on the development of novel delivery strategies of insecticide proteins targeting haemocoelic receptors.

Among the potential carrier molecules, lectins are the most extensively studied. Lectins are carbohydrate-binding and protease-resistant proteins that are widely distributed in animals, plants, and microorganisms (Vandenborre *et al.*, 2011). These proteins carry out various biological functions by reversibly binding to specific mono-saccharides or complex glycans through noncatalytic domains. In plants, lectins play an important role in defense against insect herbivores and a broad spectrum of plant lectins has been tested for insecticidal activity against agriculturally important lepidopteran, coleopteran, dipteran, and hemipteran pests (Michiels *et al.*, 2010; Vandenborre *et al.*, 2011). Along with binding to the insect gut, certain plant lectins such as the snowdrop lectin, GNA, can pass intact into the insect hemolymph following oral delivery (Fitches *et al.*, 2001). GNA binds an insect gut membrane receptor glycoprotein, aminopeptidase N (Fitches *et al.*, 2010), which may mediate entry into the cell by receptor-mediated endocytosis, followed by transcytosis of a

portion of the endocytosed lectin. Indeed, GNA has been detected in haemolymph, Malpighian tubules, fat bodies, ovarioles, and the central nerve cord (Fitches *et al.*, 2012). The movement of GNA from the gut into the hemocoel provides a mechanism for the effective oral delivery of toxins to their site of action, allowing for exploitation of insect-specific toxins that are ineffective when administered orally. Indeed, a number of scorpion and spider venoms, active on insect nervous system, can be vectored by GNA into the haemolymph (Fitches *et al.*, 2004; 2012; Wakefield *et al.*, 2010; Nakasu *et al.*, 2016). A fusion combining GNA with the insecticidal spider venom-derived neurotoxin *S. florentina* toxin 1 (SFI1) is insecticidal to both lepidopteran and hemipteran insect pests (Fitches *et al.*, 2002; 2010; Pham Trung *et al.*, 2006). Ingestion of SFI1-GNA resulted in 100% mortality of first instar larvae after 6 days of feeding, whereas no effect was observed in SFI1- or GNA-fed insects. The ability of GNA to act as a carrier protein to deliver SFI1 into the hemolymph of these insects was demonstrated by immunoblot detection of GNA-immunoreactive proteins of the same molecular mass as the intact fusion. The SFI1-GNA fusion protein was also highly toxic against *Myzus persicae* and the rice brown planthopper, *Nilaparvata lugens*. However, in the case of *N. lugens* most of the toxicity was attributed to GNA. Proteolytic degradation of GNA-based fusion proteins in the insect gut has reduced the efficiency of toxin delivery to the haemolymph (Fitches *et al.*, 2004; Pham Trung *et al.*, 2006). The insecticidal δ -hexatoxin-Hv1a (Hv1a), derived from the venom of an Australian funnel-web spider (*Hadronyche versuta*) specifically inhibits insect but not mammalian voltage-gated calcium channels (Tedford *et al.*, 2004; Chong *et al.*, 2007). Hv1a is highly toxic by injection towards many different insect pests including species from the orders Lepidoptera, Coleoptera, Diptera, and Dictyoptera, and is ineffective after oral ingestion (Fitches *et al.*, 2012; Pal *et al.*, 2013). However, Hv1a is orally toxic against one tick species (*Amblyomma americanum*), which may be related to differences in gut physiology associated with blood feeding (King, 2004). Fusion of Hv1a to GNA results in oral delivery of the toxin to its site of action, the central nervous system, in *Mamestra brassicae* (Fitches *et al.*, 2012). GNA-mediated delivery into the hemolymph and central nerve cord has been demonstrated by immunoblotting as well as fluorescence microscopy. Hv1a-GNA caused 40% mortality after 4 days in feeding bioassays, with surviving insects dying before pupation. Feeding second instar larvae cabbage leaf discs coated with 0.2% Hv1a-GNA caused 85% mortality after 10 days.

GNA has been fused to numerous toxins to increase the mortality in many insects (Fitches *et al.*, 2002; 2004; 2010; 2012; Pham Trung *et al.*, 2006; Wakefield *et al.*, 2010; Pyati *et al.*, 2014; Nakasu *et al.*, 2016).

Taken together, these studies highlight that plant lectins can be transcytosed across the insect gut epithelium, and have potential for delivery of intrahemocoelic toxins to their target sites.

In this thesis chapter, the possible role of XXX as an effective carrier of toxic domains across the gut of aphid pests is investigated, trying also to identify the shortest XXX domain able to promote transepithelial transport.

2.2 Materials and methods

2.2.1 Microbial expression systems and insects

Escherichia coli and *Pichia pastoris* were used for expression of recombinant proteins. *E. coli* Dh5 α was maintained on low salt LB (Luria Bertani) broth (1% (w/v)

Bacto-Tryptone, 0.5% (w/v) Yeast extract, 1% (w/v) NaCl). One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher) were provided for efficient transformation.

Pichia pastoris X33 was grown in 50 ml of starter culture in a 250 ml baffled flask, for 2-3 days at 30°C, under shaking. YPD (Yeast Extract-Peptone-Dextrose) media (1% (w/v) BactoYeast extract, 2% (w/v) Bacto-Peptone, 2% (w/v) Dextrose) 100 µg/ml zeocin (Thermo Fisher) was used. Yeast was maintained in an orbital shaker at 30°C and 200 rpm for 96 h.

2.2.2 Biological material: insect rearing

Acyrtosiphon pisum was maintained on broad beans (*Vicia faba* L. cv. Aquadulce) in plexiglas cages under controlled environmental conditions of $20 \pm 1^\circ\text{C}$, 70% Relative Humidity (RH), and under a 16 hour light/8 hour dark period (Guerrieri *et al.*, 2002).

2.2.3 Insect bioassays

For the bioassay on aphids artificial diet was prepared based on that described in Febvay *et al.* (1988). Adult aphids were collected from broad beans plants and transferred to 90mm diameter Petri dishes, fed with artificial diet as described by Down *et al.* (1996), and incubated for 72 h to reproduce nymph aphids. Third-instar nymphs (five per Petri dish) were collected and exposed to one of the seven treatments: (i) water, (ii) artificial diet alone, (iii) 0.1 mg/ml GNA, (iv) 0.1 mg/ml XXX, (v) 0.1 mg/ml SFI2.6/GNA, (vi) 0.1 mg/ml SFI2.6/XXX, (vii) 0.1 mg/ml SFI2.6/domain1. All the proteins were resuspended in artificial diet. Mortality was recorded daily for 8 days and diets were changed every 48 h. 30 aphids per treatment (in three Petri dishes) were used for the bioassay.

2.2.4 *Pichia pastoris* expression system

The yeast *P. pastoris*, has become a substantial workhorse for biotechnology, especially for heterologous protein production (Kurtzman, 2009).

P. pastoris is an amethylotrophic yeast, which is the eukaryotic expression system of choice for large-scale production of active recombinant fusion proteins, since prokaryotes do not fold these proteins correctly. One of the main advantages of using *P. pastoris* over *Saccharomyces cerevisiae* as a protein production host is its ability to secrete high titres of properly folded, post-translationally processed and active recombinant proteins into the culture media. This system helps to direct larger amounts of recombinant protein secreted into the culture medium. This makes the purification of the desired product straightforward (Pyati *et al.*, 2014).

For the constitutive expression, the glyceraldehyde-3-phosphate promoter (P_{GAP}) is commonly used as it allows to skip the methanol induction. The use of *P. pastoris* as an expression host has been found to be essential to produce functionally active insecticidal fusion proteins (Fitches *et al.*, 2004). The destination vector used for the expression of recombinant proteins in *P. pastoris* system is pGAPZα. The standard setup of this vector is a bi-functional system enabling replication in *E. coli* and maintenance in *P. pastoris*; pGAPZα (3.1 kb) uses the *GAP* promoter for the constitutive expression of recombinant proteins in *P. pastoris* and produces a protein that is fused to an N-terminal α-factor secretion signal. Pyati *et al.* (2014) used pGAPZα vector, a shuttle vector propagated in *E. coli*, to facilitate the insertion of multiple fusion protein cassettes into the yeast genome.

2.2.5 XXX sequence analysis

The UniProt database was used to analyse the structure (Figure 3) and sequence of XXX [XXX]. XXX is composed of three α -helical domains (I, II, and III) arranged in a heart-shaped molecule. Each domain can be divided into two subdomains (A and B) (XXX *et al.*, XXX). The whole XXX and the three separate domains coding sequences have been fused at the 3' end of the SFI2.6 neurotoxin coding sequence in the Multiple cloning sequence (MCS) of pGAPZ α vector.

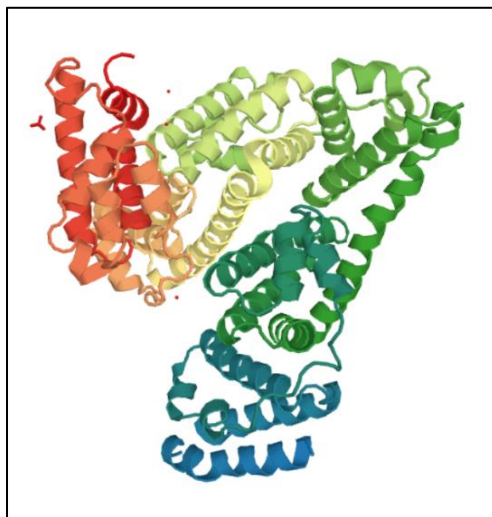


Figure 3. Crystal structure of XXX in PDB database.

2.2.6 cDNA synthesis and Polymerase Chain Reaction (PCR)

XXX total RNA (Zyagen) was retrotranscribed in cDNA using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher) following the manufacturer's instructions. The primers listed in Table 1 were used to amplify XXX and its three domains, using the synthesized cDNA, to be cloned into the pGAPZ α vector already containing the SFI2.6 toxin coding sequence (kindly donated from Angharad Gatehouse, University of Newcastle, UK). Additional sequences were added at the 5' of the primers, representing the sequences to be recognised by the restriction enzymes *NotI* and *XbaI*, to facilitate insertion of the PCR fragment into the expression vector pGAPZ α already containing SFI2.6 sequence.

| Primer name | Sequence (5'→3') | Melting temperature (T _m) |
|-----------------|------------------------------|---------------------------------------|
| XXX <i>fw</i> | XXXXXXXXXXXXXXXXXXXXXXXXXXXX | 65°C |
| XXX <i>rev</i> | XXXXXXXXXXXXXXXXXXXXXXXXXXXX | 64°C |
| Dom1 <i>fw</i> | XXXXXXXXXXXXXXXXXXXXXXXXXXXX | 65°C |
| Dom1 <i>rev</i> | XXXXXXXXXXXXXXXXXXXXXXXXXXXX | 65°C |
| Dom2 <i>fw</i> | XXXXXXXXXXXXXXXXXXXXXXXXXXXX | 64°C |
| Dom2 <i>rev</i> | XXXXXXXXXXXXXXXXXXXXXXXXXXXX | 65°C |
| Dom3 <i>fw</i> | XXXXXXXXXXXXXXXXXXXXXXXXXXXX | 64°C |
| Dom3 <i>rev</i> | XXXXXXXXXXXXXXXXXXXXXXXXXXXX | 64°C |

Table 1. Primers used for the amplification of XXX and its three domains. In red the sequences recognised by the restriction enzymes *NotI* (*fw* primers) and *XbaI* (*rev* primers).

The enzyme used was supplemented by Bioline and the PCR reaction was set up as indicated in Table 2.

| Component | Amount |
|---------------------------|---------------|
| Template | 1 µl (200 ng) |
| Primer fw (20µM) | 2.5 µl (1 µM) |
| Primer rv (20µM) | 2.5 µl (1 µM) |
| MyTaq HS Mix, 2x | 25 µl (1x) |
| Water (dH ₂ O) | up to 50µl |

Table 2. Components used in the amplification reaction.

PCR conditions were programmed to 1 minute at 95°C; 40 cycles of [15 s 95°C, 15 s 60°C, 1 min 72°C] and 15 min at 72°C.

2.2.7 Cloning of XXX and domains in SFI2.6-pGAPZα vector

In order to obtain the expression vector, the SFI2.6/XXX, SFI2.6/XXX domain1, SFI2.6/XXX domain2 and SFI2.6/XXX domain3 fusion constructs were first prepared by amplification of cDNA synthesized from XXX total RNA (Zyagen) using the primers in the Table 1.

The amplified and purified fragments were cloned in pCR 2.1 (Thermo Fisher) vector according to manufacturer's instruction and restricted with *NotI* and *XbaI* as the presence of these sites allows the insertion of the amplified fragments into the SFI2.6p-GAPZα vector.

The digestion reaction was performed using 1 µl of enzyme per µg of DNA and 10% of the final volume of 10x Fast Digest Buffer (Table 3).

| Component | Amount |
|-------------------------|---------------|
| pCR2.1 vector DNA | 1 µg |
| Fast Digest NotI | 1 µl (1 Unit) |
| Fast Digest XbaI | 1 µl (1 Unit) |
| 10x Fast Digest™ Buffer | 3 µl (1x) |
| Water | up to 30 µl |

Table 3. Digestion of pCR2.1 vector DNA.

Digestion reactions were incubated for 30 min at 37°C, the fully digested DNA was then inactivated by incubation at 80°C for 5 minutes. Restriction fragments were isolated from the agarose gel by QIAquick Gel extraction Kit (Qiagen) following manufacturer's instructions.

Then, the digested fragments were ligated to SFI2.6-pGAPZα (similarly digested) in a reaction with insert:vector ratio of 3:1 (Table 4).

| Component | Amount |
|----------------------------|---------------|
| SFI2.6-pGAPZαA vector DNA | 100 ng |
| XXX | 200 ng |
| domains | 60 ng |
| 10x Ligase Reaction Buffer | 2 µl (1x) |
| T4 DNA Ligase | 1 µl (1 Unit) |
| Water | up to 20 µl |

Table 4. Ligation reaction.

Reactions were incubated over night at room temperature to ensure complete ligation. Ligated constructs were further used to transform One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher). Transformation reaction was plated on LB agar plates (1% (w/v) Bacto-Tryptone, 0.5% (w/v) Yeast extract, 1% (w/v) NaCl, 1.5% (w/v) Agar) containing zeocin (25 µg/ml) and incubated overnight for selection of transformants containing the pGAPZα plasmid. Plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen) and positive transformants were confirmed by colony PCR and DNA sequencing.

2.2.8 Preparation of *Pichia pastoris* competent cells

The following procedure is for the preparation of competent *P. pastoris* cells produced with the *Pichia* EasyComp Kit (Thermo Fisher). A YPD Agar plate (1% (w/v) BactoYeast extract, 2% (w/v) BactoPeptone, 2% (w/v) Dextrose, 2% (w/v) Agar) was streaked with X33 *P. pastoris* strain to obtain single colonies. The plate was incubated at 30°C for 2-4 days. A single *P. pastoris* colony was inoculated in 10 ml of YPD and grown over night at 28-30°C in a shaking incubator (250-300 rpm). The overnight culture was diluted to an OD₆₀₀ (Optical Density) of 0.1-0.2 in 10 ml of YPD then grown at 28-30°C in a shaking incubator until the OD₆₀₀ reached 0.6-1.0. The cells were pelleted by centrifugation at 500 x g for 5 minutes at room temperature,

the supernatant discarded and the pellet resuspended in 10 ml of Solution I supplied by the kit. The cells were pelleted by centrifugation at 500 x g for 5 minutes at room temperature, the supernatant was discarded and the pellet was resuspended in 1 ml of Solution I.

2.2.9 Transformation of expression constructs in *P. pastoris*

Prepared constructs SFI2.6/XXX, SFI 2.6/XXX domain1, SFI 2.6/XXX domain2 and SFI2.6/XXX domain3 were transformed into *P. pastoris* for over-expression of recombinant proteins. DNA construct were linearized using *Bam*HI FastDigest (Thermo Fisher), adding 1 µl of enzyme per µg of plasmid DNA and 10% of the final volume of 10x Fast Digest Buffer.

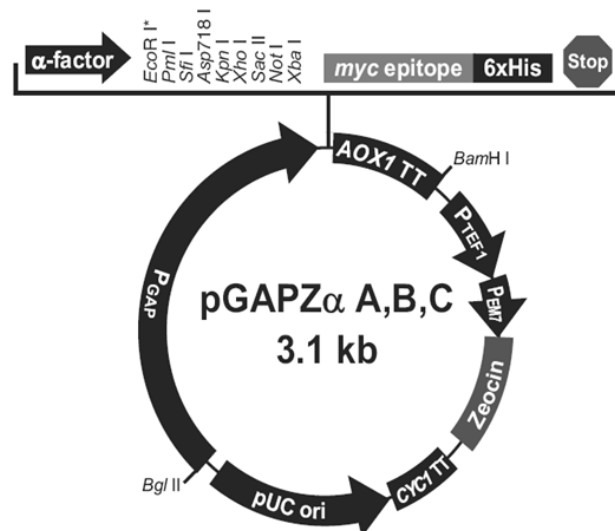


Figure 4. Schematic view of pGAPZα vectors. The pGAPZα A, B, and C (3.1 kb) vectors use the GAP promoter to constitutively express recombinant proteins in *P. pastoris*. Proteins can be expressed as fusions to a C-terminal peptide containing the myc and polyhistidine tags. In addition, pGAPZα produces proteins fused to an N-terminal peptide encoding the α-factor secretion signal.

Linear DNA can generate stable transformants of *P. pastoris* via homologous recombination between the transforming DNA and regions of homology within the genome (Cregg *et al.*, 1985; Cregg *et al.*, 1989).

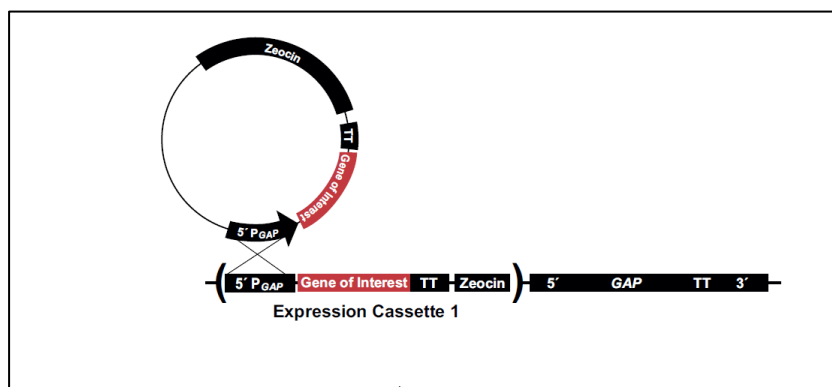


Figure 5. Homologous recombination for *P. pastoris* transformation. Gene insertion events at the GAP promoter locus from a single crossover event between the locus and the P_{GAP} region on the pGAPZα vectors.

In order to transform the yeast, 3 µg of linearized expression vector were added to the competent cells. 1 ml of Solution II was added to the DNA/cell mixture and mixed by vortexing the tube. The transformation reactions were incubated for 3 hours at 30°C in a water bath, mixed every 15 minutes by vortexing. The cells were heat-shocked in a 42°C heat-block for 10 minutes. The cells were split into 2 microcentrifuge tubes (approximately 525 µl per tube) and 1 ml of YPD medium added to each tube. Then, they were incubated at 30°C for 1 hour to allow expression of zeocin resistance and pelleted by centrifugation at 3,000 x g for 5 minutes at room temperature; after discarding the supernatant the pellet resuspended in Solution III. The last step was repeated and finally the cell pellet was resuspended in 150 µl of Solution III. The entire transformation reaction was plated on YPD Agar plates containing zeocin (100 µg/ml) and incubated for 2 to 4 days at 30°C, until colonies appeared on the plate. About 6 conspicuous colonies were picked randomly from the YPD agar plate and cultured in YPD media for 24-36 h. Glycerol stocks were prepared from these cultures and stored at -80°C until later use for protein production.

2.2.9.1 Small-scale screening for fusion protein expression

P. pastoris glycerol stocks were plated, single colonies were inoculated in 50 ml of YPD media 100µg/m zeocin and incubated at 30°C for 1-2 days on a shaker. 1 ml of culture media was collected every 48 h, centrifuged at 8,000 x g for 5 min, and the supernatant was collected. The protein expression was examined by SDS-PAGE and Western blot.

2.2.10 Protein expression and purification from *P. pastoris*

Selected positive colonies were cultured in YPD growth media together with the positive control expressing SFI2.6 toxin fused with *Galanthus nivalis* agglutinin (GNA) and grown in 200 ml of starter cultures in a 1L baffled flask for 2-3 days at 30°C with shaking. The starter culture was used to inoculate 900 ml of basal media (231 mM phosphoric acid, 6.83 mM calcium sulphate, 70 mM potassium sulfate, 60.4 mM magnesium sulfatex7H₂O, 73.6 mM potassium hydroxide, 435 mM glycerol, 0.03% (v/v) antifoam (Thermo Fisher)) in a bench top fermenter (New Brunswick Scientific Bioflo 110). The bench fermenter with basal media was calibrated with a pH probe and dissolved oxygen and sterilised in an autoclave at 121°C, 15 lbs pressure for 20 minutes. The sterilised fermenter vessel was then set up for measuring pH and temperature using a digital pH controller and digital temperature sensor. Cooling of the water supply was used to maintain the temperature at 30°C. Two 500 ml bottles were connected to the fermenter for the addition of acid and base, and one 1000 ml bottle for the addition of sterile 50% glycerol solution (v/v with distilled water), containing 9.6 ml PTM1 (24 mM cupric sulfatex5H₂O, 0.53 mM sodium iodide, 17.7 mM manganese sulfatexH₂O, 0.8 mM sodium molybdatex2H₂O, 0.3 mM boric acid, 4.9 µM cobalt chloride, 147 mM zinc chloride, 243 mM ferrous sulfatex7H₂O, 0.8 mM biotin, 51 mM sulfuric acid). After 24 hours of incubation, fermentation was initiated. Sterile media supplemented with (3.92 ml/l) PTM1 salts was inoculated with 100 ml starter cultures. Cultivation was set up with input of the following parameters: 30% dissolved oxygen, pH 4.5, 30°C. A glycerol feed (4-9 ml/h) was maintained during the fermentation process. A decrease in glycerol feed and increase in the level of dissolved oxygen makes the termination of the process.

2.2.10.1 Downstream processing of supernatant

Secreted proteins, contained in the supernatant, were separated from the cell pellet by centrifugation at 8,000 x g for 30 min at 4°C.

The supernatant was separated from the mycelium by sequential passing through a 1.7 µm, 1.2 µm, and 0.7 µm filters. Fast protein liquid chromatography (FPLC) was performed to purify the recombinant proteins. Fractions of 100 µl were collected and used for further analysis.

2.2.10.2 Ni²⁺-NTA affinity purification

Constructs were designed with a fused C-terminal 6xHis tag motif so they can be purified by affinity chromatography. It is based on the interaction between Ni²⁺ ions immobilized on a matrix and the histidine side chain on the tagged protein using Ni²⁺-NTA (nickel-nitrile-triacetic acid) affinity column. As a first step, the Ni²⁺-NTA column is charged by washing NTA resin with 50 mM EDTA (Ethylenediaminetetraacetic acid) (containing 0.1% (w/v) SDS, pH 8) followed by distilled water to remove EDTA. The column was equilibrated with 1x lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM Imidazole, pH 8), and then the supernatant diluted with lysis buffer to a final concentration of 1x was loaded onto the Ni²⁺-NTA column. Before washing the column 1x lysis buffer was loaded again. Then, the loaded column was washed with 50 ml of washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM Imidazole, pH 8). Finally, bound proteins were eluted with 15 ml of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM Imidazole, pH 8).

