USE OF RNAI TO DEVELOP NEW BIOTECHNOLOGIES FOR INSECT CONTROL

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Ai miei "pensieri felici"

INDEX

RIASSUNTO

Il controllo degli insetti dannosi in agricoltura è ancora in gran parte attuato con insetticidi chimici di sintesi. L'ampio impiego di pesticidi è notoriamente associato a numerosi problemi, quali sviluppo di resistenza negli insetti bersaglio, contaminazione ambientale, tossicità per organismi non-bersaglio, uomo incluso, e perdita di biodiversità. La ricerca di metodi di lotta alternativi ha portato allo sviluppo di nuove tecniche di natura biologica e al loro inserimento in strategie di controllo integrato (*Integrated Pest Management*, IPM), opzione fortemente promossa dalla direttiva europea 2009/128/EC sull'uso sostenibile dei pesticidi.

Il controllo biologico è basato sull'utilizzo di antagonisti naturali, ma questa definizione classica viene spesso ampliata, includendo anche l'uso di molecole e geni derivanti da antagonisti naturali o da essi modulati. Lo studio funzionale e molecolare delle relazioni antagonistiche tra fitofago e suoi nemici naturali rappresenta, pertanto, una fonte preziosa di molecole e geni coinvolti nell'induzione di alterazioni patologiche esiziali, riproducibili attraverso strategie molecolari di controllo ispirate da meccanismi naturali di soppressione degli insetti dannosi.

Gli imenotteri parassitoidi a sviluppo endofago, in particolare i coinobionti (i. e. che lasciano in vita l'ospite consentendone lo sviluppo) mostrano sofisticati adattamenti fisiologici, finalizzati a rendere possibile lo sviluppo della propria progenie all'interno del corpo dell'ospite. Tali adattamenti mirano a evadere passivamente o sopprimere attivamente il sistema immunitario dell'ospite, di cui ne alterano lo sviluppo, la fisiologia e la riproduzione, al fine di consentire la sopravvivenza e la crescita degli stadi giovanili del parassitoide. Questa regolazione è ottenuta grazie a fattori parassitari costituiti da secrezioni di origine materna (rilasciate nell'emocele dal parassitoide al momento dell'ovideposizione) e/o di origine embrionale (prodotte dalla stessa progenie).

I Polydnavirus (PDV) sono un potente fattore di regolazione materna iniettato nelle larve di lepidotteri ospiti durante l'ovideposizione da parte di parassitoidi braconidi e incneumonidi. I PDV sono integrati come provirus nel genoma del parassitoide e sono trasmessi verticalmente alla progenie attraverso la linea germinale. La loro replicazione avviene solo in alcune cellule specializzate del calice ovarico, dove i virioni prodotti si accumulano per essere iniettati nell'emocele dell'ospite al momento dell'ovideposizione. Le particelle virali infettano diversi tessuti dell'ospite, esprimendo geni che inducono, in particolare, soppressione

della risposta immunitaria e alterazione del sistema endocrino, di fondamentale importanza per la sopravvivenza e sviluppo degli stadi giovanili del parassitoide. Lo studio e l'analisi delle basi molecolari di queste interazioni antagonistiche offre l'opportunità (1) di identificare molecole e geni, candidati ideali per lo sviluppo di nuovi bioinsetticidi, e (2) di sviluppare nuove strategie di controllo basate sull'uso dell'RNA interference (RNAi), al fine di riprodurre sindromi parassitarie basate sulla riduzione di espressione di geni dell'ospite indotta da specifici fattori di virulenza.

L'RNAi è un meccanismo di regolazione dell'espressione genica mediata da piccoli RNA a doppio filamento che viene utilizzato per il controllo degli artropodi dannosi attraverso il silenziamento di geni che regolano funzioni vitali. In questo progetto di dottorato, sono stati scelti come bersaglio del silenziamento geni che regolano la risposta immunitaria, individuati da studi sulla sindrome immunosoppressiva indotta da PDV, o che sono stati acquisiti per trasferimento genico orizzontale, favorito da PDV, al fine di indurre una riduzione di immunocompetenza in grado di esaltare l'azione di biocontrollo da parte degli antagonisti naturali. I geni bersaglio così prescelti sono il *102 Sl* e *gasmin Sl*.