Each single fraction generated from the load, wash and elution steps was collected and analysed using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). After the end of protein purification, the column was stripped and rinsed following the manufacturer's recommendations. The soluble fractions of the protein were dialysed against distilled water at 4°C using dialysis tubing with a molecular weight cut off of 12-14 kDa. Dialysed proteins were transferred to a round bottom flask and then it was frozen in liquid nitrogen and freeze dried.

2.2.11 SDS-PAGE

SDS-PAGE is routinely used to separate macromolecules based on molecular mass. Samples collected at various stages of purification (10 µl) were mixed with 10 µl loading dye (40% (v/v) Glycerol, 240 mM Tris/HCl, 8% (w/v) SDS, 0.04% (w/v) bromophenol blue, 5% (v/v) beta-mercaptoethanol, in distilled water, pH 6.8), and boiled at 95°C for 10 min. The denatured samples were loaded on 12% SDS-PAGE gel and were separated in a Mini-Protean Tetra cell gel electrophoresis apparatus (BioRad) with running buffer (25 mM Tris base, 192 mM Glycine, 0.1% (w/v) SDS, pH 8.3). Proteins were run at 80 V for 15-20 min, and then the voltage was increased to 120 V for additional 45 min. The gels were then either stained with Coomassie brilliant blue staining solution (40% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (v/v) Coomassie brilliant blue (Thermo Fisher), in distilled water) overnight, at a room temperature, or used immediately for Western blot analysis. Stained gels were then immersed in destaining solution (20% (v/v) methanol, 10% (v/v) acetic acid, in distilled water) for 2-3 days at room temperature, under shaking, until a clear background was obtained. Gel images were recorded using a Gel-Doc system (BioRad).

2.2.12 Western Blotting

Following SDS-PAGE, the semi-dry method was used to transfer protein from the gel to nitrocellulose membranes. A 0.2 µm pore size membrane is suitable for use with a

low molecular weight of proteins. Blotting of the gel was done by soaking nitrocellulose membranes and pieces of blotting paper in Towbin transfer buffer (0.025 M Tris base, 0.192 M glycine, 20 % (v/v) methanol) and then assembled in the following order: cathode plate, 3 sheets of blotting paper, acrylamide gel, nitrocellulose membrane, 3 sheets of blotting paper, and anode plate; these were then placed in an electro blotter. The process of electro-blotting was done at 0.1 A for 45 minutes. The transfer process followed by blocking the membrane with 50 ml of blocking solution (5% (w/v) non-fat milk powder, 1x PBS, 0.1% (v/v) Tween-20) for 1 hour with gentle agitation at room temperature. The blocking step was followed by placing the membrane in the primary antibody, anti 6xhis (Invitrogen), at a dilution 1:2000 in blocking buffer and allowed to incubate overnight at 4°C. Removing residual unbound primary antibody was then done by washing the membrane with 1x PBS (Phosphate Buffered Saline) (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4) 3 times, for 5 minutes each time, under shaking conditions. The membrane was then left in diluted HRP-conjugated secondary antibody, goat anti-mouse antibody in blocking buffer at ratio 1:2000 (v/v), for 2 hours at room temperature. The membrane was then briefly rinsed with two changes of TBST (Tris Buffered Saline) (50 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Tween-20, pH 7.5) for 10 minutes and then thoroughly washed in distilled water. The specifically bound secondary antibody of the target protein was detected using SuperSignal West Pico Chemiluminescent Substrate (Invitrogen). Detection solution was mixed in a ratio of 1:1, poured over the membrane and incubated for 5 minutes. The membrane was then exposed to photosensitive film (Fuji-RX). Automatic X-ograph Imaging systems Compact X4 developer was used to develop the film.

The obtained bands have been evaluated taking into account the molecular mass of the fusion proteins predicted by using an online tool (<http://web.expasy.org>).

2.3 Results

Although the absorption of proteins by the insect midgut *in vivo* has been reported by several authors (Jeffers and Roe, 2008), the study of the mechanism of this process has been approached only recently. After the demonstration *in vitro* that transcytosis is the process involved in the transport across lepidopteran midgut of unprocessed large proteins (XXX and horseradish peroxidase) (XXX *et al.*, XXX; XXX), further studies on primary cultures of lepidopteran midgut cells established that internalization of XXX occurs by clathrin-mediated endocytosis and is mediated by a XXX receptor, homologous to the multiligand receptor expressed in many mammalian absorptive epithelia (XXX *et al.*, XXX). Clathrin-mediated endocytosis is also involved in XXX internalization by *S. littoralis* midgut cells (XXX *et al.*, XXX). This finding provided a new opportunity for the use of XXX as a carrier molecule for the delivery of bioinsecticidal proteins with haemocoelic targets, since the interaction with an endocytosis receptor strongly enhances protein uptake.

Since the mature XXX molecule is X kDa, a quite big size for a potential carrier molecule, here we try to identify the shortest amino acid sequence recognized and internalized by the XXX receptor. XXX and its three domains have been investigated for their ability to cross the intestinal barrier fused with one of the neurotoxins derived from *Segestria florentina* salivary glands, SFI2.6. The main purpose of this work is to establish if XXX or one of its domains are able to enhance the absorption of SFI2.6 through the insect gut.

2.3.1 Assembly of the SFI2.6-XXX/XXX domains fusion protein constructs

The sequence encoding XXX and its three domains, obtained from UniProt [XXX], were successfully amplified from the cDNA synthesized from XXX total RNA (Zyagen). The primers used, listed in the Table 1 (see materials and methods), are known to contain *NotI* and *XbaI* recognition sites at 5' ends of forward and reverse primers, respectively, to allow the ligation of the PCR products into the vector pGAPZ α , already containing the insecticidal neurotoxin SFI2.6 coding sequence, kindly donated by Professor Angharad Gatehouse (Newcastle University, UK).

The PCR products were electrophoretically separated on a 1% agarose gel and amplification of bands with the correct size was obtained (Figure 6).

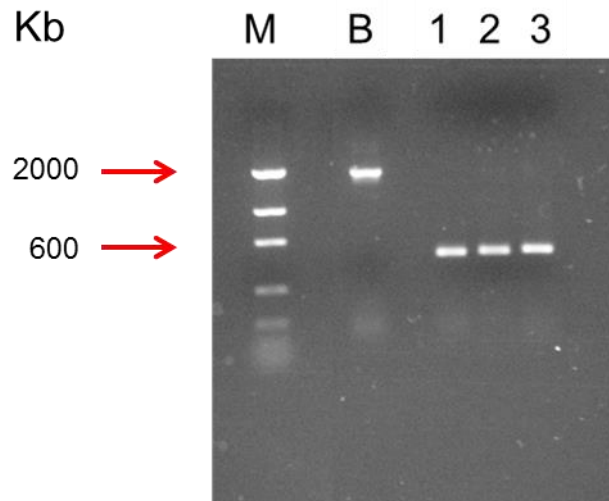


Figure 6. Amplification of XXX and XXX domains fragments. M: 100 bp marker; B: XXX; 1: XXX domain 1; 2: XXX domain 2; 3: XXX domain 3.

Amplified and purified fragments were cloned in pCR 2.1 vector and restricted with *NotI* and *XbaI* (Figure 7), to obtain the insert from the vector backbone. The presence of these sites allows the insertion of the amplified fragments into the SFI2.6p-GAPZ α final vector (Figure 8).

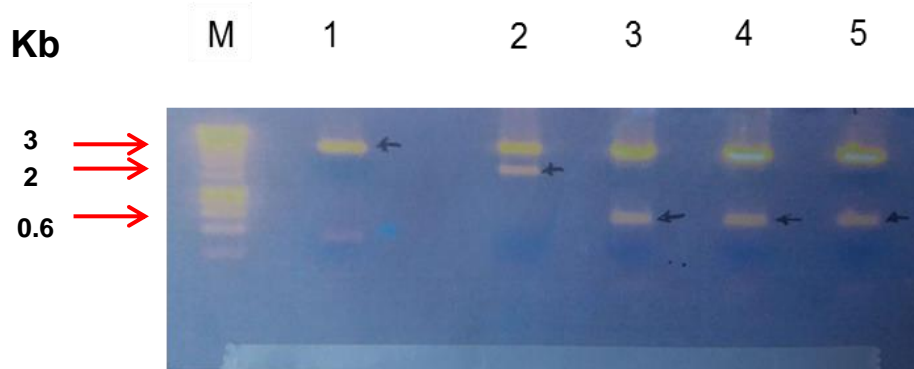


Figure 7. Agarose gel electrophoresis of double digestion with *NotI* and *XbaI* of pCR 2.1 containing XXX (lane 2) and its domains sequences (lanes 3, 4, 5). In lane 1 SFI2.6/GNA-pGAPZ α plasmid digested with *NotI* and *XbaI*.

To check the results of the reaction, digestion products were run on a 1% agarose gel. The double digestion reaction revealed the presence of the whole XXX sequence of about 2 kb (Figure 7, lane 2) and the three separate domains of about 600 bp (Figure 7, lanes 3,4,5), in addition to the pCR 2.1 “waste vector” of about 3 kb.

The destination vector was similarly digested. The destination vector used was obtained from SFI2.6/GNA-pGAPZ α , containing the SFI2.6 toxin coding sequence fused with GNA coding sequence. The latter was discarded and subsequently substituted with XXX and domains coding sequences.

The digestion revealed the presence of the SFI2.6/pGAPZ α vector of about 3 kb and the GNA sequence of about 200 bp to be discarded (Figure 7, lane 1). The digested fragments have been then recovered from the gel and purified. Then they have been successfully cloned into the expression vector SFI2.6-pGAPZ α .

The constructs for recombinant expression of SFI2.6/XXX, SFI2.6/XXX domain1, SFI2.6/XXX domain2 and SFI2.6/XXX domain3 fusion proteins were designed to link

the 3' end of the SFI2.6 toxin sequence, present inside the pGAP α vector, and the 5' of the sequence coding for XXX or its domains. In addition, the constructs are arranged in frame at the 5' with the signal peptide-encoding sequence and at the 3' with *myc* and his tags-encoding sequences (Figure 8).

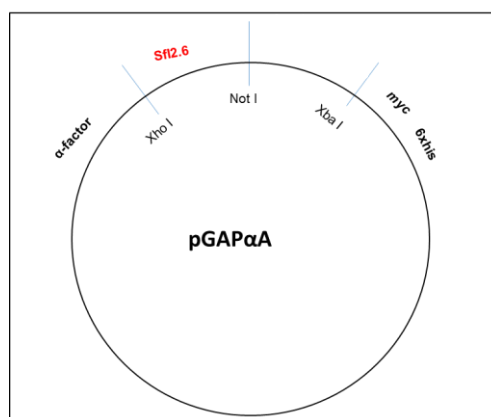


Figure 8. Schematic view of SFI2.6-XXX/XXX domains constructs.

Consequently, the protein products contain a *N*-terminal secretory signal, the yeast α -mating factor, to ensure the secretion of the translated protein into the medium and a C-terminal extension of 23 amino acids, including a *myc* epitope and 6xhis tag (Figure 9), to facilitate purification of the expressed proteins.

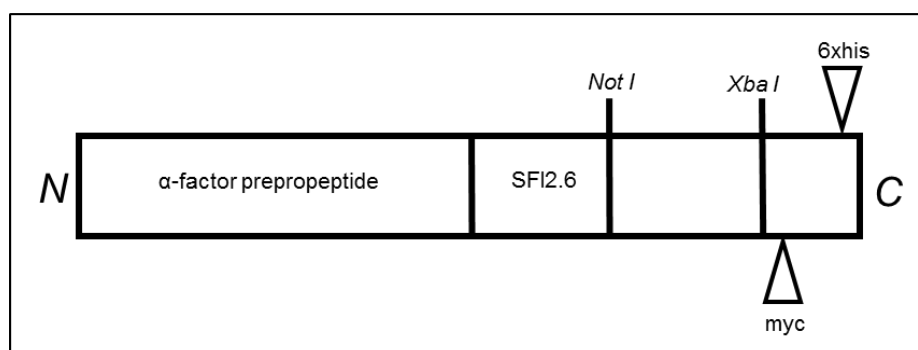


Figure 9. Schematic representation of fusion protein constructs.

Positive clones were identified by colony PCR, using the primers listed in the Table 1 (see materials and methods), and DNA sequencing was performed to check them for the correct fragment joining to ensure that no errors had occurred when the restriction and the ligation of fusion protein constructs were carried out. A PCR colony screening on all different colonies confirmed the maintenance of correct construct integrity. DNA sequencing was used to confirm maintenance of correct construct integrity and positioning at each stage. The sequence was translated using a free translation tool (<http://web.expasy.org/translate>). A schematic diagram of the expression constructs and the predicted protein sequences for the SFI2.6/XXX, SFI2.6/XXX domain1, SFI2.6/XXX domain2 and SFI2.6/XXX domain3 is shown in Figure 10.

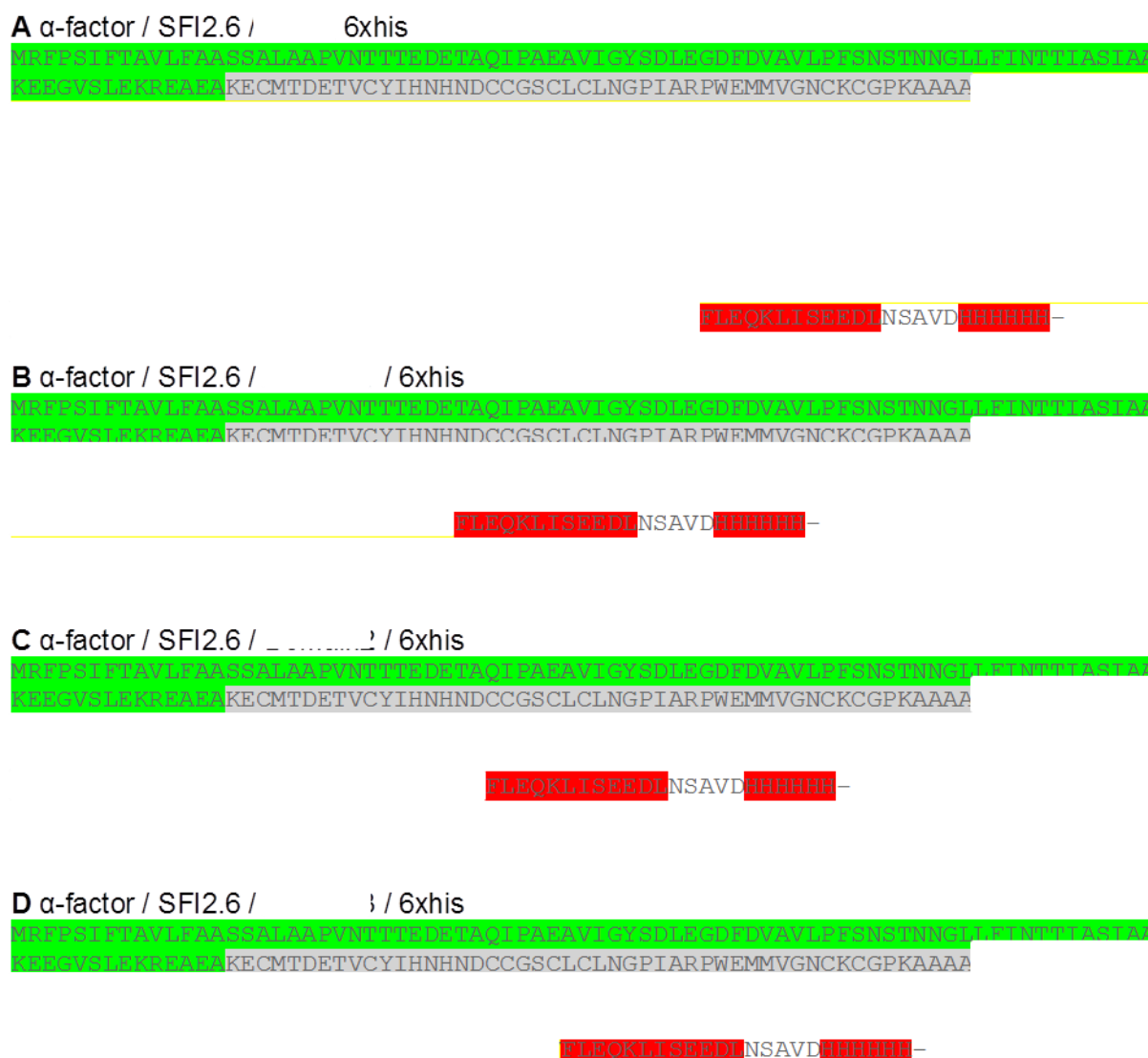


Figure 10. Presumed amino acid sequence generated from the online tool *ExPASy* produced from the fusion constructs SFI2.6/XXX (**A**), SFI2.6/XXX domain1 (**B**), SFI2.6/XXX domain2 (**C**) and SFI2.6/XXX domain3 (**D**). In green the yeast alpha factor signal sequence; in grey the SFI2.6 toxin sequence; in yellow, XXX (A), XXX domain 1 (B), XXX domain 2 (C), XXX domain 3 (D) fragments respectively; in red the myc and the 6xhis tags.

DNA from a verified clone of the complete expression constructs SFI2.6/XXXpGAPZ α , SFI2.6/XXX domain1-pGAPZ α , SFI2.6/XXX domain2-pGAPZ α , and SFI2.6/XXX domain3-pGAPZ α were linearized (Figure 11) to transform competent cells and to integrate via homologous recombination the expression cassettes into the genome of the host *P. pastoris*, X33 strain. Finally the positive clones were selected on zeocin-containing plates.

The fusion constructs were linearized at site 5' GGATCC 3' with *Bam*HI, resulting in a fragment of about 5 kb for XXX fusion construct (Figure 11, lane 1), and about 3.6 kb

for domains fusion constructs (Figure 11, lanes 3-4). This also served as a control to ensure that the *Bam*HI enzyme had worked.

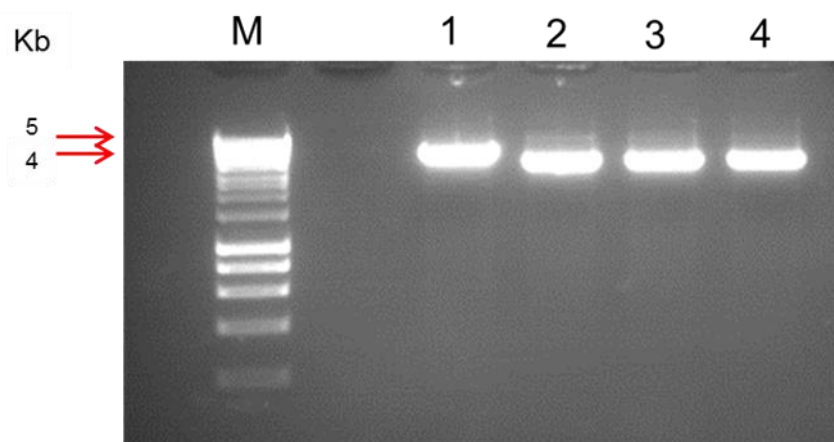


Figure 11. Agarose gel electrophoresis after of pGAP α linearization using *Bam*HI. Linearized SFI2.6/XXX-pGAPZ α (lane 1); SFI2.6/XXX domain1-pGAPZ α (lane 2), SFI2.6/XXX domain2-pGAPZ α (lane 3), SFI2.6/XXX domain3-pGAPZ α (lane 4).

2.3.2 Expression of the recombinant fusion proteins

Plasmids properly expressing the constructs SFI2.6/XXX-pGAPZ α , SFI2.6/XXX domain1-pGAPZ α , SFI2.6/XXX domain2-pGAPZ α and SFI2.6/XXX domain3-pGAPZ α were linearized and inserted into competent cells of the *P. pastoris* X33 strain. Due to the presence of a constitutive yeast promoter on the expression vector, they were selected on zeocin-containing plates. After preparation of the fusion constructs, the expression of recombinant proteins were run on a small-scale culture, allowing isolation of high level expression clones. Culture supernatants were analysed by Western blotting, using an anti-6xhis-antibody. A SFI2.6-GNA fusion protein containing 6xhis tag was used as a positive control. Results demonstrated that the fusion protein containing XXX was correctly expressed, with a molecular weight of about 80 kDa (Figure 12).

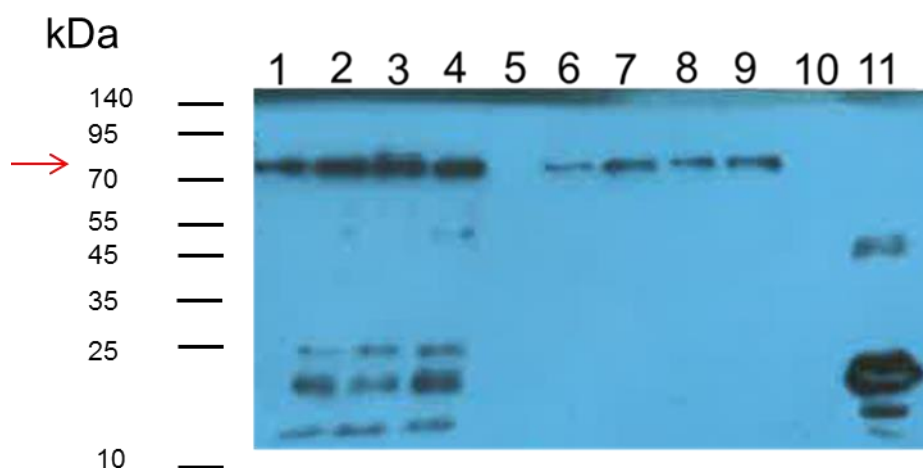


Figure 12. XXX fusion protein supernatants (lanes 1-4), XXX fusion protein pellets (lanes 6-9), SFI2.6 toxin/GNA as positive control in lane 11.

Western blot analysis of the fusion proteins from different yeast colonies demonstrated that they have been expressed with slightly different molecular mass. The fusion protein containing domain1 (Figure 13, lanes 1-3) showed a molecular mass of about 35 kDa. The fusion proteins containing domain2 (Figure 13, lanes 4-6) and domain3 (Figure 13, lanes 7-9) showed a molecular mass of about 40 kDa. The delay in the gel run is likely due to some post-transcriptional modifications including phosphorylation or glycosylation, which naturally occur when recombinant proteins are expressed in a eukaryotic system.

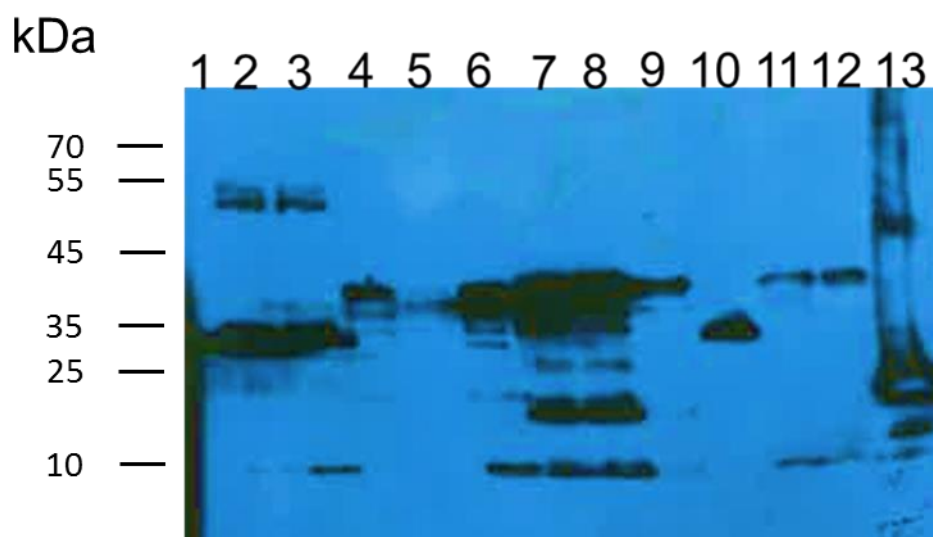


Figure 13. XXX domain1 fusion protein (lanes 1-3); XXX domain2 fusion protein (lanes 4-6); XXX domain3 fusion protein (lanes 7-9); SF12.6 toxin/GNA as positive control in lane 13. All the proteins are extracted from cell medium. In lanes 10, 11 and 12 XXX domain1, 2 and 3 fusion proteins proteins extracted from the cell pellets.