Lo studio e la caratterizzazione dei fattori di virulenza codificati dal bracovirus associato all'imenottero braconide *Toxoneuron nigriceps* hanno permesso l'identificazione del gene *102,* importante regolatore della risposta immunitaria cellulare nelle larve di *Heliothis virescens,* e del suo omologo in larve di *Spodoptera littoralis* (*102 Sl*).

Il sequenziamento di molti genomi di lepidotteri ha permesso di identificare porzioni di DNA derivanti da PDV, come il gene *gasmin Sl* di *S. littoralis*, che svolgono un ruolo chiave nella risposta immunitaria. Il silenziamento separato dei geni *gasmin Sl* e *102 Sl*, ottenuto con molecole di dsRNA prodotte *in vitro* e somministrate per via orale, induce immunosoppressione nelle larve trattate che risultano essere più suscettibili all'azione dell'entomopatogeno *Bacillus thuringiensis (Bt)*. Questo incremento di sensibilità al *Bt* rappresenta una nuova e interessante strategia di controllo biologico, basata sull'aumento dell'azione di contenimento da parte di agenti naturali. Tuttavia, restano da sviluppare adeguate strategie di rilascio ambientale di dsRNA, per limitarne la loro degradazione.

In tale contesto va a collocarsi il presente studio, il cui scopo è quello di sviluppare metodi sostenibili di rilascio ambientale di dsRNA, attraverso batteri ricombinanti e piante geneticamente trasformate, e di valutarne il loro effetto sull'efficacia insetticida dello Xentari™, un bioformulato commerciale a base di *Bt* subsp. *aizawaii*, e di differenti

tossine *Cry* (Cry1Aa, Cry1Ab, Cry1Ca, Cry1Da e Cry2Ab), sulle larve di *S. littoralis*.
Per produrre i batteri esprimenti dsRNA, cellule di *Escherichia coli* ceppo HT115 sono state trasformate con il vettore ricombinante L4440*,* contenente il frammento *102 Sl* inserito tra due promotori T7 e ottenuto con il metodo di clonaggio Gateway®. Il ceppo HT115 è particolarmente adatto alla iperespressione del dsRNA poiché è privo del gene che codifica per la RNAsi III, che ne favorirebbe la degradazione, e contiene, inoltre, una sequenza codificante per la T7 RNA polimerasi sotto il controllo del promotore del gene Lac.
La trascrizione del dsRNA viene facilmente indotta dall'IPTG (Isopropil-β-D-1-tiogalattopiranoside), un analogo funzionale dell'allolattosio, l'induttore dell'operone Lac, permettendo la trascrizione bidirezionale del frammento *102 Sl,* con conseguente produzione del dsRNA. I batteri esprimenti dsRNA sono stati sottoposti a sonicazione sia per inattivarli, prima di un loro uso sicuro dal punto di vista ambientale, sia per rompere la parete batterica e facilitare il rilascio di dsRNA nell'intestino delle larve. In un primo set di esperimenti, è stata valutata la riproducibilità del silenziamento ottenuto in uno studio precedente con dsRNA *102 Sl* sintetizzato *in vitro.* Pertanto, è stata somministrata alle larve, tramite iniezione nella cavità orale, una sospensione di batteri ricombinanti corrispondenti a 45 ng/µl di dsRNA (dose con maggior efficacia nella down-regolazione del gene *102 Sl – in vitro*). In seguito, diversi quantitativi di batteri, corrispondenti a diverse concentrazioni di dsRNA (45 ng/µl, 100 ng/µl, 200 ng/µl) sono stati somministrati tramite applicazione su dieta artificiale. In parallelo, sono stati condotti esperimenti controllo utilizzando batteri trasformati esprimenti dsRNA diretti contro il gene codificante la green fluorescent protein *(GFP),* ovviamente assente nelle larve di *S. littoralis.* Dai risultati ottenuti si evince che i batteri ricombinanti somministrati tramite dieta artificiale sono più efficaci del dsRNA sintetizzato *in vitro* in termini di silenziamento genico. Inoltre, tale silenziamento genico è risultato associato a un fenotipo immunosoppresso, come si evince dal mancato incapsulamento di sferette cromatografiche iniettate nella cavità del corpo delle larve trattate, osservando un chiaro effetto dose-risposta. Infatti, a dosi crescenti di dsRNA è stata registrata una diminuzione sia del livello di silenziamento del gene sia dell'indice d'incapsulamento. Per valutare l'impatto del silenziamento genico sull'aumento della virulenza dell'entomopatogeno *B. thuringiensis* sono stati condotti differenti biosaggi su diverse età larvali, IV e V età con dosi sub-letali del formulato commerciale Xentari™ che, da prove preliminari, hanno mostrato effetto solo sulla riduzione di peso nelle larve controllo.

Inizialmente le larve di IV età sono state alimentate con dieta artificiale ricoperta dalla sospensione di batteri esprimenti dsRNA *102 Sl* e, in seguito, una volta raggiunta la V età, la dieta è stata trattata con 12 µg/cm^2 del formulato *Bt*. Successivamente, è stata valutata la simultanea somministrazione dei batteri ricombinanti e di Xentari™ (9 µg/cm^2 per le larve di IV età e 12 µg/cm^2 per le larve di V età) per riprodurre una condizione più realistica di applicazione in campo. Dai risultati ottenuti si evince che la somministrazione orale combinata con la dieta artificiale di batteri esprimenti dsRNA e del bioinsetticida determina un notevole aumento della letalità del *Bt*, sia quando i due componenti sono somministrati contemporaneamente sia nel caso in cui il silenziamento genico viene ottenuto prima dell'esposizione a *Bt*.
La tecnica di RNAi può essere utilizzata producendo *in planta* appropriati dsRNA. Un secondo obiettivo di questo lavoro è stato valutare se la somministrazione orale di molecole di dsRNA insieme a un substrato alimentare naturale come le piante, che può influenzare il profilo enzimatico intestinale e quindi la capacità di degradare le molecole ingerite, sia efficace nell'indurre una riduzione dell'immunocompetenza, potenziando la letalità del *Bt*. Per ottenere le piante di tabacco transgeniche esprimenti le molecole di dsRNA *102 Sl*, espianti provenienti da foglie di tabacco (*Nicotiana tabacum)* sono stati co-coltivati con sospensioni di agrobatterio (*Agrobacterium tumefaciens),* in precedenza trasformato con un vettore in cui è stato clonato, tramite clonaggio Gateway®, un frammento del gene *102 Sl.* Le larve di *S. littoralis* sono state alimentate con il tessuto fogliare prodotto da piante provenienti da linee esprimenti i livelli più alti del gene 102. Per valutare se e da quale età larvale avesse inizio il silenziamento genico, è stato condotto un biosaggio, partendo con larve di *S. littoralis* di II età. Il livello del trascritto del gene *102* nelle larve alimentate con foglie di tabacco transgeniche è risultato ridotto significativamente, rispetto ai controlli alimentati con foglie di tabacco non transgeniche, a partire dalla IV età e fino allo stadio di prepupa. Il saggio d'incapsulamento ha dimostrato che il silenziamento genico è associato a un fenotipo immunosoppresso, in cui l'inibizione della risposta immunitaria cellulare, nelle larve alimentate con le due linee transgeniche, è messa in evidenza dal mancato incapsulamento da parte degli emociti di sfere cromatografiche iniettate nella cavità del corpo. Per valutare l'effetto del *Bt* sulle larve di *S. littoralis* silenziate attraverso l'utilizzo di piante transgeniche come vettore di molecole di dsRNA, sono stati condotti tre biosaggi su larve di IV e V età, trattate, rispettivamente, con 1 µg/cm^2 e 3 µg/cm^2 di Xentari™ (dosi sub-letali calcolate preventivamente per questi due stadi di sviluppo). Come negli