The expression of recombinant proteins run on a small-scale culture allowed the selection of the best expressing clone for fusion protein production by bench-top fermentation. One clone of *P. pastoris* X33 expressing each of the two recombinant proteins was used for bench-top fermentation. The growth of selected yeast clones expressing the fusion proteins was performed in a bench-top fermenter, using a start culture previously expanded for 2-3 days. During the fermentation, parameters for dO_2 and pH were closely monitored and regulated. The supernatants were then collected by centrifugation and clarified by filtration through a filter flask before mixing with a 2x binding buffer, for the loading into a liquid chromatography column.

2.3.3 Purification of recombinant proteins

The recombinant fusion proteins contained a 6xhis-tag so the proteins were purified by nickel affinity chromatography, using a HisTrap nickel column. The proteins were detected by monitoring their UV absorbance at 280 nm. The protein content of every fraction was checked by SDS-PAGE. Gels were either stained with Comassie dye or used for Western blot analysis using anti 6xhis-antibody (Figure 14).

For all constructs, the recombinant proteins were eluted with 125 mM imidazole. Eluted protein fractions were dialysed against distilled water at 4°C, using tubing with a molecular weight cut off of 12-14 kDa, to remove imidazole, free toxin, and salts from the samples. Following dialysis, the proteins were transferred into a round bottom flask, frozen in liquid nitrogen and freeze-dried.

The successful recovery of the recombinant proteins in the elution fraction was confirmed by Western blot (in figure 14 the results for SFI2.6/XXX and SFI2.6/XXX domain1 are shown). The final dried proteins were re-suspended in 100 μ L distilled water. The total concentration of protein in the samples was determined by a Bradford assay.

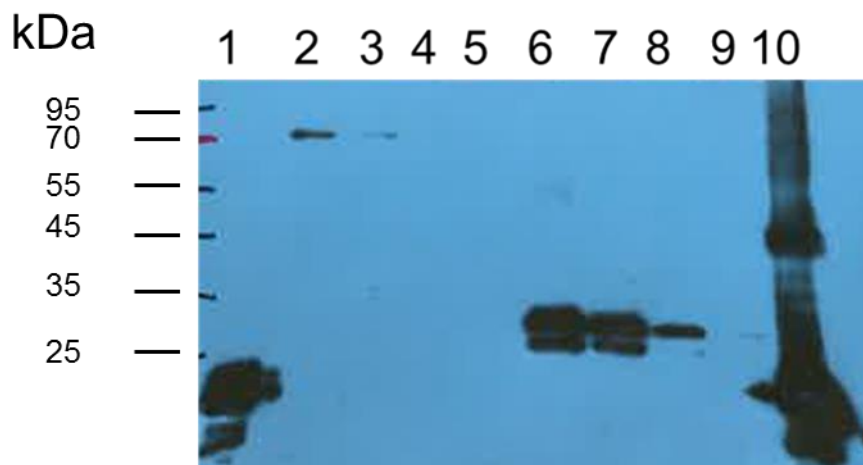


Figure 14. XXX fusion protein elution fractions (lanes 2-5); XXX domain 1 fusion protein elution fractions (lanes 6-9); SFI2.6/GNA as positive control in lane 10.

2.3.4 Biological activity of fusion proteins incorporating the SFI2.6 toxin and XXX or its domains

The amount of the fusion proteins estimated by a standard Bradford assay was quite low. In particular the protein yield of SFI2.6/XXX and SFI2.6/XXX domain1 fusion proteins was 0.1 mg, sufficient to test them in feeding bioassays on the pea aphid, *Acyrtosiphon pisum*, but not on Lepidoptera, since much higher amounts were required; while the protein yield of SFI2.6/XXX domain2 and SFI2.6/XXX domain3 was lower than 0.01 mg, not sufficient to run an adequate number of bioassay replicates.

To do this, SFI2.6/XXX and SFI2.6/XXX domain1 fusion proteins have been dissolved into *A. pisum* artificial diet, at 0.1 mg/ml. Three negative controls were used: the artificial diet alone, the same supplemented with 0.1 mg/ml XXX and with 0.1 mg/ml GNA. Water and SFI2.6/GNA incorporated into the artificial diet at the same concentration were used as positive controls. The toxicity of all the protein constructs were assayed using *A. pisum* third-instar nymphs.

30 *A. pisum* (3 independent replicates of 10 nymphs each) were fed with SFI2.6/XXX and SFI2.6/XXX domain1 recombinant proteins to assess the oral toxicity. At 0.1 mg/ml, survival of *A. pisum* fed with SFI2.6/XXX and SFI2.6/XXX domain1 was dramatically reduced when compared to the control fed with artificial diet only, 0.1 mg/ml XXX and 0.1 mg/ml GNA. Furthermore, SFI2.6/XXX and SFI2.6/XXX domain1 show higher mortality compared to the positive control SFI2.6/GNA.

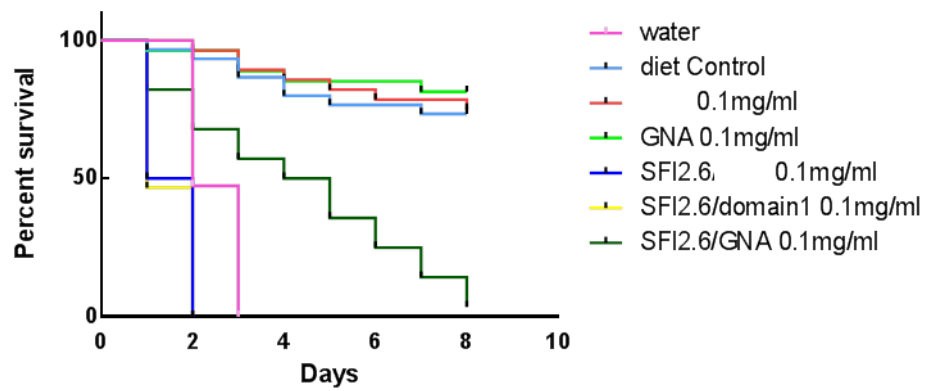


Figure 15. Kaplan-Meier survival analysis on *A. pisum* bioassay (Log-rank statistics; $P < 0.001$; $n = 30$).

2.4 Discussion

Arthropods which are natural antagonists of insects include a wide range of predators, such as spiders, scorpions and many other insects, as well as parasitoids, mostly in the orders of Hymenoptera and Diptera (Quicke, 1997). These beneficial arthropods have evolved a wealth of molecular weapons to kill or dramatically alter the host physiology, which, among others, include very potent venom blends, particularly amenable to be used as a source of new natural bioinsecticides (Windley *et al.* 2012; Pennacchio *et al.* 2012). Nevertheless, a typical crop protection application relies on oral application and venom components, often containing very active neurotoxins, are not orally active because they are evolutionary meant to be injected. In fact, after being ingested by insects, proteinaceous toxins require to escape proteolysis in the gut lumen in order to be transported across the gut epithelium, in an undegraded bioactive form, to the haemolymph, where they can reach their natural targets and exert their function (Pennacchio *et al.*, 2012).

In this thesis work, I focused on the development of an efficient delivery strategy for SFI2.6, a neurotoxin derived from the spider *S. florentina* that selectively blocks insect calcium channels. This toxin has modified C- and N- termini that promote a strong *in vivo* stability and, in addition, has several disulphide bonds contribute to the assembly of the structural motif “inhibitor cystine-knot”, that provides a constrained globular conformation to the molecule. As a consequence, SFI2.6, as many other spider toxins, is extremely resistant to proteolysis and environmental degradation. Then, the use of this toxic domain appeared particularly amenable for the design of a fusion protein facilitating its passage through the gut in an unaltered form.

Previous results have shown that the oral insecticidal activity of peptides derived from the venom of spiders and scorpions can be enhanced by fusing them to the protein carrier GNA (Fitches *et al.*, 2002; 2004; 2012; Pyati *et al.*, 2014; Nakasu *et al.*, 2016). To find out alternative carriers, I focused on the use of XXX because previous studies demonstrated in Lepidoptera that XXX is able to efficiently cross, undegraded, the gut epithelium (XXX *et al.*, XXX; XXX *et al.*, XXX). Since the mature XXX molecule is quite large as a carrier (X kDa), it appeared very attractive the idea of identifying the shortest XXX sequence maintaining the capacity to cross the gut epithelium shown by the entire protein. XXX and its three domains have thus been fused with SFI2.6, to establish if XXX or one of its domains were able to allow the absorption of the neurotoxin through the insect gut.

In the present study recombinant expression in yeast of SFI2.6/XXX, SFI2.6/XXX domain1, SFI2.6/XXX domain2 and SFI2.6/XXX domain3 fusion proteins has been performed. These latter two domains showed a molecular mass slightly exceeding the expected value, likely due to post-translational modifications. Indeed, these results are in agreement with the higher concentration on XXX domain2 and XXX domain3 of predicted sites for post-translational modifications, which can be obtained by using NetGlycate, DictyOGlyc and NetPhos (<http://www.cbs.dtu.dk>), and with the reasonable hypothesis that post-translational modifications can similarly take place on the whole protein and on its separated domains. *P. pastoris*, unlike bacterial expression systems, has the ability to perform many of the post-translational modifications usually performed in higher eukaryotes, including disulphide bond formation, O- and N-linked glycosylation, phosphorylation, and ubiquitination (Shirai *et al.*, 2008). Glycosylation is one of the most common post-translational modifications in *P. pastoris*, which may take place also on those proteins, which are not normally glycosylated by the native host, thus the addition of glycosylation

residues (Cereghino and Cregg, 2000). For example, in the case of the expression of a glucoamylase catalytic domain from *Aspergillus awamori* by *P. pastoris* the molecular weight of the secreted protein was 20 kDa heavier than the native form (Heimo *et al.*, 1997). About 10 kDa could be attributed to O-linked glycosides added by *P. pastoris*, probably consisting of 20-30 mannose residues.

P. pastoris has the capability to secrete expressed proteins into the culture medium due to the yeast alpha-factor sequence incorporated in the expression vector used, pGAP α . This process reduces contaminations and difficulties associated with the isolation of proteins from the cell lysate. Nevertheless, also some drawbacks are linked with the secretion of the fusion proteins into the medium, such as proteolysis by intracellular proteases from lysed cells and, to less extent, by extracellular proteases and cell-bound proteases (Kang *et al.*, 2000; Gellissen, 2000; Cereghino and Cregg, 2000; Jahic, 2003; Fitches *et al.*, 2004). In fact, the numerous bands with low molecular weight visible in the Western blot performed on the recombinant proteins obtained suggest that they have been partially degraded. Problems associated with proteolysis, which occurs predominantly between the insecticidal domain and the carrier protein, can be foreseen in the production of recombinant proteins in *P. pastoris*, such as the reduction of product yield and decrease of bioinsecticidal activity as suggested from Fitches *et al.* (2004).

The integrity of the expressed fusion proteins could be enhanced by the addition of linker regions between the toxin and the carrier coding sequences as suggested in Gustavsson *et al.* (2001), or by genetic modification with site-directed mutagenesis to remove potential cleavage sites, as suggested in Pyati *et al.* (2014).

Because the fermentation process was successfully performed with XXX and its domain1, while for the other domains the protein yield was not sufficient to run an adequate number of bioassay replicates, I focused the functional work on SFI2.6/XXX and SFI2.6/XXX domain1 fusion proteins. However, although these fusion proteins were successfully expressed, the yield of production was quite low.

Optimization of *P. pastoris* media components and culture conditions, such as temperature, are possible solutions to improve fusion proteins expression. Furthermore, another possible strategy could be the assembling of double copies of fusion constructs into the expression cassette inserted in the pGAP α vector as suggested in Pyati *et al.* (2014). The assembling of double copies construct is more reliable than the earlier method of screening large numbers of transformants for high copy strains resulting from multiple integrations of a transforming plasmid (Higgins and Cregg, 1998). Due to the low protein yield, the obtained amounts of fusion proteins were not sufficient for feeding bioassays with lepidopteran larvae. Then, we switched to aphids, which in preliminary trials proved to have a gut epithelium permeable to XXX (unpublished data), and require much lower amounts of recombinant protein to be dissolved in liquid diet.

This choice is further motivated by the fact that aphids are very important agricultural pests. Among the many pest aphids, the pea aphid, *Acyrtosiphon pisum*, is probably one of the most intensively studied and adopted for laboratory and genetic studies. The nutritional physiology of aphids do also offer additional elements supporting their use for feeding bioassays, even at low concentration. These insects exclusively feed on plant phloem sap that contains much sugar and some nonessential amino acids, but is poor in lipids and proteins; it is largely accepted that aphids substantially have no intestinal digestion of proteins (Rispe *et al.*, 2008). Indeed, a number of studies have proved oral insecticidal activity on aphids of peptides derived from the venom of spiders and scorpions fused with GNA

(Fitches *et al.*, 2002; 2004; 2010; 2012; Trung *et al.*, 2006; Wakefield *et al.*, 2010; Pyati *et al.*, 2014; Nakasu *et al.*, 2016).

The results obtained from bioassays on the pea aphid suggest that XXX and domain1 are extremely efficient carriers compared with GNA, used as positive control. It will be worthy to test in future the oral toxicity of SFI2.6/XXX domain2 and SFI2.6/XXX domain3 as well.

Even though the toxicity of the orally administered fusion proteins containing XXX and domain1 demonstrates that they are transported across the gut of the aphids, and then released to the haemolymph where the toxins can reach their targets and exert their function, the mechanism involved is still unknown and these aspects are worth of future investigations.

In conclusion, the toxicity of orally administered fusion proteins containing XXX and domain1 fused to SFI2.6 toxin strongly suggests that both XXX and its domain1 have the potentiality to act as molecular shuttles for toxins with haemocoelic targets. Considering the total number of species that produce insect-specific toxins and the variety of toxins within each venom type, the potential for the development and application of novel biopesticides, based on the proposed delivery strategy, using XXX or its domains as carriers, appears virtually limitless.

3 THE USE OF BACTERIA FOR THE ORAL DELIVERY OF DSRNA

3.1 Introduction

3.1.1 RNA interference (RNAi)

RNA interference (RNAi) is a mechanism of transcriptional, post-transcriptional and translational regulation of gene-expression, which is highly conserved among higher eukaryotes (Carthew and Sontheimer, 2009; Berezikov, 2011). A messenger RNA (mRNA), in the presence of complementary RNA (endogenous or exogenous), forms a very stable double-stranded structure. This leads to specific degradation of mature mRNA and, therefore, the block of gene expression. The level and complexity of gene regulation by RNAi appears to be much more intricate than originally anticipated, which has led to the opinion that this process is the principle means of fine tuning protein levels in cells (Bartel, 2009).

The main endogenous effectors of the RNAi phenomenon are small endogenous single-stranded non-coding RNA molecules (about 20-22 nucleotides long), encoded by the eukaryotic nuclear DNA, and mainly active in the regulation of gene expression at the transcriptional and post-transcriptional level. In the first case they are useful for the normal turnover of endogenous mRNAs, necessary to ensure a possible rapid change in the expression profiles of these molecules; whilst at the post-transcriptional level it involves a suppression of translation, or degradation of the target molecule (Ambros, 2004). Moreover, in nature, the RNAi phenomenon seems to play an important protective role of the genome from instability caused by the accumulation of transposons and repeated sequences and it may constitute part of a defence mechanism against viral infections, as well as playing a role in developmentally regulated translational suppression (Ding, 2010).

3.1.1.1 Small RNAs (sRNA) in RNAi mechanism

Small RNAs (sRNAs) can be classified into three categories according to the origin (endogenous or exogenous), their precursors structure and the pathway through which they are processed: short interfering RNA (siRNA), microRNA (miRNA) and piwi-interacting RNA (piRNA).

Short interfering RNA or siRNA: short fragments of dsRNAs of about 20-25 nucleotides (Caplen *et al.*, 2001) that can be synthetically produced, from long molecules of dsRNA. The biological functions of endo-siRNAs include repression of transposable elements, chromatin organisation as well as gene regulation at transcriptional and post-transcriptional level (Brennecke *et al.*, 2007; Chung *et al.*, 2008; Ghildiyal *et al.*, 2008; Tam *et al.*, 2008; Watanabe *et al.*, 2008; Fagegaltier *et al.*, 2009; Ghildiyal and Zamore, 2009; Piatek and Werner, 2014).

Micro interfering RNA or miRNA: oligomers of about 22 nucleotides that contain non-coding inverted and repeated regions allowing the formation of a double-stranded hairpin able to trigger the mechanism of RNAi (Sledz and Williams, 2005). A microRNA molecule is transcribed by RNA polymerase II from the genomic DNA as a primary long RNA transcript known as pri-miRNA. The pri-RNA has a double-stranded structure, recognized by the protein-based microprocessor complex,

containing proteins that bind the double-stranded RNA, called Pasha (Drosha in invertebrates); the complex inside the nucleus chops the hairpin-loop structure to produce a characteristic repetitive secondary 70-base pair structure, the pre-miRNA (Lee *et al.*, 2003). At the end of the nuclear processing the pre-miRNAs are exported towards the cytoplasm, where they will then be further processed by RNase III (Dicer) in mature miRNA in the cell cytoplasm (Han *et al.*, 2006). The cleavage produces double-stranded RNA molecules of about 22 base pairs: thus starting from a double-stranded complex (pre-miRNAs), a short half-life intermediate, one of the strands is degraded by a nuclease while the other strand represents the mature miRNA (Lund and Dahlberg, 2006).

Piwi-interacting RNA or piRNA: 24-31-nucleotide-long fragments that differ from miRNA and siRNA for three main features. First, it is generally known that piRNA are produced from primary transcripts encoded by single-stranded defined genomic regions (Aravin *et al.*, 2007a). Second, they require the presence of Piwi proteins and the mechanism appears to be independent from Dicer activity (Vagin *et al.*, 2006 and Houwing *et al.*, 2007). In addition, they contribute to the silencing of transposable elements exclusively in the gonads (Aravin *et al.*, 2003, 2007b; Vagin *et al.*, 2006; Brennecke *et al.*, 2007; Olivieri *et al.*, 2010), where they determine the silencing of transposons and retrotransposons in the germ cells (Lin, 2007). Furthermore, as recently described in Ross *et al.* (2014) Piwi proteins have some peculiar roles also in somatic cells, such as genome rearrangement and epigenetic programming.

3.1.1.2 The molecular mechanism of RNAi

In many organisms, intergenic or antisense transcription produces different types of small RNAs and long non-coding RNAs (lncRNAs) that have emerged as key regulators of gene expression. In addition to their roles in RNA degradation and translational repression, small RNAs interfere with chromatin structure and target gene expression via RNA interference pathways triggering co-transcriptional silencing mechanisms (Holoch and Moazed, 2015).

The silencing process may be schematically divided into two steps (initiator and effector steps) common in fungi, plants, nematodes, insects and vertebrates. The small RNAs production involves different proteins, among these Dicer and Argonaute respectively represent the core of the initiator phase, which leads to the sRNA production and the effector phase.

Initiator phase: processing of dsRNA (or pre-miRNA)

The dsRNA molecules are recognized and cut into sRNA from Dicer, a dimer which belongs to the RNase III family (Sledz and Williams, 2005). Crystallographic studies show two catalytic domains with endonucleasic activity (RNase III), an helicase ATP-dependent NH₂-terminal domain and a COOH-terminal domain with specific sites for dsRNA binding (Filipowicz, 2005), in addition to a PAZ domain (Piwi/Argonaute/Zwille) (Moss, 2001). Dicer functions as a monomer containing a single center for the ATP-dependent cleavage of dsRNA to produce the sRNA (microRNAs and siRNAs) (Moss, 2001).

Effector phase: incorporation of sRNA (siRNA or miRNA) in a protein complex

The sRNAs, produced by Dicer, are incorporated into a nuclease complex composed by multiple subunits that, according to the species and nature of sRNA (siRNA or miRNA), change in the structure and the effect induced on the target transcripts.

The multiprotein complex RISC (RNA Interference Silencing Complex) is fundamental for the specific recognition and degradation of target transcript

(Hutvagner and Zamore, 2002; Meister and Tuschl, 2004). The central element of the RISC complex is a member of the Argonaute gene family (AGO). AGO has a high content of basic amino acids and its nuclease activity is involved in the mRNA cleavage. AGO proteins are in all eukaryotic organisms (Filipowicz, 2005), they have a molecular weight of about 100 kDa and contain two domains, one in the *N*-terminal position, called PAZ, and the other in *C*-terminal position, called PIWI (Carmell *et al.*, 2002), which is involved in the interaction with Dicer protein (Meister and Tuschl, 2004). The initial RISC complex remains inactivated until the unfolding of the two siRNA strands (or miRNA) convert it into an active form, by means of the helicase activity of the same complex (Sledz and Williams, 2005). At this point RISC interacts only with one of the two strands of siRNA or miRNA, starting the sequence-specific degradation of mRNA complementary (siRNA) or partially complementary (miRNA) to the "guide" sequence (Martinez *et al.*, 2002). This process involves, respectively, the mRNA degradation or inhibition of translation (Sledz and Williams, 2005). Experimental evidence has shown that miRNAs are also able to block the elongation of the amino acid chain or its termination (Meister and Tuschl, 2004). The remarkable efficiency of RNAi has been associated to the amplification of the interference mechanism. It has been shown that the target mRNA, complementary to siRNA, functions as a primer for RNA-dependent RNA-polymerase (RdRP), which transforms the mRNA into double-stranded RNA, which in turn will be the Dicer substrate. This step thus amplifies the RNAi response, which can fuel itself until all of the target mRNA is degraded. RdRP is not present in insects. The gene silencing mediated by RNAi is therefore one of the most elegant and efficient gene silencing mechanisms existing in nature (Baulcombe, 2005; Campbell and Choy, 2005).

3.1.1.3 RNAi in insects

In insect science the gene silencing mediated by dsRNA revolutionized the study of gene function in different insect orders including Diptera (Misquitta and Paterson, 1999; Dzitoyeva *et al.*, 2001, Torres *et al.*, 2011), Coleoptera (Bucher *et al.*, 2002; Tomoyasu and Denell, 2004; Tomoyasu *et al.*, 2008), Hymenoptera (Amdam *et al.*, 2003; Gatehouse *et al.*, 2004), Orthoptera (Dong and Friedrich, 2005; Marshall *et al.*, 2009), Blattidae (Cruz *et al.*, 2006; Martin *et al.*, 2006), Lepidoptera (Rajagopal *et al.*, 2002; Turner *et al.*, 2006, Yang *et al.*, 2010) and Hemiptera (Araujo *et al.*, 2006; Mutti *et al.*, 2006; Jaubert-Poss *et al.*, 2007). In RNAi shows a huge potential for the control of insect pests linked to the high specificity of action of administered dsRNA molecules (Borovsky, 2005; Gordon and Waterhouse, 2007; Price and Gatehouse, 2008; Gu and Knipple, 2013). Obviously, in order to develop an efficient strategy for insects control mediated by RNAi, the optimal condition is the oral administration of the dsRNA along with the food and the internalization into midgut cells or the absorption by the gut, depending on the site of silencing. Therefore, understanding the mechanisms of dsRNA absorption and the development of efficient oral delivery strategies are critical to use the RNAi methodology for plant protection.

3.1.1.4 Cellular internalization and export of dsRNA

Whangbo and Hunter (2008) defined different RNAi mechanisms. In cell-autonomous mechanism the silencing process is limited in the cells that produce dsRNA or in those where dsRNA was inserted. On the other hand, in non cell-autonomous mechanism the interference effect of dsRNA is propagated to other cells, different from the production/primary internalization site. The last mechanism includes both systemic and environmental RNAi. In the systemic RNAi, which by definition occurs only in multicellular organisms, silencing signals can pass through the barrier formed by the cells and spread throughout the insect's body. Instead, the environmental RNAi refers to the process by which the gene silencing of specific sequences occurs in response to an environmental exposure to exogenous dsRNA (for example, the ingestion followed by the target gene silencing). The environmental silencing in multicellular organisms therefore involves the uptake of dsRNA by a first group of cells (for example the intestinal cells) followed by the silencing spread in a second group of cells or tissues (Figure 16).

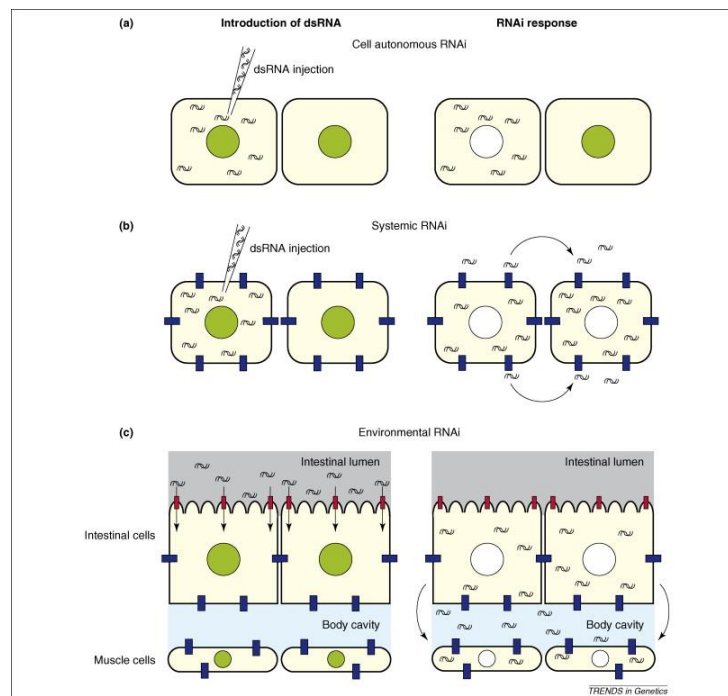


Figure 16. Non cell-autonomous, systemic and environmental RNAi (Whangbo and Hunter, 2008).

3.1.1.5 dsRNA transport in *Caenorhabditis elegans*

The most exploited model to study the transport of dsRNA molecules is the warm *Caenorhabditis elegans*. Several studies suggest that *sid-1* and *sid-2* genes in *C. elegans* encode for transmembrane proteins involved in respectively systemic (Feinberg and Hunter, 2003) and environmental (Winston *et al.*, 2007) RNAi. The *sid-2* protein, localized on the apical membrane of intestinal cells, is required for the initial entry of dsRNA molecules from the intestinal lumen and the *sid-1* transmembrane ubiquitous protein (except for the nervous system cells) functions as a channel for dsRNA (Whangbo and Hunter, 2008).

3.1.1.6 dsRNA transport in insects: uptake mechanism endocytosis-mediated

Almost all species of insects possess *sid-1* homologs (with the exception of Diptera) but not *sid-2*. Counterparts of *sid-1* gene have been identified in some insects such as *Tribolium castaneum*, *Bombyx mori* (Tomoyasu *et al.*, 2008) and *Apis mellifera* (Aronstein *et al.*, 2006), but not in *Drosophila* genome (Winston *et al.*, 2002). These proteins, however, are not involved in the uptake of dsRNA and in RNAi process in insects (Gordon and Waterhouse, 2007; Miller *et al.*, 2008, Tomoyasu *et al.*, 2008). In fact, the systemic RNAi is very efficient in *T. castaneum* (Tomoyasu *et al.*, 2008) even after silencing the three counterparts of *sid-1* identified in this species. The hypothesis is further supported by the fact that, despite the presence of three homologues of *sid-1*, in *B. mori* the systemic RNAi is a rare event. This suggests that in insects there is an alternative system for the absorption of dsRNA. Little is known about the transport of dsRNA in insects, with the exception of two studies in *D. melanogaster*, in which systemic RNAi does not happen. In *D. melanogaster* homologs of the *sid* genes have not been identified. Two different studies revealed that, in *D. melanogaster* embryonic S2 cells (Schneider 2) the uptake of dsRNA takes place thanks to scavenger receptor-mediated endocytosis, and that endocytosis is strongly conditioned by the length of the dsRNA fragment (Saleh *et al.*, 2006; Ulvila *et al.*, 2006).

3.1.1.7 Systemic RNAi in insects

Studies conducted on the complete genome of *D. melanogaster* revealed the absence of genes encoding the RdRP enzyme required for the amplification of the siRNA and crucial to make RNAi effects persistent and systemic (Sijen *et al.*, 2001), the fundamental prerequisites for the application of RNAi in insect control. Systemic RNAi in insects has been observed for the first time in the beetle *T. castaneum*, in two independent studies, which have shown, respectively, that the RNAi persists also in the following developmental stages (Tomoyasu and Denell, 2004) and even in the next generation (Bucher *et al.*, 2002). Currently, in insects there is no evidence of the presence of RdRP (Roignant *et al.*, 2003; Jose and Hunter, 2007; Gordon and Waterhouse, 2007; Richards *et al.*, 2008; Tomoyasu *et al.*, 2008), therefore the amplification of the RNAi phenomenon is based on a different mechanism compared to the one observed in *C. elegans*. The first evidence of the existence of a similar system has been observed in studies showing that RNA polymerase II exhibits a RdRP-similar activity in *D. melanogaster* embryonic cells. This enzyme is involved in RNAi-mediated antiviral immunity and transposon suppression (Lipardi and Paterson, 2009). This protein is also present in other animals (Lipardi and Paterson, 2009).

3.1.1.8 Factors affecting the RNAi in insects

According to the current literature, Huvenne and Smagghe (2010) defined five key factors that have a crucial role in the efficiency of silencing:

- Concentration of dsRNA. For every target gene and organism an optimal concentration has to be determined to induce optimal silencing. It is not true that exceeding that optimal concentration results in a more effective silencing (Meyering-Vos and Muller, 2007; Shakesby *et al.*, 2009).
- Nucleotide sequence. The sequence used will determine possible off-target effects in the target organism, but also in other insects. Off-target silencing is reported in the triatomid bug *Rhodnius prolixus*: together with the targeted nitroporin 2, two highly homologous nitroporin genes were silenced (Araujo *et al.*, 2006). Vacuolar H⁺ ATPase dsRNA of the Colorado potato beetle (*Leptinotarsa decemlineata*) also

silenced the ortholog gene in *Diabrotica virgifera virgifera*, although higher dsRNA concentrations were necessary for efficient RNAi in *D. virgifera virgifera* compared to the Colorado potato beetle (Baum *et al.*, 2007).

- Length of the dsRNA fragment. This is a determinant of uptake and silencing efficiency in intact organisms (Mao *et al.*, 2007) and cell lines (Saleh *et al.*, 2006). In feeding experiments most sequences range between 300 and 520 bp. However, there is a study using only one siRNA (Kumar *et al.*, 2009). The choice to use siRNA is probably based on the success of siRNA in clinical research (Castanotto and Rossi, 2009; Kurreck, 2009). In the case of S2 cells, Saleh *et al.* (2006) reported that the length of the dsRNA should be minimally 211 bp.

- Persistence of the silencing effect. The effect of silencing may be transient as a function of the target protein turnover rate. The silencing effect on aquaporin in *Acyrtosiphon pisum* persists for 5 days and is then reduced (Shakesby *et al.*, 2009). As reported by Turner *et al.* (2006) this transient effect of dsRNA against the pheromone binding protein, in the light brown apple moth (*Epiphyas postvittana*), may be correlated with the turnover rate of the target protein.

- Life stage of the target organism. Although older life stages are more efficient for handling, the younger stages often show larger silencing effects. For instance, no silencing effect was observed after treating fourth instars of *R. prolixus* with nitrophenol 2 dsRNA compared to 42% silencing when using second instars (Araujo *et al.*, 2006). Also in the case of the fall armyworm (*Spodoptera frugiperda*) a stronger silencing effect was observed in fifth instar larvae compared to adult moths (Griebler *et al.*, 2008).

3.1.1.9 Delivery of dsRNA in insects

The identification of specific target genes and appropriate delivery strategies for the dsRNA first in the laboratory and then on a large scale are necessary features for the use of the dsRNA to control pests. Efficiency and low costs are prerequisites of RNAi technology for pest control and the choice of proper delivery strategies for dsRNA molecules strongly influences the efficiency of gene silencing (Yu *et al.*, 2013). Therefore, it is important to find simple and reliable administration methods of dsRNA.

The three main techniques, briefly described below, for the administration of dsRNA applied in the entomological research are micro-injection, feeding and soaking (Yu *et al.*, 2013). In the case of nematodes, they are all effective delivery methods (Jose and Hunter, 2007).

3.1.1.9.1 Delivery of dsRNAs by microinjection

The term "microinjection" is intended to indicate the direct injection of dsRNA into the insect haemocoelomic cavity. This type of administration is the most efficient for systemic RNAi and has been successfully used in many insects belonging to different orders. A study on the moth *Plodia interpunctella* reveals the efficiency of this protocol in the eggs for the silencing of larval genes (Fabrick *et al.*, 2004). The administration of the dsRNA through microinjection has been proved to be a powerful tool for the study of gene function in different species of insects and in particular in model organisms such as *D. melanogaster* and *T. castaneum*. The dsRNA injection has many advantages: high efficiency in gene inhibition, direct and immediate entry in the target tissue avoiding barriers like the integument or the intestinal epithelium, and lastly the amount of injected dsRNA, which reaches the target, is precisely known (Yu *et al.*, 2013). Nevertheless, this method has some limitations. The

injection causes an undesired stimulation of the immune system and it is not applicable in the field for the insect control.

3.1.1.9.2 Delivery of dsRNAs by soaking

The soaking requires the immersion of the insect in solutions containing dsRNA. It has been proved to be effective in the inhibition of gene expression in *D. melanogaster* embryos and cells (Eaton *et al.*, 2002; March and Bentley, 2007). The efficiency of this method is comparable to the one obtained by micro-injection, but it requires lower concentration of dsRNA. However, the soaking is only applicable for some cells and some insect tissues, and only in some developmental stages, when they can absorb dsRNA from a solution. The dsRNA insertion in the cells through transfection is a system of gene transfer mediated by electroporation or incorporation into nanoparticles, which allows a more efficient response compared to simple immersion, probably because it helps the entry into the cells (Valdes *et al.*, 2003; Yu *et al.*, 2013).

3.1.1.9.3 Delivery of dsRNA by oral ingestion

Feeding of dsRNA seems to be the most practical method due to its ease, cost effectiveness, time saving, less invasiveness, and above all, its natural route of entry. This method also allows to administer more easily the dsRNA to high numbers of insects as well as to small insects, for which it is difficult to use the method of microinjection. Oral delivery of dsRNA was first demonstrated in *C. elegans* (Timmons and Fire, 1998), and thereafter, it has been tested in a number of insect species. RNAi in *C. elegans* was also observed when worms were fed on the bacteria engineered to express large quantities of dsRNA. RNAi in *Reticulitermes flavipes* and *Diatraea saccharalis* was also successful via feeding (Zhou *et al.*, 2008; Yang *et al.*, 2010). The dsRNA feeding method is comparatively attractive as it is convenient, easy to manipulate, causes less damage to the insect, and a more natural method of introducing dsRNA into insect body (Chen *et al.*, 2010). It also has its merits in small insects that are more difficult to manipulate using microinjection. The dsRNA could also be fed by either expressing dsRNA in bacteria or by *in vitro* synthesis. Early insect RNAi feeding studies did not give the desired knockdown effects as the injection of dsRNA effectively silenced the aminopeptidase gene *slapn*, which is expressed in the midgut of *S. littoralis*, but feeding with dsRNA did not achieve RNAi (Rajagopal *et al.*, 2002). However, further studies on feeding dsRNA revealed effective gene knockdown effects in many insects, including insects of the orders Hemiptera, Coleoptera, and Lepidoptera (Mao *et al.*, 2007). Feeding dsRNA to *E. postvittana* larvae has been shown to inhibit the expression of the carboxylesterase gene *EposCXE1* in the larval midgut and also inhibit the expression of the pheromone-binding protein EposPBP1 in adult antennae (Turner *et al.*, 2006). The feeding of dsRNA also inhibited the expression of the nitrophorin 2 gene in the salivary gland of *R. prolixus*, leading to a shortened coagulation time of plasma (Araujo *et al.*, 2006). The RNAi pathway is a well-conserved mechanism in insects and holds high potential as an insect control technology as many insects have been found to be susceptible to orally ingested dsRNA (Belles, 2010). The RNAi effects with dsRNA via oral ingestion are widespread in insects; however, the effect is not universal, and there are variations in sensitivities across taxa (Wangbo and Hunter, 2008). The optimization of the used concentration of dsRNA to trigger RNAi is important for oral delivery (Turner *et al.*, 2006). The efficiency of RNAi by ingestion of dsRNA varies between different species. After oral delivery of dsRNA, it is hard to determine the amount of dsRNA brought inside the insect through ingestion

(Surakasi *et al.*, 2011). Another complication with this dsRNA delivery method is the requirement of greater amount of material for delivery (Chen *et al.*, 2010). This phenomenon has been observed after ingestion of *CELL-1* dsRNA by the termite *R. flavipes* (Zhou *et al.*, 2008), *TPS* dsRNA in *Nilaparvata lugens* nymphae (Chen *et al.*, 2010), and *Nitrophorin 2* dsRNA by *R. prolixus* (Araujo *et al.*, 2006). Moreover, different species of insects have different sensitivities to RNAi molecules when delivered orally, for example, *Glossina morsitans* fed with dsRNA may effectively inhibit the expression of *TsetseEP* in the midgut, but cannot inhibit the expression of the transferrin gene *2A192* in fat bodies due to lack of transfer capacity between tissues (Walshe *et al.*, 2009). The mechanisms associated with the transfer of gene expression through feeding delivery method still need further investigations. The delivery mode of dsRNA in insects could be modified by protecting the dsRNA to enhance its uptake in the gut and ultimately could increase efficiency of the silencing as this method was tried in the delivery of siRNA to mammalian cells and specific tissues (Kurreck, 2009). Coating of dsRNA is very important for its protection from endogenous nucleases of insects. In addition, coating can be helpful in protecting dsRNA designed for spray formulations on aerial plant parts.

Transgenic plants are an ideal system for oral delivery of dsRNAs for pest control, as they allow a cheap production of these molecules, either alone (Jiang *et al.*, 2016; Malik *et al.*, 2016; Yue *et al.*, 2017) or pyramided with other insecticidal compounds, such as *Bt* toxins (Ni *et al.*, 2017).

3.1.1.10 Bacteria to orally deliver dsRNA molecules

In most RNAi studies of non-model insects, RNAi reagents are produced through *in vitro* enzymatic reverse transcription or chemical synthesis. However, this is impractical for field application for pest control because of its high cost. An alternative way of inducing RNAi is to express the dsRNA *in vivo* via vector constructs harboring segments of target gene sequence, such as bacteria. This promising technology was developed at the end of the last century for the silencing in *C. elegans* (Timmons and Fire, 1998). Notably, a particular RNase-free *Escherichia coli* strain has been transformed for the production of dsRNA. The killed bacteria have been added in the growth medium and after being ingested by the nematode they were able to cause the degradation of the target mRNA. Since then, the RNAi mediated by bacteria has been successfully also applied for insects (Tian *et al.*, 2009; Li *et al.*, 2011; Zhu *et al.*, 2011; Kontogiannatos *et al.*, 2013, Yang and Hang, 2014; Kim *et al.*, 2015). Furthermore, expressing dsRNA in bacteria can give dual benefits in terms of dsRNA synthesis and stability in a kind of bioformulation, in fact bacterial cell protects the molecules from both environmental degradation, and the degradation within the insect intestinal lumen (Whyard *et al.*, 2009).

These studies employing transgene mediated RNAi represent significant progress toward developing RNAi approaches for pest management. In this system, dsRNAs of the targeted insect genes are expressed from a plasmid with *T7* promoters in inverted orientation flanking the inserted partial cDNA sequence of the target gene in an *E. coli* strain. Thus, dsRNA is produced in the bacterial cells by a process similar to *in vitro* synthesis. The ingestion of such bacteria expressing dsRNA has been shown to produce robust RNAi responses at both transcriptional and phenotypic levels in many insects, such as *S. frugiperda* (Tian *et al.*, 2009), *Bactrocera dorsalis* (Li *et al.*, 2011), and *L. decemlineata* (Zhu *et al.*, 2011), *S. exigua* (Kim *et al.*, 2015). Notably, all these investigations showed gene silencing effects induced in tissues

beyond the gut, i.e., systemic RNAi. These dsRNA expressing bacteria could potentially serve as novel biological insecticides.

3.1.2 Immune genes as possible targets for RNAi-based pest control

The RNAi technique can be used in the entomological field for the functional analysis of genes playing important roles in physiological homeostasis and in the reproduction and in strategies for the control of harmful species in agriculture due to its high specificity (Gordon and Waterhouse, 2004; Price and Gatehouse, 2008; Yu *et al.*, 2013). This approach can be pursued either directly, by suppressing functions in the target insect host that generate lethal phenotypes, or, indirectly, by enhancing the impact and biocontrol efficiency of natural pathogens, as a consequence of the immune disruption syndrome of the host (Washburn *et al.*, 2000; Fath-Goodin *et al.*, 2006). The immunosuppressed host is made more susceptible to a wide range of other pathogens including fungi, viruses, and bacteria. This offers the possibility to develop new integrated control strategies not exclusively relying on toxic molecules, but aiming at achieving sustainable manipulation of biocontrol agents.

3.1.2.1 The insect immune system

The insect immune system consists of a humoral and a cellular response, which are directly involved in the protection against pathogens and parasites. The humoral response consists in the expression of antimicrobial peptides and activation of enzymatic cascades regulating the haemolymph coagulation and melanization. On the other hand, the cellular response is mediated by haemocytes, cells circulating in the haemolymph that, depending on the nature of the pathogen, are able to eliminate it through phagocytosis, nodulation or encapsulation. Following the entry in the haemolymph of pathogens or parasites and their recognition by haemocytes, the immune cells are involved in three possible ways (Satyavathi *et al.*, 2014):

3.1.2.1.1 Phagocytosis

Phagocytosis begins with the recognition of the invading pathogen, followed by its internalization and resulting in haemocyte-mediated intracellular degradation. In insects, phagocytosis is mainly performed by plasmatocytes and granulocytes circulating in the haemolymph.

3.1.2.1.2 Nodulation

This kind of cell-mediated defense is realized when the phagocytosis alone is insufficient to deal with the infection. Nodulation involves the entrapment of microorganisms mediated by aggregates of haemocytes. The non-self element, closed inside the nodule, is killed due to the *in situ* production of cytotoxic molecules. From a quantitative point of view nodulation is the most important mechanism of defense against viral, bacterial and fungal infections in the insects and in other invertebrates.

3.1.2.1.3 Encapsulation

The encapsulation occurs when the non-self intruders are bigger, such as parasites, protozoa and nematodes. The encapsulation can be observed for example in case of parasitoid eggs laid in the host insect haemolymph. Following the binding with the target, the haemocytes produce a multicellular capsule around the invader, with a subsequent melanization of the structure. The non-self element, closed inside the capsule, is killed due to the *in situ* production of cytotoxic molecules.

3.1.2.2 The functional role of the 102 protein in *Heliothis virescens* and *S. littoralis* cellular immune response

Toxoneuron nigriceps (Hymenoptera, Braconidae) is an endoparasitoid parasitoid koinobiont of *H. virescens* larvae (Lepidoptera, Noctuidae). *T. nigriceps* is able to actively block the immune response of their hosts mainly due to maternal factors, such as the poison and the fluid of ovarian calyx, which contains a symbiont bracovirus (*TnBV*), essential for the immunosuppression of the host and in the regulation of many other vital functions (Tanaka and Vinson, 1991; Pennacchio *et al.*, 1997; Pennacchio *et al.*, 1998; Pennacchio *et al.*, 2001; Pennacchio and Strand, 2006). The polydnviruses, to which *Bracovirus* and *Ichnovirus* belong, are obligate symbionts stably integrated into the genome of ichneumonid and braconid Hymenoptera parasitizing lepidopteran larvae, capable of replication only in the female ovaries and once injected into the host during oviposition infect different cell types, without replicating. Infected tissues express the genes responsible for the main changes in host physiology, in particular of the immune response, whose fulfillment is essential for the survival of an endoparasitoid. *TnBV* is a polydnvirus belonging to the bracovirus genus (BV). Its genome (consisting of 29 double-stranded DNA circles) has a large non-coding region, a high content of A-T (> 68%) and a coding region corresponding to about 22% of the entire genome (unpublished data). It is characterized by the presence of genes in multiple copies, sometimes clustered in members of gene families. The expression of several genes of this virus leads to structural and functional alterations in the haemocytes and in the humoral immune response, which is recorded from the early hours after parasitization (Malva *et al.*, 2004; Ferrarese *et al.*, 2005). Therefore, *TnBV* is the most important factor in the induction of parasitic immunosuppressive syndrome observed in parasitized *H. virescens* larvae. The study of immunosuppressive syndrome in hosts parasitized by *T. nigriceps* allowed the identification of a host gene that is negatively modulated soon after oviposition.

This gene, called *102*, has been identified and functionally characterized (Falabella *et al.*, 2012) as part of a broad effort aiming to discover *TnBV* encoded virulence factors and their mechanism of action. The *102* gene plays an important role in the immune response in *H. virescens*, encoding a key protein for the encapsulation of non-self intruders. The *102* protein is involved in the formation of haemocytic aggregates by giving rise to amyloid fibrils coating the non-self intruders, around which the deposition of cell layers and the production of melanin and other toxic compounds results strictly localized. Indeed, the fibrils function as a molecular scaffold for the melanin synthesis and other toxic metabolites which are then restricted to the target site, where they play their function, causing the death of the covered non-self objects, preventing a lethal systemic diffusion. Moreover, its silencing leads to an immunosuppressed phenotype probably reproducing the syndrome observed in parasitized larvae from *T. nigriceps*. In particular, in the *H. virescens* larvae treated with *102* dsRNA a reduced level of encapsulation is observed (Falabella *et al.*, 2012). A homologous gene of *H. virescens 102* was detected in *S. littoralis* (Lepidoptera, Noctuidae). Also in this insect, the gene is directly involved in the regulation of encapsulation and in the melanization reaction (Di Lelio *et al.*, 2014). In this study it has been shown that oral administration of *Sl 102* dsRNA in *S. littoralis* larvae causes an inhibition of the cellular immune response, as highlighted by the failure in encapsulation of chromatographic beads injected in the haemocoel of larvae. Further studies have demonstrated the involvement of this gene in the nodulation response against bacteria (Caccia *et al.*, 2016).

This makes this gene an ideal candidate for RNAi based pest control strategies based on immunosuppression, since encapsulation and nodulation responses are key-elements of insect immune barriers.

3.1.2.3 *gasmin* gene in lepidopteran species

It has been recently demonstrated that bracoviruses can mediate gene flux between a parasitoid wasp and Lepidoptera. The acquisition of bracovirus sequences through this original mechanism of horizontal gene transfer (HGT) could result in adaptive advantages for the host, in particular in the immune response against pathogens (Gasmi *et al.*, 2015).