esperimenti con i batteri, è stata condotta una prima prova che prevedeva la somministrazione del dsRNA e del *Bt* non contemporanea. Le larve di IV età di *S. littoralis* sono state alimentate con foglie di tabacco trasformato per tre giorni, fino al raggiungimento della V età e, successivamente, trattate con Xentari™ per altri tre giorni. Le larve controllo sono state alimentate con foglie trattate solo con acqua. Inoltre, per riprodurre i possibili effetti di una condizione di campo, in cui le piante vengono trattate con *Bt* in seguito al rinvenimento di fitofagi a livello critico, sono state condotte prove di somministrazione del *Bt* a tempi più brevi, valutando il livello di silenziamento genico. L'intervallo minimo di alimentazione su foglia trasformata necessario ad indurre un incremento significativo dell'efficacia del successivo trattamento con *Bt* è risultato essere di 24 ore e dipendente dal quantitativo di foglia ingerito.

Al fine di valutare la possibile presenza di un effetto sinergico basato sul silenziamento in *S. littoralis* di geni che controllano diverse risposte immunitarie cellulo-mediate, sono stati silenziati contemporaneamente due geni immunitari, *102 Sl* e gasmin *Sl*, che controllano nodulazione/incapsulamento e fagocitosi, rispettivamente. I risultati ottenuti hanno mostrano che la somministrazione combinata dei dsRNA è in grado di regolare negativamente la trascrizione di entrambi i geni e che i livelli di silenziamento genico e di immunosoppressione sono comparabili a quelli ottenuti con i dsRNA somministrati individualmente. In linea con questi risultati è risultato il mancato effetto sinergico del doppio silenziamento sull'incremento di mortalità associato a trattamenti con dosi sub-letali di *Bt*.

In conclusione, i batteri ricombinanti e le piante transgeniche rappresentano una strategia efficiente per il rilascio di dsRNA in grado di innescare un'inibizione specifica dell'espressione di geni target con conseguente riduzione dell'immunocompetenza. I livelli di silenziamento genico sono stati più elevati rispetto a quelli ottenuti con molecole di dsRNA nude prodotte *in vitro,* in quanto, verosimilmente, più esposte alla degradazione ambientale e nel lume intestinale. I risultati ottenuti pongono le basi per un possibile uso futuro di queste strategie di somministrazione orale, sia perché permettono di indurre l'ipersensibilità degli insetti dannosi agli antagonisti naturali potenzialmente presenti nell'ambiente, sia perché, la loro somministrazione in combinazione con composti ad attività bioinsetticida (tossine del *Bt*) ne aumenta notevolmente l'efficacia.

SUMMARY

The identification of new bioinsecticides and of their efficient delivery strategies is one of the current approaches to reduce the use of synthetic chemicals in agriculture. The use of natural antagonists as a source of virulence factors or of molecular technologies that mimic their negative effect on the host insects paves the way toward the development of new bioinspired tools of pest control. To this aim, RNA interference (RNAi) can be used to artificially down-regulate host genes negatively targeted by virulence factors of natural antagonists, providing new opportunities for pest control. Recently, it has been demonstrated that RNAi-mediated silencing of an immune gene (*Sl 102*), to reproduce the negative effect of a polydnavirus associated with a parasitic wasp, generates an immunosuppressed phenotype in *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae, making them more susceptible to the entomopathogen *Bacillus thuringiensis*.