HGT occurred from *Cotesia congregata* to lepidopteran genomes. In particular, insertions of DNA from the CcBV (*Cotesia congregata* bracovirus) in the genomes of non-hosts, the monarch (*Danaus plexippus*), the silkworm (*B. mori*), the beet armyworm (*S. exigua*) and the fall armyworm (*S. frugiperda*), have been identified (Gasmi *et al.*, 2015). All these insertions were characterized by the presence of large stretches of nucleotide sequences strikingly similar to those of bracoviruses (close to 90% identities at the nucleotide level) flanked by lepidopteran-specific sequences. Insertions include genes but also in some cases parts of bracovirus circles, the organization of which has been conserved, indicating the direction of HGT was from bracovirus to Lepidoptera. Moreover, in one insertion a regulatory signal involved in dsDNA circle production in the wasp has been retained, constituting an unambiguous signature of the bracoviral origin of the sequence since bracovirus replication is non-autonomous and occurs exclusively in the wasp ovaries (Gasmi *et al.*, 2015).

Functional analyses have been performed on some transferred genes giving insights on their possible role as domesticated genes in Lepidoptera, which is probably related to their function during parasitism. A sequence (1548 bp long) highly similar to CcBV25 (90% sequence identity at the nucleotide level), one of the CcBV genes highly expressed in parasitized host (*Manduca sexta*) fat body and haemocytes (Chevignon *et al.*, 2014). In *S. exigua* BV2-5 (called *gasmin*) is highly expressed in haemocytes. As a first approach to provide some indication about the role of BV2-5, a recombinant baculovirus producing the transferred bracovirus protein BV2-5 was used to infect *S. frugiperda* cultured cells. Cellular localization of the gene product by immunofluorescence revealed that BV2-5 had a negative impact on cytoskeleton rearrangement and motility during baculovirus infection, thereby reducing replication of pathogenic baculovirus in infected lepidopteran cells (Gasmi *et al.*, 2015). The results therefore suggest the transferred bracovirus genes could confer a partial protection towards baculovirus infections. Unexpectedly, complementary functional analyses of *S. exigua* infected with baculovirus producing BV5-2 protein, showed a higher susceptibility to *B. thuringiensis* entomopathogenic bacteria (Gasmi *et al.*, 2015; 2016).

The silencing of this gene could nicely complement the RNAi mediated suppression of *SI 102*, by disrupting phagocytosis.

In this thesis chapter, the production and delivery of dsRNA, as part of RNAi based insect control strategies, is covered and its use proposed for targeting immune genes. In particular, these genes have been identified by studying their role in parasitism; their silencing by RNAi aims at mimicking the immunosuppressive syndrome generated in insect hosts by parasitic wasps and associated viral symbionts, in order to make them more susceptible to natural antagonists.

3.2 Materials and methods

3.2.1 Biological material: insect rearing

S. littoralis larvae were reared on artificial diet (47.25 g/L wheat germ, 67.3 g/L brewer's yeast, 189 g/L corn meal, 6.75 g/L ascorbic acid, 0.75 g/L cholesterol, 0.5 g/L propyl 4-hydroxybenzoate, 3 g/L methyl 4-hydroxybenzoate, 1.25 g/L wheat germ oil, 33.75 g/L agar and 3 g/L vitamin mix (1.2 g/Kg vitamin B1, 2.6 g/Kg vitamin B2, 2.5 g/Kg vitamin B6, 40 g/Kg choline, 10 g/Kg pantothenic acid, 32 g/Kg inositol, 0.25 g/Kg biotin, 2.5 g/Kg folic acid, 5 g/Kg 4-aminobenzoic acid, 0.5 mg/Kg vitamin B12, 10 g/Kg glutathione, 2.1 g/Kg vitamin A, 0.25 g/Kg vitamin D3, 24 g/Kg vitamin E, 0.25 g/Kg vitamin K, 25 g/Kg vitamin C in dextrose), at $25 \pm 1^\circ\text{C}$, $70 \pm 5\%$ R.H., and under a 16:8 h light/dark period.

3.2.2 Tissue sample collection for DNA and RNA extraction

S. littoralis larvae were anaesthetized on ice and surface-sterilized with 70% ethanol (v/v with distilled water) prior to dissection. Larval haemolymph was collected from a cut of the abdominal leg and haemocytes were separated from plasma by centrifugation for 5 min, $500 \times g$, at 4°C . Midgut and fat body were isolated after cutting the larval abdomen lengthwise. The rest of the body was also collected to be analyzed.

After isolation, samples for RNA extraction were immediately put into TRIzol reagent (Thermo Fisher) and kept at -80°C until total RNA extraction that was performed, according to manufacturer's instructions. DNA was extracted from haemocytes using the protocol described in Cubero *et al.*, 1999, with minor modifications. The concentration of extracted DNA or RNA was assessed by measuring the absorbance at 260 nm, with a Varioskan™ Flash Multimode Reader (Thermo Fisher), and sample purity was evaluated by assessing 260/280 nm absorbance ratio. RNA quality was checked by electrophoresis on 0.8% agarose gel.

3.2.3 Identification of *Sl gasmin* cDNA, sequencing and cloning of the ORF sequence

A partial *Sl gasmin* cDNA (Accession Number FQ973054.1) was identified by BLAST analyses (Gish and States, 1993; Altschul *et al.*, 1997) in a public database of EST sequences from *S. littoralis* female antenna, using as query the sequence of the *S. exigua gasmin* gene, complete coding sequence (Accession Number KP406767.1). The predicted *Sl gasmin* protein, gamin protein (Accession Number AKP99421.1) and hypothetical protein CcBV_25.3 (Accession Number YP184865.1) were aligned using the Clustal Omega algorithm.

For the determination of *Sl gasmin* ORF (open reading frame), total RNA was extracted from haemocytes of *S. littoralis* sixth instar larvae as described in 3.2.2. After quantification and check of RNA quality, RNA was subjected to retro-transcription, using the Ambion RETROscript® kit (Life Technologies). A 1038 bp long *Sl gasmin* ORF cDNA fragment was obtained by PCR using the *Sl gasmin* ORF forward primer (ATGTTGCCTATTACCATACTAACG) in combination with the *Sl gasmin* ORF reverse primer (ATACTGGAATTGGACATATTTGAGC) amplified with Phusion High-Fidelity DNA Polymerase (Thermo Fisher). PCR conditions were programmed to 30 s at 98°C ; 40 cycles of [10 s 98°C , 30 s 60°C , 1 min 72°C] and 15 min at 72°C . After amplification, the obtained PCR product was separated by gel electrophoresis and the visible band of the expected size was purified with a Quick gel extraction & PCR purification COMBO Kit (Thermo Fisher). The PCR product was additionally cloned into Zero-Blunt TOPO vector using the Zero-Blunt TOPO PCR

Cloning Kit (Thermo Fisher), according to the manufacturer's instructions. After transformation of One Shot TOP10 chemically competent *Escherichia coli* cells (Thermo Fisher), the transformants were incubated overnight at 37°C on LB plates containing 50 µg/mL kanamycin. Bacterial colonies containing the fragment of the appropriate size were selected by colony PCR using Phusion High-Fidelity DNA Polymerase (Thermo Fisher) and M13 Forward (-20)/M13 reverse primers (Thermo Fisher), and grown overnight in LB kanamycin (50 µg/ml) medium. The plasmid DNA was extracted from 4 ml of bacterial culture using a ChargeSwitch-Pro plasmid miniprep Kit (Thermo Fisher) as instructed by the manufacturer. The plasmid DNA was sequenced at Eurofins Genomics.

3.2.4 qRT-PCR for the measurement of *Sl gasmin* expression

Total RNA was isolated as described in 3.2.2. Differential relative expression of studied genes was measured by one-step qRT-PCR, using the SYBR Green PCR Kit (Applied Biosystems), according to the manufacturer's instructions. *S. littoralis* β -actin gene (Accession Number Z46873) was used as endogenous control for RNA loading (β -actin RT fw: CCGTCTTCCCATCCATCGT; β -actin RT rev: CCTTCTGACCCATACCAACCA). All primers were designed using Primer Express, version 1.0 software (Applied Biosystems). Relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001; Pfaffl *et al.*, 2002).

qRT-PCR for measurement of *Sl gasmin* expression was carried out by using specific primers (*Sl gasmin* RT fw: AGTCGTTTCAGAATGGTAACA; *Sl gasmin* RT rev: GACGCATTGAAGCCAATCAT), designed to detect a segment of the *Sl gasmin* mRNA external to the segment targeted by the dsRNAs. For validation of the $\Delta\Delta Ct$ method the difference between the Ct value of *Sl gasmin* and the Ct value of β -actin transcripts [$\Delta Ct = Ct(Sl\ gasmin) - Ct(\beta\text{-actin})$] was plotted versus the log of ten-fold serial dilutions (2000, 200, 20, 2 and 0,2 ng) of the purified RNA samples. The plot of log total RNA input versus ΔCt displayed a slope less than 0.1 ($Y = 1.149 + 0.0133X$, $R^2 = 0.0493$), indicating that the efficiencies of the two amplicons were approximately equal.

For validation of the $\Delta\Delta Ct$ method the difference between the Ct value of AMPs and *lysozyme 1a* the Ct value of β -actin transcripts [$\Delta Ct = Ct(\text{sample}) - Ct(\beta\text{-actin})$] was plotted versus the log of two-fold serial dilutions (200, 100, 50, 25 and 12.5 ng) of the purified RNA samples. The plot of log total RNA input versus ΔCt displayed a slope lower than 0.1 (*attacin*: $Y = 1.3987 + 0.0144X$, $R^2 = 0.0565$; *gloverin*: $Y = 1.3567 + 0.013X$, $R^2 = 0.0715$; *lysozyme 1a*: $Y = 1.690 + 0.0124X$, $R^2 = 0.0313$), indicating that the efficiencies of the two amplicons were approximately equal.

3.2.5 Expression profile of *Sl gasmin* in response to different pathogens

To analyze *Sl gasmin* expression in response to pathogens, *S. littoralis* fifth instar larvae, surface-sterilized with 70% ethanol (v/v in distilled water) and chilled on ice, received an intra-haemocoelic injection of 2×10^7 *E. coli*, 3×10^8 *Staphylococcus aureus* or 2×10^7 *Saccharomyces cerevisiae* cells, suspended in 5 µl of PBS. Injections were performed through the neck membrane, using a Hamilton Microliter syringe (1701RNR 10 µl, gauge 26s, length 55 mm, needle 3). At the injection time and at different time points after injection, larvae (n=8 for each experimental sample) were dissected and haemocytes were collected and processed for total RNA extraction as described above; the relative expression of *Sl gasmin* was assessed by q-RT-PCR, as described in 3.2.4.

3.2.6 dsRNA *in vitro* synthesis

Total RNA purified from hemocytes of *S. littoralis* sixth instar larvae was retro-transcribed with the RETRO script Kit (Thermo Fisher) and a 789 bp long *Sl gasmin* cDNA fragment was obtained by PCR, using the ds *Sl gasmin* forward primer (GCCGGCATGTTGTCTATTACC) in combination with the ds *Sl gasmin* reverse primer (TCCTTCCAGCTTCTGAGTCA). This cDNA fragment was used as template for a nested-PCR reaction, performed with primers containing at their 5' ends the T7 polymerase promoter sequence (T7-*gasmin* *Sl* fw: TAATACGACTCACTATAGGGAG-TTCGAGGATACAAGCAGAG; T7-*gasmin* *Sl* rev: TAATACGACTCACTATAGGGAG-GGATGCTCAGGATATCTGTTAC). The resulting PCR product served in turn as template to synthesize dsRNA (522 bp long), using the Ambion MEGAscript[®] RNAi Kit (Thermo Fisher), according to the manufacturer's instructions. dsRNA preparations were quantified by measuring their absorbance at 260 nm with a Varioskan[™] Flash Multimode Reader (Thermo Fisher) and purity was evaluated by assessing 260/280 nm absorbance ratios. Products were run on 0.8% agarose gels to confirm their integrity.

3.2.7 Administration of dsRNA to *S. littoralis* larvae and silencing of *Sl gasmin*

Sl gasmin dsRNA (522 bp long) and *control* dsRNA (obtained from the control template supplied by the kit used for dsRNA preparation, 500 bp long) were synthesized as described above. *S. littoralis* fourth instar larvae (first day) were anaesthetized on ice and 1 µl of *Sl gasmin* dsRNA or *control* dsRNA in PBS was poured into the lumen of the foregut by means of a Hamilton syringe (1701 RN SYR 10 µl, gauge 26s, length 55 mm, needle 2). dsRNA treatments consisted of one oral administration of 150 ng per day, for 3 days (from fourth to fifth instar). After the last dsRNA administration and prior to any further experiment, the haemolymph from 3 treated larva was used for qRT-PCR analysis to confirm the occurrence of gene silencing.

3.2.8 Detection of actin filaments in haemocytes

Newly molted fifth instar larvae of *S. littoralis*, treated with *Sl gasmin* dsRNA or *control* dsRNA as described above, were surface-sterilized with 70% ethanol (v/v in distilled water) and chilled on ice. Larval haemolymph from individual larvae was collected from a cut of the abdominal proleg and was placed on glass slides for 10 min, so that the haemocytes would settle and attach to the glass. Then, haemolymph was gently removed and haemocytes were rinsed gently 3 times with PBS. Attached cells were fixed for 10 min in 4% (w/v) paraformaldehyde in PBS, washed 3 times in PBS and permeabilized for 4 min with 0.1% (v/v) Triton-X100 in PBS. Haemocytes were washed 3 times in PBS and then incubated for 20 min with 4 µg/ml TRITC-phalloidin (Tetramethylrhodamine B isothiocyanate-phalloidin). After 3 rinses in PBS, the samples were mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories) and examined under a fluorescence microscope (ZEISS Axiophot 2 epifluorescence microscope).

3.2.9 Encapsulation, nodulation and phagocytosis assays

To assess the effect of *Sl gasmin* dsRNA administration on *S. littoralis* haemocytes functionality, their ability to form capsules, nodules and to perform phagocytosis of microorganisms were evaluated.

Encapsulation response was assessed as described in Di Lelio *et al.*, 2014. Briefly, 12 h after the last dsRNA administration (*Sl gasmin* dsRNA or *control* dsRNA) larvae were anesthetized by immersion in water for 5-10 min, sterilized in 70% ethanol (v/v

in distilled water) and washed in sterile water. After being dried on autoclaved filter paper, the larvae were placed on parafilm with the back facing upwards. Larvae were injected with a Hamilton microliter syringe (702RNR 25ul, gauge 22s, August 55 mm length, tip 3) with 10 µl of PBS 1x containing about 40 CM Sepharose Fast flow chromatographic beads (Pharmacia). Injections were performed piercing the membrane of the neck and holding the syringe in a position parallel to the body of the larva, to prevent damage to the gut. Larvae were then gently transferred into a tube containing diet and placed in a climatic chamber. 24 hours after the injection the beads were recovered by dissection. Briefly, the larvae, anesthetized as described above, have been sectioned in 500 µl of anticoagulant solution MEAD (20 mM sodium phosphate pH 7.3; 150 mM NaCl). The beads, identified under a stereomicroscope, were recovered with a Gilson pipette, placed in 300 µl of PBS into the wells of a plastic plate, and observed under a light microscope (Leica DM IRB Microsistem) to calculate the level of encapsulation. The encapsulation index (indicated as I.I.) was expressed by using five levels in an arbitrary scale of encapsulation (Figure 17), defined as follows:

- 0 - no cells adherent to the beads
- 1 - up to 10 adherent cells
- 2 - more than 10 adherent cells but with less than a complete layer
- 3 - one or more complete layers without melanization
- 4 - one or more complete layers with melanization

The encapsulation index has been calculated with the following formula (Li *et al.*, 2007):

$$I.I.(%) = [\sum (\text{encapsulation level} \times \text{total beads of this level}) / \text{total beads} \times 4] \times 100$$

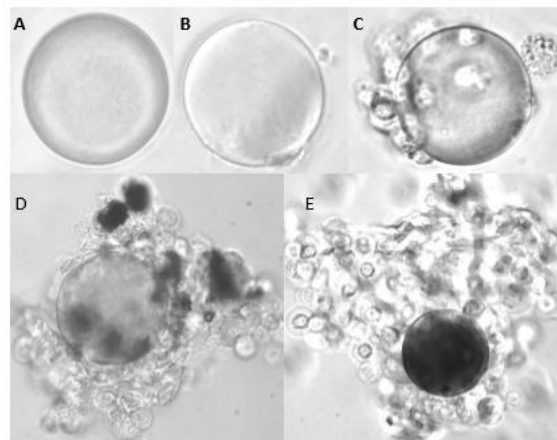


Figure 17. Encapsulation level: **A)** 0 (no cells adherent to the beads); **B)** 1 (up to 10 adherent cells); **C)** 2 (more than 10 adherent cells but with less than a complete layer); **D)** 3 (one or more complete layers without melanization); **E)** 4 (one or more complete layers with melanization).

For the nodulation assay, 12 h after the last dsRNA administration (*Sl gasmin* dsRNA or *control* dsRNA) *S. littoralis* larvae were chilled on ice, surface-sterilized with 70% ethanol (v/v in distilled water) and intra-haemocoelically injected with 5 µl of a PBS suspension of 2×10^6 *E. coli* cells. Injections were performed through the neck membrane using a Hamilton syringe (1701 RN SYR 10 µl, gauge 26s, length 55 mm, needle 3). 18 h after bacteria injection, haemolymph was collected from a cut of the thoracic proleg into an equal volume of ice-cold MEAD. Nodules in haemolymph samples were then counted under a microscope (Axioskop, Carl Zeiss Microscopy, Germany) by means of a Bürker chamber.

To measure phagocytosis competence of *S. littoralis* haemocytes an *in vitro* assay was performed. Haemolymph samples were extracted 24 h after the last dsRNA administration (*Sl gasmin* dsRNA or *control* dsRNA) from *S. littoralis* larvae chilled on ice from a cut of the thoracic leg into ice-cold PBS (2:1 v/v) and added with 4 µl of a PBS suspension of 2×10^7 fluorescein conjugated *E. coli* cells (K-12 strain BioParticles®, fluorescein conjugate, Thermo Fisher) or 2×10^7 *S. aureus* (Wood strain, BioParticles® fluorescein conjugate, Thermo Fisher) and incubated for 15 min. Samples were loaded into a Bürker chamber where total and fluorescent haemocytes were counted under a fluorescence microscope (Axioskop 20, Carl Zeiss Microscopy, Germany).

Haemocyte viability in haemolymph samples was assessed by trypan blue assay. Briefly, an haemolymph aliquot was mixed with 0.4 % (w/v) trypan blue (Sigma-Aldrich) (2:1). Viable and dead cells were counted under the microscope using a Bürker chamber. Viable cells were close to 98 % in all haemolymph samples (data not shown).

For rescue experiments of haemocytes from silenced larvae, haemolymph samples were extracted 24 h after the last dsRNA administration (*Sl gasmin* dsRNA or *control* dsRNA) from *S. littoralis* larvae chilled on ice. Samples were centrifuged 5 min at 500 x g, at 4°C. The plasma was kept on ice and haemocytes were resuspended in PBS and centrifuged as previously described. PBS was then removed and haemocytes from larvae treated with *Sl gasmin* dsRNA were resuspended in the plasma isolated from larvae treated with *control* dsRNA, while haemocytes from larvae treated with *control* dsRNA were resuspended in the plasma isolated from larvae treated with *Sl gasmin* dsRNA. The measure of phagocytosis competence was then performed as described above.

3.2.10 Bioassays with Cry1Ca for LC₅₀ calculation

Cry1Ca toxin was kindly supplied by Professor Juan Ferré (University of Valencia, Spain). Prior to use, Cry1Ca was dialyzed overnight, at 4°C in 50 mM sodium carbonate buffer, pH 9.0. After dialysis, toxin concentration was determined by the Bradford assay (Bradford, 1976), using bovine serum albumin as standard.

Six h after the last dsRNA administration, the newly molted fifth instar larvae were confined individually in plastic trays (Bio-Ba-32, Color-Dec, Italy) covered with perforated plastic lids (Bio-Cv-4, Color-Dec Italy) and exposed to surface-treated artificial diet under the same environmental conditions reported above. For the first three days, the upper surface (1 cm²) of the artificial diet (0.3 cm³) was uniformly overlaid with 50 µl of purified Cry1Ca toxin dissolved in 50 mM sodium carbonate buffer at pH 9.0. Control larvae were reared on artificial diet overlaid with 50 µl sodium carbonate buffer. Experimental larvae were maintained on artificial diet, replaced every 24 h, and daily inspected for survival, until pupation. To determine the 50% lethal concentration (LC₅₀) of Cry1Ca toxin, the bioassay described above was carried out at 5 different concentrations plus a control using 16 larvae for each experimental condition. Mortality was assessed after 10 days and Probit analysis (Finney, 1971) was performed with the POLO-PC program (LeOra Software, Berkeley, CA), to determine LC₅₀ values, 90% fiducial limits and toxicity increase ratio (TI) for each experimental treatment.

3.2.11 Cloning of *SI 102* and *SI gasmin* into *L4440* vector for dsRNA synthesis *in vivo*:

3.2.11.1 cDNA synthesis and PCR amplification

The RNA extracted from *S. littoralis* haemocytes was subjected to retro-transcription, using the Ambion RETROscript® kit (Life Technologies). The obtained cDNA was quantified with Varioskan™ Flash Multimode Reader and used to perform the PCR amplifications using a couple of primers (Table 5) to amplify *SI 102*, *SI gasmin*, and *GFP* as negative control.

| Name | Sequence (5' → 3') | Tm |
|----------------------|------------------------------|------|
| <i>fw SI 102</i> | CACCAACCTCCTGAGCGTGCCT | 66°C |
| <i>rev SI 102</i> | CGGAGTGCTGCTTCAGAATC | 66°C |
| <i>fw GFP</i> | CACCAAGTGGAGAGGGTGAAGGTG | 60°C |
| <i>rev GFP</i> | GGGCAGATTGTGTCGACAG | 60°C |
| <i>fw SI gasmin</i> | CACCATGTTGCCTATTACCATACTAACG | 60°C |
| <i>rev SI gasmin</i> | ATACTGGAATTGGACATATTTGAGC | 60°C |

Table 5. Primers used for the amplification of *SI 102*, *SI gasmin* and *GFP* fragments.

PCR conditions were programmed to 30 s at 98°C; 35 cycles of [10 s 98°C, 30 s 66°C, 1 min 72°C] and 10 min at 72°C. To verify the outcome of the PCR reactions the samples were run on 0.8% agarose gel and visualized on a transilluminator. The PCR amplification products were then purified using PureLink Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher). PCR products were then cloned into pENTR/D-TOPO vector (Thermo Fisher) compatible with the Gateway Technology for the transfer into the definitive *L4440* Gateway vector. The plasmid DNA was sequenced at Eurofins Genomics.

3.2.11.2 Gateway cloning technology

The Gateway Technology is a universal cloning method that takes advantage of the site-specific recombination properties of the bacteriophage lambda (Landy *et al.*, 1989) to provide a rapid and highly efficient way to move the gene of interest into multiple vector systems (Figure 18).

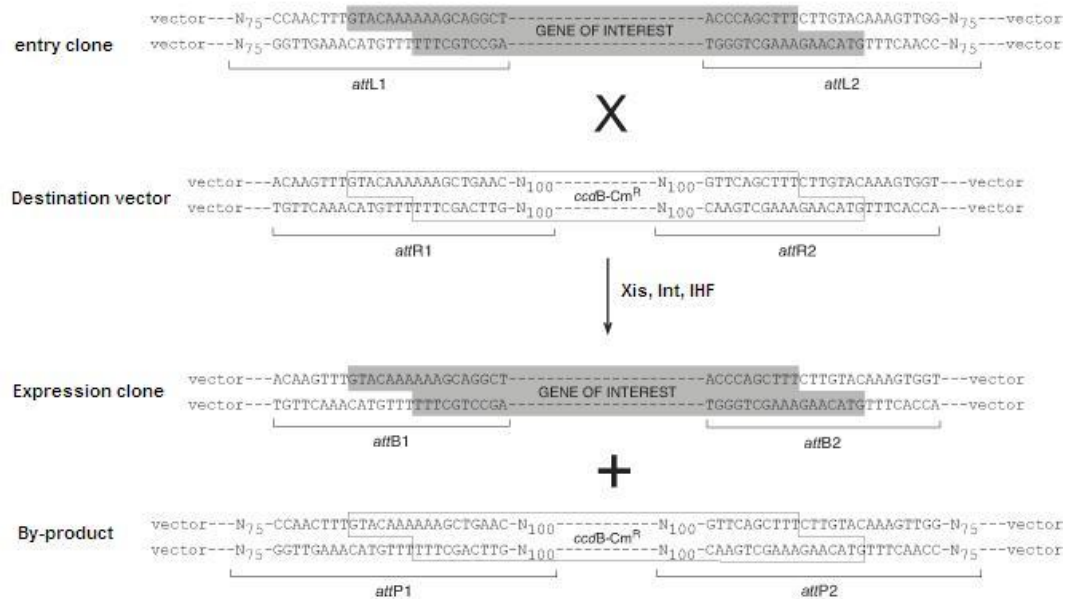


Figure 18. The recombination region of the expression clone resulting from gateway *L4440* × entry clone is shown. Shaded regions between *Att* sites correspond to DNA sequences transferred from the entry clone into *L4440* vector by recombination.

A brief outlined description on how to obtain the expression vector with the Gateway technology is described as follows:

1. The genes were cloned into pENTR/D-TOPO (Thermo Fisher), a Gateway entry vector to create the entry clone.
2. The *L4440* vector (Figure 19), kindly donated by Elia Di Schiavi (Consiglio Nazionale delle Ricerche, Naples) was converted into a Gateway destination vector.
3. The expression clones were generated by performing a LR recombination reaction between the entry clones and *L4440* Gateway destination vector.

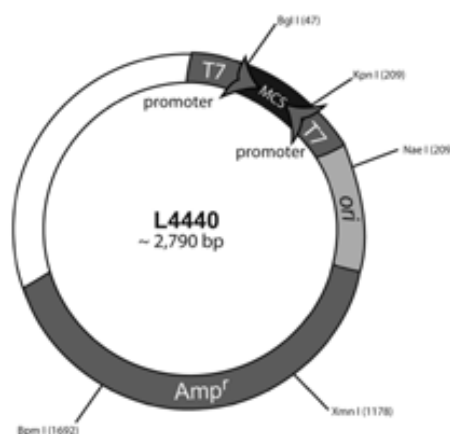


Figure 19. The multicloning site of *L4440* vector is bidirectionally flanked by *T7* promoters driving the synthesis of RNA complementary strands (i.e. dsRNA).

3.2.11.3 Generation of the ENTRY clone

To recombine the gene into the Gateway converted *L4440* vector, for the expression of dsRNA in bacteria, Gateway entry clones containing the *Sl 102* and *Sl gasmin* PCR products have been produced. The vector chosen was pENTR/D-TOPO (Figure 20). The TOPO cloning reaction allows the insertion of the PCR product into the entry vector pENTR/D-TOPO using TOPO assisted directional cloning. The reaction has been incubated 5 min at room temperature (Table 6).

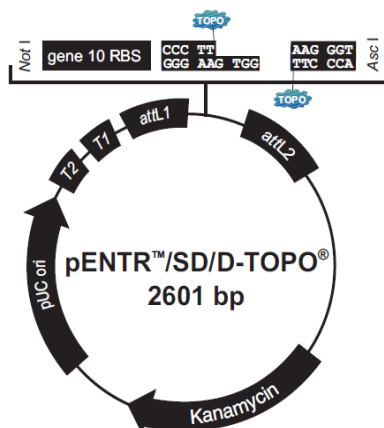


Figure 20. pENTR/SD/D-TOPO vector scheme.

| Component | Volume |
|---------------------|--------|
| Salt solution | 1 µl |
| Water | 3 µl |
| pENTR/D-TOPO vector | 1 µl |
| PCR product | 1 µl |

Table 6. TOPO cloning reaction.

Following the reaction, One Shot TOP10 chemically competent *E. coli* cells *E. coli* cells were transformed with the reaction products, and the next day the plasmids were extracted from different colonies using ChargeSwitch-Pro plasmid miniprep Kit (Thermo Fisher) as instructed by the manufacturer. The plasmid DNA was sequenced at Eurofins Genomics.

3.2.11.4 Conversion of *L4440* into a Gateway vector

L4440 vector was converted into a Gateway destination vector by using the Gateway vector conversion system, ligating a blunt-ended cassette containing attR sites flanking the *ccdB* gene and the chloramphenicol resistance gene into the multiple cloning site of *L4440* vector, creating a Gateway destination vector for the expression of dsRNA.

The overview of the experiments performed was as follows:

1. The genes of interest were cloned into a Gateway entry vector to create entry clones.
2. Expression clones were generated by performing an LR recombination reaction between the entry clones and the converted Gateway destination vector.
3. Competent cells were transformed with the expression clones.

To convert the *L4440* into a Gateway vector, 5 µg of *L4440* were double digested with *KpnI* and *BglII* (both supplied by Thermo Fisher). The reaction has been incubated 2 h room temperature (Table 7). Those enzymes were chosen to remove as many of the MCS restriction sites as possible. This will minimize the number of additional nucleotides on the expressed dsRNA.

| Components | Volumes |
|---------------------|---------------|
| <i>KpnI</i> | 2 µl (2 Unit) |
| <i>BglII</i> | 2 µl (2 Unit) |
| 10x buffer T | 4 µl (1x) |
| 0.1% XXX | 4 µl (0.01%) |
| <i>L4440</i> vector | 25 µl (1µg) |
| Water | Up to 40 µl |

Table 7. Digestion of *L4440* vector.

The ends of the vector were converted to blunt double-stranded DNA using T4 DNA polymerase (Thermo Fisher) to ligate the blunt-ended cassette containing attR sites. The reaction mixture was incubated 2h room temperature (Table 8).

| Component | Volume |
|-----------------------------|--------------|
| T4 DNA Polymerase | 2 µl |
| 5x T4 DNA Polymerase Buffer | 20 µl |
| 10 mM dNTP mix | 1 µl |
| Linearized DNA | 40 µl (2 µg) |
| Water | Up to 100 µl |

Table 8. Components to convert the vector's ends in blunt-ended ends using T4 DNA polymerase.

The phosphate groups at 5' ends were removed from the bunt-ends with calf intestinal alkaline phosphatase (CIAP) (Thermo Fisher), to decrease the background associated with self-ligation of the vector. The reaction was incubated 1h at 50°C (Table 9).

| Component | Volume |
|------------------------------|----------------|
| DNA | 10 µl (1 pmol) |
| 10x Dephosphorylation buffer | 4 µl |
| CIAP | 1 µl (1U/ µl) |
| Water | Up to 40 µl |

Table 9. Components for dephosphorylation of vector's ends using CIAP enzyme.

CIAP was heat-inactivated for 15 minutes at 65 °C. The DNA was adjusted to a final concentration of 50 ng/μl in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). The reaction has been incubated 1h room temperature (Table 10).

| Component | Volume |
|---------------------------------|---------------|
| Dephosphorilated vector | 3 μl (50 ng) |
| 5X T4 DNA ligase buffer | 2 μl |
| Gateway® reading frame cassette | 2 μl (10 ng) |
| T4 DNA ligase | 1 μl (1 unit) |
| Water | Up to 10 μl |

Table 10. Ligation reaction.

The ligated vector was used to transform *ccdB* Survival 2 T1^R and TOP10 competent cells (both supplied by Thermo Fisher) for the positive selection. The cassette cloned into the *L4440* vector contains the *ccdB* gene, whose product is toxic for *E. coli* cells except for the One Shot *ccdB* Survival 2 T1^R, a genetically modified strain, resistant to the toxic effects of the *ccdB* gene (Bernard and Couturier, 1992; Bernard *et al.*, 1993). The positive selection occurs because the destination vector gives 10,000 times more colonies in the modified strain compared to the DH5α TOP10 cells. In fact, any ratio less than 10,000 indicates either an inactive *ccdB* gene or contamination of the plasmid prep with another antibiotic-resistant plasmid.

3.2.11.5 LR recombination

Finally, the clone expressing dsRNA was created by swapping the death gene present in the Gateway converted *L4440* vector (*ccdB* gene) with the *SI 102* and *SI gasmin* genes previously inserted into the entry clones. The exchange between the death gene and the two genes of interest was achieved using a transposition reaction catalyzed by the LR clonase enzyme (Thermo Fisher) (Table 11). Following the reaction, competent TOP10 *E. coli* cell strains were transformed with the reaction products, and the next day the plasmids were extracted from different colonies using ChargeSwitch-Pro plasmid miniprep Kit (Thermo Fisher). The plasmid DNA was sequenced at Eurofins Genomics.

| Component | Volume | Incubation |
|-----------------------------|---------------|-------------|
| Gateway <i>L4440</i> vector | 1 μl (150 ng) | 1 h 25°C |
| ENTRY vector | 1 μl (150 ng) | |
| 1x TE, pH8 | up to 8 μl | |
| LR clonase mix | 2 μl | |
| Proteinase K | 1 μl | 10 min 37°C |

Table 11. LR recombination reaction.

This will result into *SI 102* and *SI gasmin* fragments being cloned inside the *L4440* vector between the *T7* convergent promoter for the expression of dsRNA.

Moreover, the conversion of *L4440* into a Gateway vector is very useful for a quick and easy cloning reaction of any other PCR product.

3.2.12 Transformation of bacteria and expression of dsRNA

E. coli cells of the *HT115* strain, kindly donated by Elia Di Schiavi, have been transformed with the *SI 102 L4440* recombinant vector. This strain is particularly suitable for the dsRNA overexpression because it lacks the RNase III gene, so the expressed RNA molecules are less susceptible to degradation. Moreover, this strain carries the *T7* RNA polymerase gene under control of *Lac* gene promoter, so that the transcription of dsRNA can be easily induced by IPTG (Isopropyl β -D-1-thiogalactopyranoside), the molecular mimic of allolactose, the inducer of the *Lac* operon. In order to produce dsRNA, the bacteria transformed with *SI 102 L4440* were grown in the liquid broth Luria-Bertani (LB) containing 100 μ g/ml ampicillin and 12,5 μ g/ml tetracycline at 37 °C for 16 h with 250 rpm shaking rate. Then, 5 ml of cultured broth were added to 500 ml of fresh LB medium and allowed to grow until OD_{600} =0.6-0.7. Expression of *T7* RNA polymerase gene was induced by addition of 1 mM IPTG and the bacteria were incubated with shaking overnight at 37°C.

3.2.13 Pre-treatments of the transformed bacteria

The bacteria expressing dsRNA specific to *SI 102* gene were cultured at 37°C and the dsRNA over-expression occurred as described above. Bacterial cells were harvested by centrifugation at 12,000 x g for 1 min at 4 °C and resuspended in PBS. The bacterial cells underwent a sonication pre-treatment (Bandelin Sonoplus, Berlin, Germany), with 95% intensity and 10 cycles of sonication (59 sec on/2 sec off) to facilitate the dsRNA release. The vitality of bacteria after the treatment was evaluated by spreading them on Petri dishes containing LB agar (supplied with 100 μ g/ml ampicillin and 12.5 μ g/ml tetracycline).

3.2.14 Oral administration of transformed bacteria expressing dsRNA to *S. littoralis* larvae and silencing of *SI 102*

To assess the silencing activity of the bacteria expressing dsRNA specific for *SI 102* gene, *S. littoralis* fifth instar larvae were fed with 2 μ l (about 4×10^7 cells) of sonicated bacteria expressing dsRNA twice per day, for two consecutive days. Control larvae were fed with bacteria expressing *GFP* dsRNA. To evaluate the effect followed by the ingestion of dsRNA on *SI 102* gene expression, the treated larvae were dissected and haemocytes were collected and processed for total RNA extraction as described above; the relative expression of *SI 102* was assessed by q-RT-PCR, as described in 3.2.4 by using specific primers (*SI 102* RT fw: GGCGGTGTCGTCGATTATG; *SI 102* RT fw: GAGCGAGGAAATGTTCAAT).

3.2.15 *In vivo* encapsulation assay

The *SI 102* dsRNA or *GFP* dsRNA was administered as described above to 10 *S. littoralis* larvae in the first day of fifth instar. After 12 h from the last administration, the encapsulation assay was performed as described in 3.2.9.

3.2.16 Administration of transformed bacteria expressing dsRNA to *S. littoralis* larvae on artificial diet and silencing of *SI 102*

To assess the insecticidal activity of the bacteria expressing a dsRNA targeting *SI 102* gene in *S. littoralis* larvae, a feeding bioassay on artificial diet has been set up. Newly molted fourth instar larvae were confined individually in plastic trays as described in 3.2.10 and alimented with surface-treated artificial diet under the same

environmental conditions reported in 3.2.7. For the first three days, twice per day, the upper surface (1 cm^2) of the artificial diet (0.3 cm^3) was uniformly overlaid with $30\text{ }\mu\text{l}$ of *Sl 102* dsRNA-expressing bacteria treated as described above (3.2.15), corresponding to approximately 10^8 cells equivalents. Control larvae were reared on artificial diet overlaid with $30\text{ }\mu\text{l}$ of *GFP* dsRNA-expressing bacteria. Experimental larvae were maintained on artificial diet, replaced every 12 h.

24 h after the last application the larvae were dissected and haemocytes were collected and processed for total RNA extraction as described above; the relative expression of *Sl 102* was assessed by qRT-PCR, as described in 3.2.4.

3.3 Results

RNA interference (RNAi) is a mechanism of sequence-specific suppression of gene expression, mediated by small double stranded RNA (dsRNA), which is highly conserved among eukaryotes. Administration of dsRNA targeting transcripts encoding essential proteins results in insect mortality and this evidence has opened the door to RNAi-based pest management (Zhang *et al.*, 2013; Scott *et al.*, 2013; Yu *et al.*, 2016).

RNAi can be pursued either directly, by suppressing functions in the target host that generate lethal phenotypes or, indirectly, by enhancing the impact of natural antagonists as a result of reduced immunocompetence. In the current work the second approach was tested on larvae of *S. littoralis*, one of the most damaging insects for agriculture.

Among the numerous genes involved in different aspects of insect immune response I focused in particular on *SI 102* and *SI gasmin* genes.

SI 102, recently identified and isolated in my host laboratory, is involved in encapsulation and nodulation responses, and its silencing promotes an enhancement of entomopathogen toxicity (Di Lelio *et al.*, 2014; Caccia *et al.*, 2016).

Whereas *SI gasmin*, an homologous of *gasmin*, a gene involved in immune responses in *S. exigua* larvae, has been identified in *S. littoralis* and molecularly and functionally characterized for the first time in the current thesis work, showing a role of the gene in pathogen phagocytosis.

These results suggest that both genes represent good candidates in the use of RNAi-based pest management and thus suitable delivery strategies have to be developed. For this purpose in this work, bacteria have been used as a biofactory for cheap production and protected release into the environment of bioactive dsRNA molecules. Recombinant vectors for the *in vivo* dsRNA expression containing fragments of *SI 102* and *SI gasmin* genes (and *GFP* gene, absent in insects, in control bacteria) were successfully constructed. The bacteria used to produce dsRNA, a particular RNase-free *E. coli* strain, were transformed with *SI 102* recombinant vector and orally administrated both with direct in-mouth injection and applied on artificial diet to evaluate the silencing and phenotypic changes associated with it. The system chosen to perform the cloning steps was based on the Gateway Technology because it is a powerful new methodology that greatly facilitates the cloning of PCR products by replacing restriction endonucleases and ligase with efficient site-specific recombination.

3.3.1 Identification and functional characterization of *SI gasmin*:

3.3.1.1 *S. littoralis gasmin* gene (*SI gasmin*)

A partial cDNA showing 87% sequence identity with the *S. exigua gasmin* gene was retrieved from an EST library of *S. littoralis*. An open reading frame (ORF) in the *S. exigua* DNA sequence was identified using the online tool <https://www.ncbi.nlm.nih.gov/orffinder>. Based on the alignment between *S. littoralis* and *S. exigua* sequences, highly conserved sequences have been found and chosen to design the couple of primers used to amplify the *SI gasmin* ORF (Table 5, materials and methods).

This cDNA encodes a predicted protein (*SI gasmin*) of 346 amino acids (aa), with a putative signal peptide of 21 aa, that shows 94% and 86% sequence identity with *S. exigua gasmin* and the protein expressed by CcBV (*BV_25.3*) respectively (Figure 21 and 22).

| | | | |
|----------------------|-----|--|-----|
| <i>S. littoralis</i> | 1 | MLPITILTLTLLITSIIISRIQAEHEELKAELSGMRTPAYKNDRI | 45 |
| <i>S. exigua</i> | 1 | MLPITILTLTLLITGIIISRIQAEHEELKAELSGMRTPAYINDRVM | 45 |
| <i>CcBV_25.3</i> | 1 | MLSITVATLLVTLSNAIPRIQAEQKEQKAQLNGMRTPVYKSDHVI | 45 |
| <i>S. littoralis</i> | 46 | EALMRQKRDTLKIVPKSYVEQTNHVDLQRGKRLRRGLPKFDNFL | 90 |
| <i>S. exigua</i> | 46 | EALMRQKRDTLKIVPKSYVEQTNHVDLQRGKRLRRGLPKFDNFL | 90 |
| <i>CcBV_25.3</i> | 46 | EALMRQKRDITINIVPESYVEQTNLADKSRGNRLRRDLPKFDNFQ | 90 |
| <i>S. littoralis</i> | 91 | SGLDKKLSSHDIITSNRTGLVNYFASRLYQENVTFPENEGKNDVYG | 135 |
| <i>S. exigua</i> | 91 | SGLEKKLSSHYITSNRTGQVNYFASRLYQENFTFPENEGKNDVYG | 135 |
| <i>CcBV_25.3</i> | 91 | EGLDKKFSSFDAPSNRTGQVNYFASRVNQKDITFSESDGKNIVYG | 135 |
| <i>S. littoralis</i> | 136 | SRDVQIGNTFSDNPNGTNVYGPILVQNNIYRGKNKNAQYGASIT | 180 |
| <i>S. exigua</i> | 136 | SRDVQIGNTYSNPNNGTNVYGPILVQKNNYRQKNKNAQYGASIT | 180 |
| <i>CcBV_25.3</i> | 136 | SQDVQIGNTFSDNPNGTNVYGPILVQENNTYQQKNEAQYGASIT | 180 |
| <i>S. littoralis</i> | 181 | QIGNRYPEHPGINDIYTGVVVQNGNTYSVNPNGNKTSAFAINVQSN | 225 |
| <i>S. exigua</i> | 181 | QIGNRYPEHPGKNDIYTGVVVQNGNTYSVNPNGNKTSAFAINVQSD | 225 |
| <i>CcBV_25.3</i> | 181 | QIGNRYPEHPGKNDIYTGVVVQNGNTYPVNPNGNKTSAFAINQFN | 225 |
| <i>S. littoralis</i> | 226 | NTYPESIENNNDALKPIIHQSGNFYPSNDSEAGMRTKYNTESQG | 270 |
| <i>S. exigua</i> | 226 | NTYPESIENNKDALKPIIHQSGNFYPSNDSEAGRNTKNNTESQG | 270 |
| <i>CcBV_25.3</i> | 226 | NTYPKSIENNTDALKPIIYHSGNFYPSNDSEAGMNTKNNTKSGQ | 270 |
| <i>S. littoralis</i> | 271 | GSVEQNTSDSQGNSSSTNYDSDSSSHIESTTLRYEEREAYANKHT | 315 |
| <i>S. exigua</i> | 271 | GSVEQNTSDSQGNSSSTNYDSDSSSHIESTTLRYEEREAYANKHT | 315 |
| <i>CcBV_25.3</i> | 271 | GSVEQNTSDSQGNSSSTNYDSDSSSHIESTTPHNGEREAYANKHT | 315 |
| <i>S. littoralis</i> | 316 | KPNGQISLSSNVFYSLYSFIIILLKYVQFQY | 346 |
| <i>S. exigua</i> | 316 | KPNGQISLSSNVFYSLYSFIIILLKYVQFQY | 346 |
| <i>CcBV_25.3</i> | 316 | EPNGQISSFARFSVEVYLFIAFFKFTIFDS | 345 |

Figure 21. Alignment of *S.* gasmin, gasmin and hypothetical protein CcBV_25.3 protein sequences. Alignment was performed using the Clustal W algorithm; black and grey shading indicates identity and high conservation of amino acids, respectively.

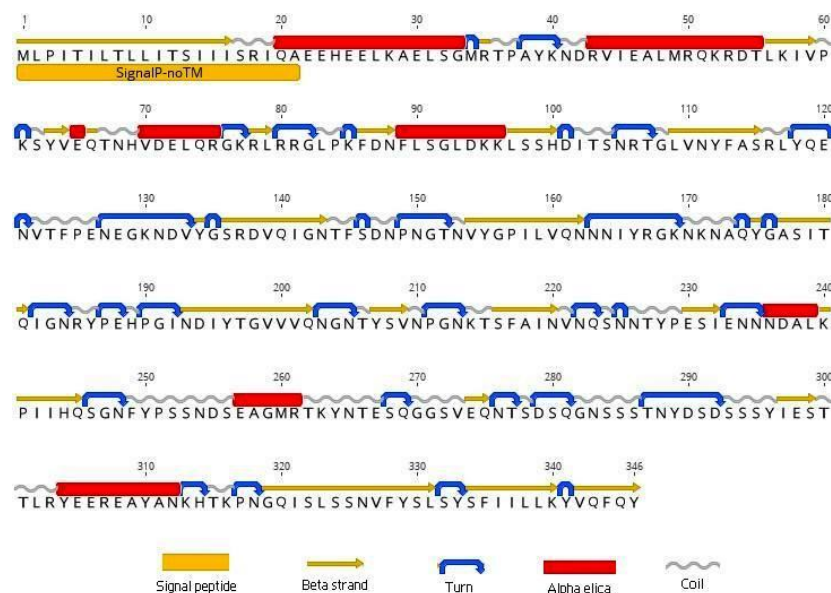


Figure 22. Predicted secondary structure and sequence conservation of *S.* gasmin. Secondary structure prediction of *S. littoralis* gasmin was carried out with the EMBOSS: Garnier algorithm; the InterProScan tool identified a potential signal-peptide. All bioinformatic analyses were performed using Geneious v6.1.6 (Biomatters, available from www.geneious.com).

3.3.1.2 Expression profile of *Sl gasmin* in different tissues and after immune challenge

The expression profile of *Sl gasmin* gene in different tissues of *S. littoralis* larvae was analyzed by qRT-PCR (Figure 23). The haemocytes were by far the most active site of transcription, suggesting a key-role of this gene in immune response. To confirm this hypothesis, the *Sl gasmin* transcription was assessed upon microbial challenge. The injection of both Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*), as well as of the yeast *Saccharomyces cerevisiae*, significantly enhanced the transcription of *Sl gasmin* in the haemocytes, with slightly different temporal profiles (Figure 24). The expression level of the studied gene rapidly started to increase after bacteria injection, showing a significant increase in the first 30 minutes and a more rapid recovery to basal expression levels in the case of *E. coli*. *S. cerevisiae* artificial infection triggered a comparatively slower response soon after injection, followed by a gradual decrease to the basal level 12 h post-injection.