To exploit this novel pest control tool, it is essential to limit the environmental and insect gut degradation of double strand RNA (dsRNA) molecules. Here we contribute to this research goal by developing two delivery strategies, based on the expression of dsRNA molecules in *Escherichia coli* and transgenic tobacco plants. Experimental larvae ingesting bacteria or plant tissue expressing *Sl 102* dsRNA showed marked transcriptional down-regulation of the targeted gene and both enhanced the killing activity of a *Bt*-based biopesticide (Xentari™), demonstrating that these two delivery strategies were both effective. Moreover, to further enhance the immunosuppression and the resulting biocontrol level by *Bt*, we concurrently silenced an additional immune gene (*Sl gasmin*), which encodes a protein acting as an opsonizing factor promoting phagocytosis. The double silencing was successfully observed and was associated with a significant impairment of both encapsulation/nodulation and phagocytosis. However, this double immune deficiency did not induce a synergistic response, further enhancing the killing activity by *Bt*, which, indeed, was similar to that observed when only *Sl 102* gene was silenced.

In conclusion, bacteria and transgenic plants expressing *Sl 102* dsRNA appear to be two promising delivery strategies for field application. Their use to induce immunosuppression offers the possibility to pursue a new bio-inspired strategy to suppress pests based on the enhancement of their sensitivity to natural antagonists and, thus, reinforcing the important ecosystem service they provide.

1 INTRODUCTION

1.1 Biological control

The control of pest insects in intensive agriculture is still largely dependent on the use of synthetic chemical pesticides. The indiscriminate use of chemicals has generated several problems, such as insecticide resistance, environmental contamination, toxicity for non-target organisms, and biodiversity loss (Mahmood *et al.,* 2016). To design cropping systems less dependent on synthetic pesticides, it is necessary to define protocols of Integrated Pest Management (IPM), an ecosystem-based strategy that focuses on long-term prevention of pests or their damage through a combination of techniques such as biological control, habitat manipulation, modification of cultural practices, use of resistant varieties, and the use of low-risk compounds (Baker *et al.,* 2020). Pesticides should be used as a final alternative, when all the other approaches fail, and treatments are made with the goal of removing only the target organism, according to established guidelines, when present at a level that can cause economic damage. This is a promising approach to minimize losses in crop quality and quantity caused by pests, which reduces the negative impact of pest management on human health, environment and nontarget organisms. Biological control should be a core component of sustainable IPM plans, increasingly based on the protection and use of natural enemies, such as pathogens, parasitoids, and predators, to regulate pest densities and keep them below the economic threshold (DeBach, 1964).

In addition to the use of living organisms, it is possible to use nature-based substances, such as, for example, plant-derived molecules (Isman 2006), semiochemicals (Bruce et al. 2005; Witzgall et al. 2010), protein applications (Thakur and Sohal 2013), and RNA interference (Koch et al. 2016; Zhu et al. 2011). The International Biocontrol Manufacturers' Association (IBMA) promotes the broader term *bioprotection,* which includes the use of both biocontrol agents and non-living plant protection tools originated from nature (Stenberg *et al*., 2021). The growing knowledge on the functional basis of biological control allows to include in this definition also the use of molecules/genes deriving from natural antagonists, which are able to reproduce the lethal syndrome they induce in the target pests they use as hosts (Bale *et al.,* 2008; Pennacchio *et al.,* 2012; Mahmood *et al.,* 2016;). A large number of bioactive molecules, that regulate insect antagonistic interactions, have been already isolated and characterized

from plants and microorganisms (bacteria, fungi and viruses), to obtain insecticides of natural origins (Kachhawa, 2017), and many untapped sources are available in nature.
Parasitic wasps (Insecta, Hymenoptera) are the most effective natural enemies of insects. The astonishing diversity of developmental patterns and host regulation strategies displayed by these parasitic Hymenoptera provides the opportunity to identify new molecules and genes involved in a number of pathologies induced in the host (Beck *et al*, 2000; Pennacchio and Strand, 2006), which are ideal candidates for the development of new bioinsecticides (Bravo *et al.,* 2007; Pennacchio *et al.,* 2012). This novel approach expands the concept of biological control, since it is based on the use of natural antagonists beyond the organism level, as a source of bioinspired tools and strategies for crop protection.