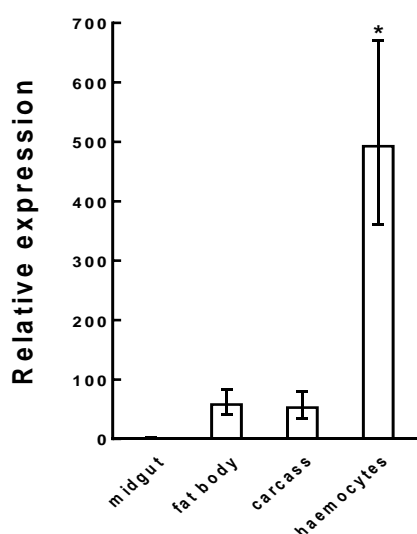


Figure 23. *Sl gasmin* transcript levels in different tissues of *S. littoralis* larvae. *Sl gasmin* gene expression as determined by RT-qPCR was significantly higher in the haemocytes compared with the other tissues analyzed. The relative expression was calculated based on the value of the lowest expression, which was ascribed an arbitrary value of 1. The values reported are the mean ± S.E. (* $P < 0.001$, One-Way ANOVA, followed by Bonferroni test, $n=9$).

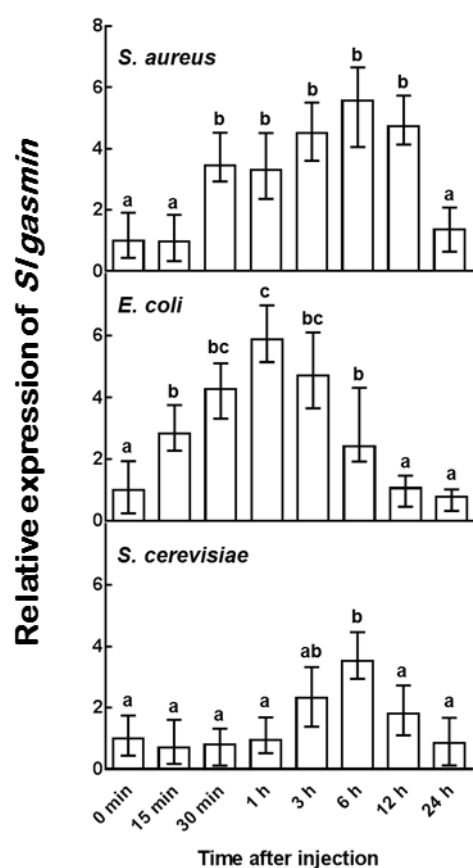


Figure 24. Relative expression levels of *Sl gasmin* in haemocytes after pathogen challenge. Transcript level of *Sl gasmin* as determined by RT-qPCR was significantly up-regulated in *S. littoralis* larvae by injection of the Gram positive bacteria *S. aureus*, the Gram negative bacteria *E. coli* bacteria, and the yeast *S. cerevisiae*. The relative expression was calculated based on the value of the expression level immediately before pathogen injection, which was ascribed an arbitrary value of 1. Data are means ± S.E. of three biological replications. Different letters above each bar indicate significant differences ($P < 0.05$, based on an One-Way ANOVA, followed by Bonferroni test).

3.3.1.3 Characterization of *Sl gasmin* immune functions by RNAi

The very high transcription level in the haemocytes and its enhancement by microbial challenge strongly suggested an important role of *Sl gasmin* in the cellular immune response mounted by *S. littoralis* larvae. To unravel its functional features, RNAi was used to study the phenotypic changes associated with gene silencing. Oral treatment of experimental larvae with *Sl gasmin* dsRNA was effective in silencing the target gene from the first day of the fifth instar to the prepupal stage (Figure 25). This prolonged and stable gene silencing allowed the study of the phenotypes associated with the reduced expression of *Sl gasmin*.

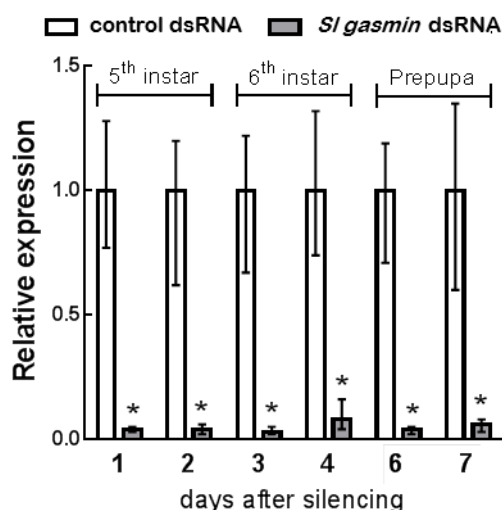


Figure 25. *Sl gasmin* silencing in haemocytes of *S. littoralis* larvae. After oral administration of control or *Sl gasmin* dsRNA during fourth instar, the expression of *Sl gamin* was significantly down-regulated until pupation. The values reported are the mean \pm standard error (* $P < 0.001$, Student's *t* test).

In particular, the role of this gene in cellular immune response was assessed, by studying the changes in nodulation, phagocytosis and encapsulation capacities associated with gene silencing.

Nodulation capacity against bacteria and yeasts was not altered by gene silencing (Figure 26 B and C). On the contrary, phagocytosis was strongly inhibited in experimental larvae treated with *Sl gasmin* dsRNA, as their haemocytes were nearly completely unable to internalize both Gram positive (*S. aureus*) and Gram negative (*E. coli*) bacteria (Figure 26 D and E, Figure 27). Encapsulation and melanization of chromatography beads injected into experimental larvae were not affected by gene silencing (Figure 28).

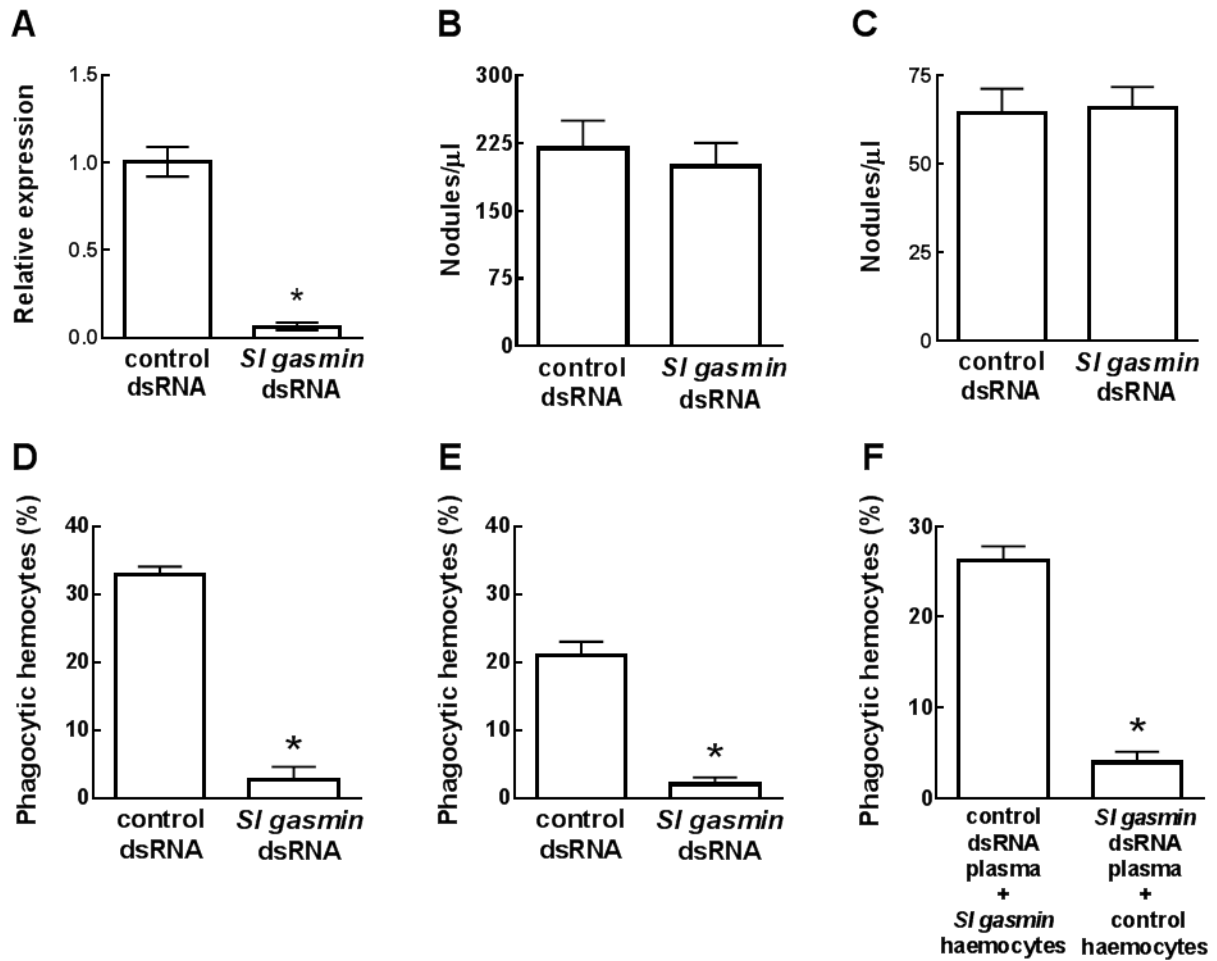


Figure 26. Cellular immune response by *S. littoralis* larvae as affected by RNAi mediated silencing of the gene *Sl gasmin*. The transcript level of *Sl gasmin* was significantly down-regulated by oral administration of *Sl gasmin* dsRNA in haemocytes of *S. littoralis* larvae used for the experiments (A). Nodulation of the Gram negative bacteria *Escherichia coli* and the yeast *Saccharomyces cerevisiae* were not influenced by RNAi mediated silencing of *Sl gasmin* gene (B and C respectively), while gene silencing significantly reduced the phagocytic capacity of *E. coli* and Gram positive (*Staphylococcus aureus*) bacteria by haemocytes (D and E respectively). Phagocytosis of *E. coli* by haemocytes from control larvae resuspended in haemolymph from silenced larvae was significantly impaired in respect to phagocytosis by haemocytes from silenced larvae resuspended in haemolymph from control larvae (rescue experiment) (F). (* $P < 0.001$, Student's *t* test).

Figure 27. Phagocytosis of bacteria by haemocytes from *S. littoralis* larvae as affected by RNAi mediated silencing of the gene *Sl gasmin*. Brightfield (A, C, E, G) and fluorescence (B, D, F, H) micrographs of haemocytes incubated in the presence of fluorescently labeled Gram negative (*E. coli*) and Gram positive (*S. aureus*) bacteria (A-D and E-H respectively). Phagocytosis was effective in larvae orally treated with control dsRNA (A, B, E, F,) while completely impaired in larvae fed with *Sl gasmin* dsRNA (C, D, G, H). Bars: 10 μ m.

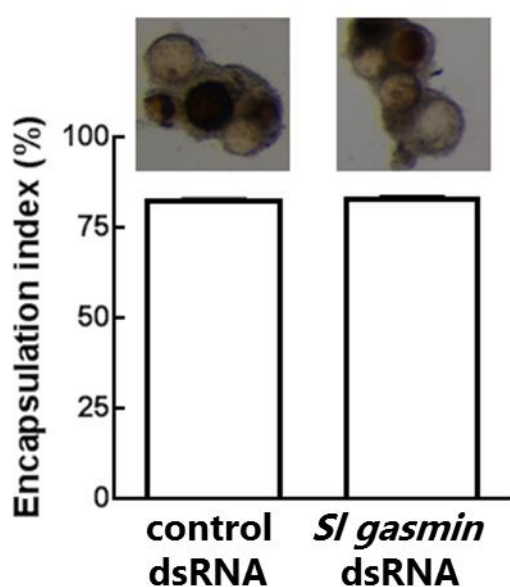
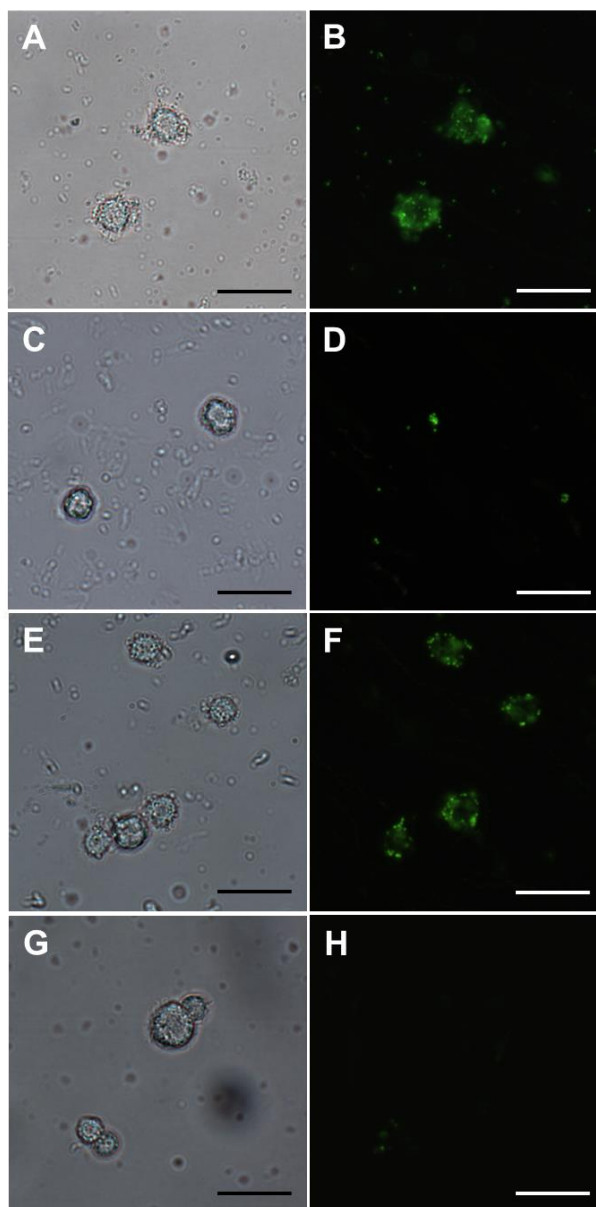


Figure 28. Encapsulation assay in *S. littoralis* larvae. Chromatography beads injected into *S. littoralis* larvae orally treated with control and *Sl gasmin* dsRNA (figure 26A) were encapsulated and melanized (insets). The encapsulation index (percentage of coated beads recovered from single larvae) was similar in both experimental larvae. The values reported are the mean \pm standard error (Student's *t* test).

To check if the disruption of phagocytosis was due to a negative effect of gene silencing on cytoskeleton architecture and dynamics, the distribution and polymerization capacity of actin in *S. littoralis* haemocytes was checked. F-actin staining clearly showed similar networks of the actin cytoskeleton in haemocytes extracted from controls and silenced larvae (Figure 29). Moreover, adhesion properties of haemocytes, which strongly depend upon proper actin cytoskeletal dynamics, were also unaffected by silencing. Indeed, haemocytes extracted from both controls and silenced larvae showed intense aggregation and adhesion on glass surface, displaying evident lamellipodia and filopodia extensions (Figure 29).

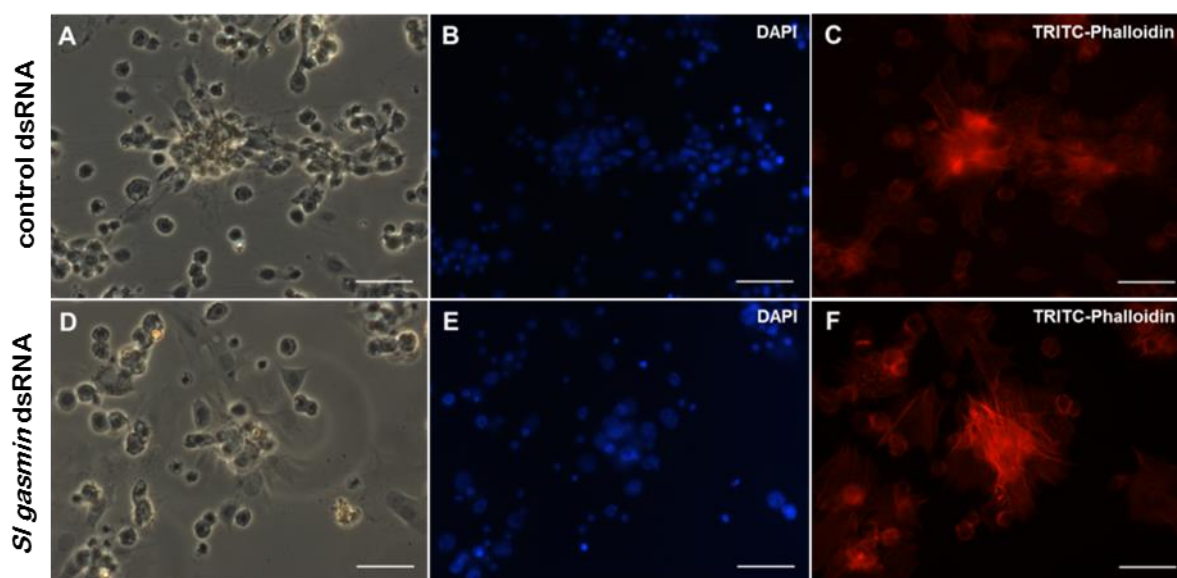


Figure 29. Actin cytoskeleton in adherent haemocytes. Brightfield images (A, D), DAPI (nuclear DNA) (B, E) and TRITC-Phalloidin (F-actin) (C, F) staining of haemocytes extracted from *S. littoralis* larvae orally treated with control or *Sl gasmin* dsRNA. Haemocytes from both experimental larvae adhered and spread on glass slides (A, D), showing evident lamellipodia (A, D, C, F) and similar actin networks (C, F). Bars: 30 μ m.

3.3.1.4 Role of *Sl gasmin* as haemolymphatic protein in immune response

The presence of a signal peptide in the protein codified by *Sl gasmin* and the phagocytosis failure associated with *Sl gasmin* silencing strongly suggested a functional role exerted in the extracellular environment, likely by interacting with pathogens entering the body cavity, to facilitate their immediate detection and subsequent phagocytosis. To test this hypothesis, a rescue experiment was designed by exposing the haemocytes obtained from silenced larvae (unable to perform phagocytosis) to the plasma of controls: this allowed to restore phagocytosis of bacteria *in vitro* (Figure 26F). In contrast, haemocytes obtained from control larvae lost their phagocytic activity when resuspended in plasma obtained from silenced larvae not expressing *Sl gasmin*. Thus, *Sl gasmin* present in the haemolymph is essential to mediate phagocytosis by haemocytes.

3.3.1.5 Effect of *Sl gasmin* silencing *in vivo*

Recently, we have demonstrated the importance of septicaemia in driving *Bt* killing activity, which resulted strongly influenced by host immunocompetence, in particular by the disruption of nodulation response (Caccia *et al.*, 2016). Here, by lowering the phagocytic activity through *Sl gasmin* silencing, we wanted to assess *in vivo* the

occurrence of a reduced clearance of midgut bacteria invading the haemocoel through *Bt*-induced midgut lesions, by scoring larval mortality as affected by gene silencing. *Sl gasmin* dsRNA treatment significantly enhanced the mortality of *S. littoralis* larvae treated with Cry1Ca toxin (Table 11), supporting the key-role played by this gene *in vivo*.

| Treatment [*] | LC ₅₀ [†] | TI [‡] |
|------------------------|-------------------------------|---------------------------|
| | <i>control</i> dsRNA | <i>Sl gasmin</i> dsRNA |
| Cry1Ca | 6.8 (4.5-10.0) | 2.7 (1.6-4.3) |
| | | 2.5 (1.2-5.2) |

Table 11. Enhancement of Cry1Ca toxicity by host immunosuppression: ^{*}Toxicity of Cry1Ca toxin were obtained on *S. littoralis* larvae after RNAi-mediated silencing of an immune gene (*gasmin* dsRNA), and in immune competent controls (*control* dsRNA). [†]Concentration ($\mu\text{g Cry1Ca}/\text{cm}^2$ diet) killing 50% of experimental larvae, with 90% fiducial limits reported in parentheses. [‡]The toxicity increase (TI) is calculated as the ratio between LC₅₀ values scored in *control* dsRNA control larvae, and in larvae treated with *Sl gasmin* dsRNA; 95% fiducial limits are reported in parentheses.

3.3.2 Construction of *L4440* recombinant vectors:

3.3.2.1 RNA extraction and RT-PCR

Starting from the total RNA extracted from haemocytes of *S. littoralis* larvae, where both *Sl 102* and *Sl gasmin* genes are highly expressed, cDNA was prepared, performing a RT-PCR. The cDNA fragment to be used for the dsRNA production was amplified by PCR, using the primers listed in Table 5 (materials and methods). The *Sl 102* sequence chosen to be amplified is based on the silencing efficiency obtained with dsRNA complementary to this sequence and prepared *in vitro* (Di Lelio *et al.*, 2014).

To evaluate the success of the amplification and the quality of the amplification products, the products were loaded on 0.8% agarose gels. In figure 30 single fragments of the expected size of 469 (A) and 1052 bp (B) respectively are visible.

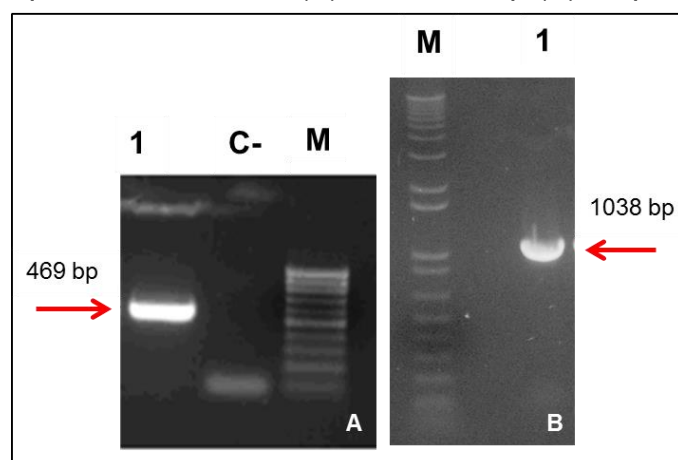


Figure 30. A) Amplification of the 469 bp sequence. 1: the fragment amplified with *fw Sl 102* e *rev Sl 102* primers; C-: PCR negative control (reaction mix without template); M: marker. **B) Amplification of the 1038 bp sequence.** 1: the fragment amplified with *fw Sl gasmin* e *rev Sl gasmin* primers; M: marker.

3.3.2.2 Gateway cloning of *SI 102* sequence

The cDNA fragments of the *SI 102* and *SI gasmin* genes have been successfully cloned in the *L4440* vector. The vector has been converted in a Gateway plasmid by inserting an expression cassette containing the *ccdB* gene and the gene for the chloramphenicol resistance, in the MCS region, flanked by *attR* sites (Figure 31) (see paragraphs 3.2.11).

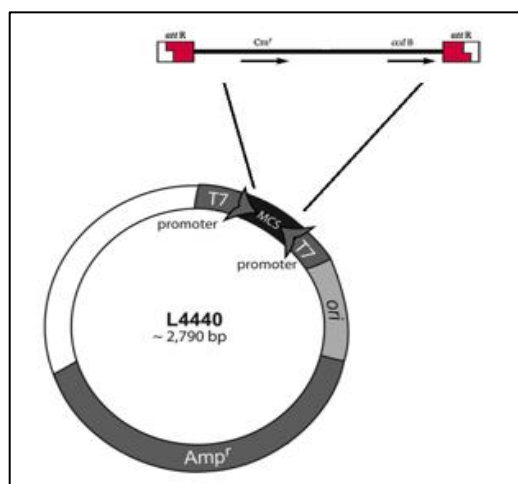


Figure 31. Gateway *L4440* vector.

The cloning method chosen was the Gateway system. It is extremely efficient since it facilitates the cloning of any PCR fragment skipping the classical restriction and ligation steps, which in most of the cases represent the limiting factors for the success of the cloning.

The first step to clone *SI 102* and *SI gasmin* sequences inside the Gateway *L4440* vector was the creation of entry clones. The forward primers, used for the cDNA amplification, have four nucleotides (CACC) at the 5' end to facilitate the cloning of the sequences of interest into pENTR/D-TOPO vector, the entry clones, containing the complementary sequence GTGG. Thanks to the presence of *AttL* sites, pENTR/D-TOPO vector is compatible with the Gateway *L4440* destination vector. The final *L4440* vector, able to express dsRNA thanks to the presence of the T7 promoters bidirectionally transcribing the sequence of interest, were obtained by exchanging lethal gene present in the Gateway *L4440* vector (*ccdB* gene) with *SI 102* and *SI gasmin* sequences, previously inserted into the donor vectors.

3.3.3 Production of *SI 102* dsRNA-expressing bacteria

The *HT115 E. coli* strain has been transformed with the recombinant vector containing the fragment of *SI 102* gene to produce *SI 102* dsRNA.

The selection of host cells containing the recombinant vector was carried out by plating 150 μ l of the transformation reaction on LB agar plates supplemented with ampicillin and tetracycline, since the *L4440* vector has the gene for the resistance to the first antibiotic and the bacterial *HT115* strain has an endogenous resistance to the second one. The transformed bacteria were grown as described in paragraph 3.2.12 (materials and methods) and then induced with isopropyl- β -D-1-thiogalattopiranoside (IPTG). IPTG binds the repressor present on the promoter in the bacterial genome that controls T7 polymerase expression. The enzyme acts on the convergent T7 promoters on the *L4440* vector, resulting in bidirectional transcription of the *SI 102* sequence, and thus achieving the production of *SI 102* dsRNA. At the

end of the induction the OD₆₀₀ was 1.2 (10⁹ cells/ml). The quantification of dsRNA molecules expressed from recombinant bacteria was not possible.

3.3.4 Bioassay on *S. littoralis* larvae

3.3.4.1 Bacteria inactivation and bioassay

Bacteria expressing *SI 102* dsRNA and expressing *GFP* dsRNA (control bacteria) were sonicated in order to inactivate them and break the bacterial cell walls to facilitate the dsRNA release in the insect gut. The bacteria were exposed to different sonication treatments (different duration of sonication cycles) to identify the optimum cycle for their inactivation. The inactivation was then verified by plating the sonicated suspension on LB agar plates supplemented with appropriate antibiotics (ampicillin and tetracycline) and observing the growth of colonies. The tests were carried out with 95% of sonicator maximum intensity, 130 watts and with the application of the following cycles (Figure 32):

- a) 5 sonication cycles (10 sec on/ 15 sec off);
- b) 10 sonication cycles (10 sec on/15 sec off);
- c) 10 sonication cycles (10 sec on/ 2 sec off);

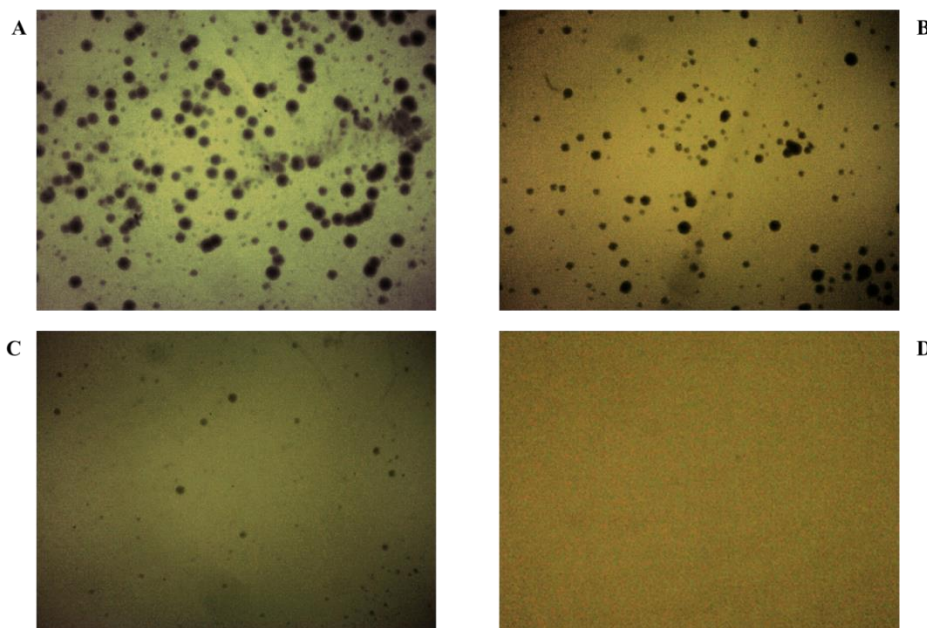


Figure 32. Sonication treatments. **A)** non treated bacteria; **B)** 5 cycles (10 sec on/15 sec off); **C)** 10 cycles (10 sec on/15 sec off); **D)** 10 cycles (10 sec on/2 sec off).

The results showed lack of growth of bacteria treated with the D cycle. Prior to bioassay, transformed bacteria were thus inactivated applying the D cycle. Following inactivation, bacterial suspension was orally administered to *S. littoralis* fifth instar larvae, and for two consecutive days as described in materials and methods (paragraph 3.2.7). Two protocols of administration were used: dsRNA was directly injected into the mouthparts of the larvae or administered through artificial diet.

3.3.4.2 Transcriptional analysis of *Sl 102* gene using qRT-PCR

To evaluate whether the direct oral administration and application on artificial diet of bacteria expressing dsRNA targeting *Sl 102* gene causes the silencing of the target gene in *S. littoralis* larvae, qRT-PCR experiments were conducted using the relative quantification method of total RNA extracted from larvae. For the validation of the $\Delta\Delta C_t$ method, 5 serial dilutions of total RNA (500; 50; 5; 0.5 and 0.005 ng) were compared with the ΔC_t values of *Sl 102* and β -actin. The resulting curve has a slope <0.1 , and therefore the method is valid (Slope = 0.0154; $R^2 = 0.0776$). In the larvae fed with bacteria expressing *Sl 102* dsRNA by direct oral administration or through artificial diet, the level of the target transcript is significantly reduced compared to control larvae ($P < 0.0001$; $t = 34.964$; $df = 32$) (Figure 33 and 34 respectively).

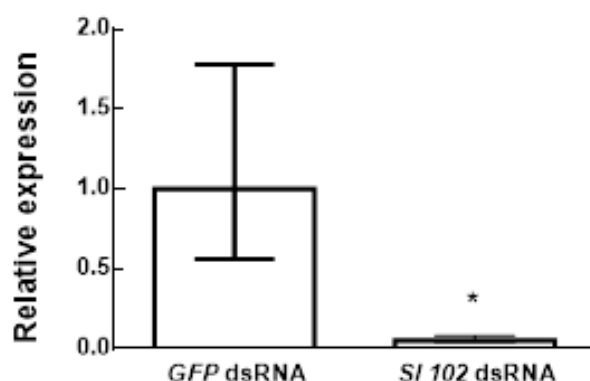


Figure 33. Silencing caused by oral injection of bacteria. The transcriptional level of *Sl 102* gene in larvae treated with bacteria expressing *Sl 102* dsRNA is statistically different compared to control larvae treated with bacteria expressing *GFP* dsRNA (* $P < 0.0001$, Student's t test).

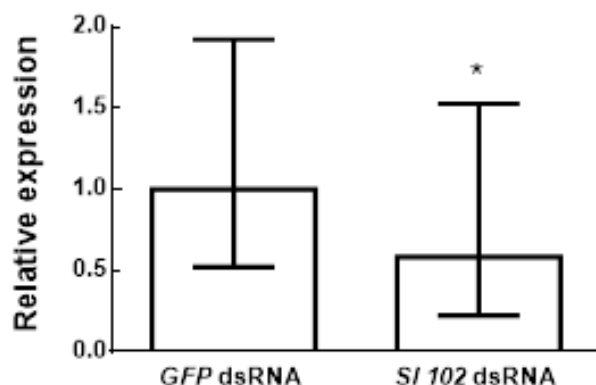


Figure 34. Silencing caused by bacteria applied on artificial diet. The transcriptional level of *Sl 102* gene in larvae treated with bacteria expressing *Sl 102* dsRNA on artificial diet is statistically different compared to control larvae treated with bacteria expressing *GFP* dsRNA (* $P < 0.0001$, Student's t test).

3.3.4.3 Analysis of the immune response in *S. littoralis* larvae treated with transformed bacteria

To assess whether following the silencing of *SI 102* gene obtained by the oral administration of transformed bacteria corresponded an alteration of the immune response and in particular of the capacity of the hemocytes to encapsulate the non-self intruders, an encapsulation assay has been performed using chromatographic beads (Di Lelio *et al.*, 2014) on larvae treated with dsRNA-expressing bacteria.

S. littoralis fifth instar larvae were fed with bacteria expressing *SI 102* dsRNA twice per day, for two consecutive days. Control larvae were fed with bacteria expressing *GFP* dsRNA. After 12 h from the last administration, the encapsulation assay was performed as described in materials and methods (paragraph 3.2.9). After 24 h, beads were recovered upon larval dissection and scored to evaluate their encapsulation rate, which was expressed with an index taking into account both the encapsulation degree of each recovered bead and the relative abundance of beads with a given encapsulation degree (Li *et al.*, 2007). As expected, 24 hours after oral injection of inactivated bacteria, the beads isolated from the haemolymph of control larvae (Figure 35a) had been completely encapsulated while this was not observed in the larvae fed with bacteria expressing *SI 102* dsRNA (Figure 35).

The encapsulation index in the silenced larvae is significantly reduced compared to controls (Figure 35).

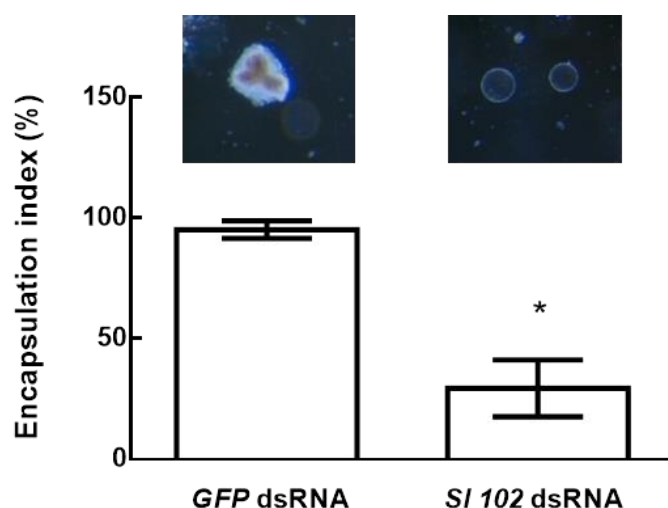


Figure 35. Oral administration of dsRNA molecules targeting the transcript of *SI 102* gene inhibits the encapsulation response in *S. littoralis* larvae. (* $P < 0.0001$, Student's *t*-test).

Treated larvae survived to the treatment and did not show morphological changes (Figure 36).



Figure 36. The picture shows the control larvae (left) compared to the larvae treated with *SI 102* dsRNA (right). Evident morphological changes were not observed in silenced larvae.

3.4 Discussion

The potential of RNAi as a mechanism to control insect pests has received remarkable attention, stimulated by the pioneering work showing the possibility to obtain gene silencing by feeding intake of dsRNA targeting genes affecting insect survival (Baum *et al.*, 2007; Mao *et al.*, 2007; Chen *et al.*, 2010). This new tool for insect control is still not fully exploited. Indeed, the future success of RNAi and the spreading on the market of new pest control technologies based on its use will depend upon identification of suitable target genes that if suppressed generate lethal phenotypes or enhance the impact and biocontrol efficiency of natural antagonists, as a consequence of the immune impairment of the host (Chen *et al.*, 2015; Caccia *et al.*, 2016). This latter approach has a strong environmental implications, as it favors the maintenance of ecological services underpinning the operation of agricultural and forestry ecosystems. In other words, by allowing the maintenance *in situ* of more susceptible hosts, natural antagonists will have the opportunity to persist and spread more efficiently.

The second fundamental aspect to take into account for the development of a RNAi strategy for pest control is the identification of appropriate delivery strategies for the dsRNA, as this aspect strongly influences the efficiency of gene silencing (Yu *et al.*, 2013). Any crop protection application relies on oral application, which is the most practical method due to its ease, cost effectiveness, time saving, less invasiveness, and above all, its natural route of entry (Tian *et al.*, 2009). One of the aims of this thesis was to develop an efficient delivery strategy for dsRNA molecules able to interfere with the immune responses of *Spodoptera littoralis* larvae, as a tool to increase the impact of entomopathogens. *S. littoralis* is one of the most damaging insects for agriculture, able to cause significant economic losses attacking a wide range of crops (Hill, 1987).

Among the numerous genes involved in the regulation of *S. littoralis* immune response I focused in particular on *Sl 102* and *Sl gasmin* genes.

Sl 102, recently identified and isolated in my host laboratory, is involved in the encapsulation and nodulation responses, and its silencing promotes an enhancement of entomopathogen-induced mortality (Di Lelio *et al.*, 2014; Caccia *et al.*, 2016). *Sl gasmin* has been isolated from *S. littoralis* and characterized at functional and molecular level in the present thesis. This is a homologue of *gasmin*, which occurs in *S. exigua* as a result of a horizontal gene transfer from the parasitic wasp *C. congregata*, mediated by *C. congregata* bracovirus (CcBV) (Gasmi *et al.*, 2015; 2016). The functional characterization of *Sl gasmin* in the current work allowed to establish its key-role in *S. littoralis* immune response. Indeed, *Sl gasmin* expression is high in haemocytes, as for *gasmin* in *S. exigua* (Gasmi *et al.*, 2016) and is enhanced by immune challenge mediated by the entrance of microbial pathogens in the haemocoel. The encoded protein is extracellularly secreted and acts as opsonizing factor promoting phagocytosis.

Interestingly, the immunosuppression induced in *S. littoralis* larvae treated with *Sl gasmin* dsRNA led to an increase in their sensitivity to pathogens, in particular to *B. thuringiensis* toxin Cry1Ca, significantly enhancing the mortality, as similarly obtained with the silencing of *Sl 102* (Caccia *et al.*, 2016). This result corroborates the immune role of *Sl gasmin* and indicates that it can be an additional target gene for RNAi based silencing strategies aiming at enhancing the impact of entomopathogens. However, the development of an adequate delivery strategy for field application is necessary.

The production of RNAi reagents in the laboratory is conducted *in vitro* by enzymatic reverse transcription or chemical synthesis. However, this is impractical for field applications because of its high cost and the high susceptibility to degradation; in fact dsRNA incubated with gut content is degraded within minutes while greater stability is achieved for haemolymph (Liu *et al.*, 2012). Degradation of dsRNA in the gut has been reported in several insects including *B. mori* (Liu *et al.*, 2013) and *Lygus lineolaris* (Allen and Walker, 2012). In addition, salivary secretion during feeding may secrete the dsRNA-degrading factor as seen in a sucking insect, *A. pisum* (Christiaens *et al.*, 2014). Thus, the delivery efficiency of dsRNA in insects could be significantly increased by protecting the dsRNA from degradation in order to enhance its uptake by the gut. A promising strategy for oral delivery of dsRNA involves the expression of dsRNA in bacteria and the administration of killed bacteria to the insects. Indeed, the dsRNA administered in this way appears to be particularly stable, because the bacterial cell protects the molecule (Whyard *et al.*, 2009) from both environmental degradation, and the degradation within the insect intestinal lumen. The resulting stability of the dsRNA and its low cost make this technology attractive for the possible field application of dsRNAs. This promising technology was first developed for the silencing in *C. elegans* (Timmons and Fire, 1998) and then has been successfully applied also to insects (Tian *et al.*, 2009; Li *et al.*, 2011; Zhu *et al.*, 2011; Kim *et al.*, 2015).

On the basis of these premises, the constructs encoding dsRNAs targeting the selected immune genes were cloned through a novel strategy here defined, based on the use of the *L4440* vector, which was, converted into a Gateway vector. The Gateway Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989). This system proved to be very efficient, by allowing a fast and easy cloning of both *Sl 102* and *Sl gasmin* and can be easily adopted for cloning a wide range of dsRNA molecules. The transformed bacteria expressing *Sl 102* dsRNA, when killed by sonication and delivered along with artificial diet were able to specifically inhibit the transcription of a target in *S. littoralis* larvae. Sonication was necessary to disrupt the bacterial cell wall and membrane (Kim *et al.*, 2015) and thus to allow the release of dsRNA synthesized by recombinant bacteria in the insect gut. Moreover, sonication, by killing bacteria, allows to meet in part the environmental safety requirements for any use under field conditions. Interestingly, oral administration of bacteria was able to reproduce the high level of gene silencing observed with dsRNA obtained *in vitro*, along with the immunosuppressive phenotype induced (i.e a decreased capacity to encapsulate non-self beads).

Silencing capacity of sonicated *Sl 102* dsRNA-expressing bacteria applied on artificial diet was also observed. This preliminary bioassay is essential to validate this delivery strategy for field application and, although this protocol led to a 2-fold decrease of *Sl 102* transcript level in treated larvae compared with the controls, the strategy is promising and improvable. The most likely cause of this lower silencing effect compared to direct injection in the mouthparts is likely due to an incomplete consumption of the artificial diet on which bacteria were applied. Anyway, this limitation could be overcome by applying higher concentrations of recombinant bacteria.

The results obtained in this work also showed the occurrence of systemic RNAi following ingestion of inactivated bacteria expressing *Sl 102* dsRNA. In fact the inhibition of transcription of a gene expressed in the haemocytes, located in the haemocoel, imply that dsRNA released into the larval midgut is transported beyond

the intestinal barrier. This result is in accordance with the capacity of *S. littoralis* midgut cells to internalize dsRNA molecules (Di Lelio *et al.*, 2014). After oral administration and internalization in midgut cells, *Sl 102* dsRNA synthesized *in vitro* was able to silence *Sl 102* gene in haemocytes, suggesting the occurrence of haemocoel entry of dsRNA molecules.

In *C. elegans*, where RNAi was first discovered, the transmembrane proteins SID-1 and SID-2 play important roles in dsRNA uptake (Winston *et al.*, 2002; McEvan *et al.*, 2012), but the relevance of *sid-1* and *sid-2* gene orthologs and/or endocytosis processes in insect RNAi is still debated. It is also currently unclear if the silencing signal is amplified in insects, as it happens in *C. elegans* and plant RNAi (Pak and Fire, 2007). The lack of consolidated knowledge in insects of both these aspects makes, at the present, highly unpredictable the degree of success of RNAi methodologies used in functional genomics studies or novel pest control strategies. To overcome these problems, it is mandatory to understand how the RNAi molecular machinery works in insects, and the differences among the different taxonomic entities, which appear to be relevant (Price and Gatehouse, 2008; Huvenne and Smagghe, 2010; Xue *et al.*, 2012; Yu *et al.*, 2013).

The results obtained in this work strongly suggest that both *Sl 102* and *Sl gasmin* are very promising candidates to develop new integrated control strategies through the use of RNAi targeting genes that if silenced are able to enhance the susceptibility to a wide range of pathogens including fungi, viruses, and bacteria. Similar studies to those performed with *Sl 102* are needed to assess the effect of killed bacteria expressing *Sl gasmin* on *S. littoralis* larvae. The possibility to increase the sensitivity of the noctuid to *Bt*, through the oral administration of bacteria expressing both *Sl 102* and *Sl gasmin* dsRNAs, could trigger a synergistic effect increasing significantly the effectiveness of the commercial formulations of *Bt* already on the market.

In conclusion, the use of bacteria for the expression of dsRNA molecules is a very promising technology both for the low-cost production, and the conferred protection against degradation. Indeed, the dsRNA is made particularly stable because the bacterial cell protects the molecule (Whyard *et al.*, 2009) from both environmental degradation, and the degradation within the insect intestinal lumen. These characteristics make the proposed technology very attractive for field applications, to be carefully evaluated on a large scale experiments.

4 CONCLUSIONS

It is now universally recognized, at both the European and Global level, that pest management must be undertaken in a much more sustainable manner. This requires a shift in approach from the current extreme reliance on synthetic chemical control products for pests and diseases to methods that include the much greater exploitation of antagonists of natural origin. This change is necessary as, despite the fact that chemical-based pest control has unequivocally become much safer in the last 20 years or so, significant concerns still remain over the safety of many synthetic pesticides. These concerns include direct effects on human health as well as impacts on non-target organisms, and soil and aquatic ecosystems. As a result, the more effective and extensive exploitation of biological control, which include, as outlined above, also the use of natural antagonists beyond the organism level, will directly address these concerns and, if implemented widely enough, will significantly contribute to a reduction in the environmental impacts of current pest and disease management practices.

Among the most promising alternatives to chemical pesticides is the use of biological control agents and, in particular, the natural materials that they produce. In contrast to broad-spectrum chemical insecticides, these “insecticides” can specifically target pest species, reducing adverse impacts on food safety, non-target organisms, and the environment. Therefore, by isolating these natural bioinsecticides and understanding the molecular mechanisms underlying their impact on target insect pests it is possible to develop new and highly novel tools and strategies for crop protection. However, the large majority of these natural bioinsecticide molecules have target receptors in the body cavity and, then, their activity upon oral uptake requires that they are absorbed and transported across the gut epithelium. It is, therefore, mandatory to define appropriate delivery strategies to significantly enhance the activity and impact of these molecules.

The present PhD thesis contributes to fill this research gap by developing new delivery strategies for bioinsecticide macromolecules. The attention has been focused on the delivery both of toxic proteins/peptides and of dsRNAs used for RNAi based pest control technologies. The take home messages arising from the work done can be summarized as follows:

- ✓ XXX and one of its structural domains is an effective carrier of toxic protein domains across the midgut barrier of the pea aphid, *Acyrtosiphon pisum*. This reinforces and further expands the impact of previous research work showing a very active transepithelial transport by XXX in insects and paves the way for future work aiming to develop new natural bioinsecticides active against different target pests.
- ✓ The use of killed bacteria as “cell capsules” for field delivery of dsRNA molecules is an effective strategy for protection against environmental degradation and to allow activation of a RNAi response.
- ✓ The proposed targeting of insect immune genes to induce immunosuppression proves to be efficient in indirect pest suppression, by enhancing the impact of natural antagonists, and particularly interesting from an ecological point of view. Indeed, with this bioinspired approach, mimicking the strategy adopted by insect antagonists to overcome the defense barriers of their hosts, we propose not to kill our target by using toxic molecules, but to expose them to a more aggressive action of entomopathogens and natural enemies, which, in this way are more

likely maintained in the environment, preventing undesired population crashes and consequent ecological instability of agroecosystems.

5 APPENDIX

List of communications:

-**2015** - Oral delivery of bioinsecticide molecules targeting haemocoelic receptors. European PhD Network in Insect Science - 6th Annual Meeting and SEI-SIPaV Joint Workshop (11th-13th November. Florence, Italy). **Oral communication.**

-**2016** - Caratterizzazione molecolare e funzionale di un gene di *Spodoptera littoralis* coinvolto nella risposta immunitaria. XXV Congresso nazionale italiano di Entomologia, 20th-24th June. Padova, Italy). **Oral communication.**

-**2016** - Oral delivery of bioinsecticides. European PhD Network in Insect Science - 7th Annual Meeting (27th-29th October. Le Colle sur Loup, France). **Oral communication.**

Experience in foreign laboratories:

School of Biology, Newcastle University, UK (21st April-20th October 2016). The work was supervised by Professor Angharad MR Gatehouse and Dr Martin Edwards.

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