1.2 Parasitoids

Parasitoids are largely represented in Hymenoptera (Quicke, 1997), although they can be found in other several orders of insects, such as Coleoptera, Diptera, Lepidoptera, Trichoptera, Neuroptera, Strepsiptera (Pennacchio and Strand, 2006). The high species diversity is almost matched by an equally wide spectrum of interactions with hosts, as indicated by the fact that parasitoids have evolved an astonishing number of symbiotic relationships with their hosts, which has allowed a spectacular adaptive radiation and the colonization of a wealth of ecological niches (Godfray, 1994; Pennacchio and Strand, 2006).
Parasitoid wasps are entomophagous insects that are free-living during their adult stage, while their juveniles have a parasitic life habit and feed on all different life stages of insect hosts, showing a wide range of exploitation strategies of the living food source they colonize and regulate to their own advantage (Harvey, 2005; Pennacchio and Strand, 2006).
According to the different host regulation strategies, two categories of parasitoids have been described: idiobionts and koinobionts (Askew e Shaw, 1986); and, based on their site of feeding, internally or externally of the host's body, can be divided into endoparasitoids and ectoparasitoids, respectively. Idiobionts are parasitoids that rapidly block and kill the host by injection of venom at the oviposition. They are in most cases ectoparasitoids that lay their eggs on the external cuticle of their hosts and larvae perforate the host body to feed on internal tissues, establishing a limited interaction with the internal milieu; in a

very few cases, idiobionts are endoparasitoid of non-growing host stages, such as eggs or pupae (Godfray, 1994). Therefore, hosts parasitized by idiobionts are static resources in which the quality (defined as the condition of resources that affects parasitoid growth, development, survival, and hence fitness) is positively correlated with host size, and may decline as hosts age (Harvey, 2005; Pennacchio *et al.,* 2014).
In contrast, most koinobionts parasitoids are endoparasitoids of insect larval stages, that continue to develop, feed and grow, even though they show severe immune suppression and neuroendocrine alterations, which underly the observed disruption of growth and reproduction (Beck *et al.,* 2000; Pennacchio and Strand, 2006).
The evident host paralysis and killing effects, induced by most idiobiont parasitoids at oviposition, are, therefore, replaced by a wealth of more subtle and specialized physiological alterations in the case of koinobiont endoparasitoids that represent the most sophisticated forms of adaptation to parasitic life, characterized, in many cases, by considerable levels of specialization. The successful parasitism usually depends on genes and gene products that the adult wasp injects at oviposition or that offspring produce during the course of development (Vinson and Iwantsch, 1980; Beckage and Gelman, 2004; Pennacchio and Strand, 2006). The host regulation factors injected by the ovipositing wasp females are venom, ovarian proteins and symbiotic viruses (Quicke, 1997). Among these factors, virus and virus-like particles described in many parasitoid wasps have a crucial role in the disruption of the lepidopteran host immunity and development; Polydnaviruses associated with some families of endoparasitoid wasps are a good example (Beckage and Gelman, 2004, Beck and Strand, 2007, Strand and Burke 2013, Strand and Burke, 2015, Wang *et al.,* 2018; Shi *et al.,* 2019; Strand and Burke, 2019).

1.3 Polydnaviruses

1.3.1 Genome structure and host regulation

Polydnaviruses (PDV) are symbiont viruses of parasitic wasps attacking lepidopteran larvae, which belong to two separate genera, *Bracovirus* (BV) and *Ichnovirus* (IV), respectively associated with braconid and ichneumonid parasitoids (Stoltz *et al.,* 1995; Strand and Burke, 2013). The absence of PDVs in basal lineages of Hymenoptera strongly suggests that the association of BV with braconids and IV with

ichneumonids arose independently (Huguet *et al.,* 2012), driven by strong evolutionary constraints leading to unique cases where a eukaryotic organism uses a virus to genetically manipulate the physiology of another eukaryote (Drezen *et al.,* 2003). Indeed, to ensure the survival and development of their offspring, parasitic wasps inject into the host these symbiotic viruses, which integrate into the host genome (Strand and Burke, 2012, Strand and Burke 2019) and express several virulence genes that result in numerous alterations, essential for the success of parasitism (Beckage and Gelman, 2004, Webb *et al.,* 2006, Strand and Burke 2019). The most evident pathogenic alteration induced by PDV on the larval host is the suppression of its immune response, which is under strong selection pressure, since it is essential for parasitoid survival and development (Shelby and Webb, 1999, Strand and Burke, 2012; Gueguen *et al.,* 2013; Strand end Burke, 2015; Gauthier *et al.,* 2018; Shi *et al.,* 2019; Strand and Burke 2019).

The wealth of virulence factors required for successful parasitism target different functions in the host and makes the PDV a natural source of potential bioinsecticides molecules (Pennacchio *et al.,* 2012).

The study of the mechanism of action of these virulence factors offers the possibility not only to exploit them as new bioinsecticides but also to develop new control strategies based on the use of molecular tools (e.g. RNAi and genome editing) to target the same functions which are disrupted in naturally parasitized hosts (Price and Gatehouse, 2008; Pennacchio *et al.,* 2012; Perkin *et al.,* 2016).

BV and IV are morphologically and genetically dissimilar but, however, both the life cycle and the organization of the genome show various similarities. This suggests that a common selective pressure led to a convergent evolution; in fact, they derive from the independent adaptation of different viruses used as tools to provide virulence genes in the parasitized host cell (Webb, 1988, Stoltz *et al.,* 1995, Webb and Strand, 2005).

The PDVs are characterized by a segmented genome made of double-stranded DNA circles that are largely noncoding and vertically transmitted through the germline, from one parasitoid generation to the other (Fleming and Krell 1993, Stoltz, 1993, Webb and Strand 2005; Strand and Burker, 2012, Strand and Burke, 2013). This ‘injected circular form’ is replication deficient but is essential for the physiological regulation that leads to parasitoid survival.

PDV have evolved a life cycle and organizational features that reflect their evolution into beneficial symbionts, which fully depend on wasps for transmission. Reciprocally, wasps fully depend on PDV virions for delivery of virulence genes to hosts. PDV are present in the germ line

and somatic cells of parasitic wasps as integrated proviruses. Replication, in contrast, only occurs in pupal/adult stage females in the nuclei of calyx cells that are located in the ovary (Webb and Strand, 2005), where virions (containing DNA encoding virulence genes) are assembled. In the case of BVs, calyx cells lyse to release single-enveloped virions that accumulate in the lumen of the reproductive tract where eggs are stored. During wasp oviposition virions are injected into the body cavity (haemocoel) of a host and rapidly infect and discharge their DNAs into the nuclei of host cells, which is followed by the expression of virus-encoded genes (Drezen *et al.,* 2003, Kroemer and Webb, 2004, Strand and Burker, 2012) (Fig. 1).

The proviral genome consists of two components: 1) the core genes that code for essential replication machinery, and 2) regions of DNA that contain virulence genes that are amplified, excised from the wasp genome, and packaged into virions. Each proviral DNA packaged into virions also possesses conserved flanking motifs that identify the site of integration/excision from the wasp genome during DNA replication, whereas the core gene containing domains lack these motifs. Thus, core genes are expressed in calyx cells to produce virions, but their transmission is entirely vertical and independent of any amplification or encapsidation (Bezier *et al.,* 2009; Beck *et al.,* 2011, Burke and Strand 2012). The genomic DNA packaged into virions contain a second conserved domain named the host integration motif, which mediates rapid integration into the genome of host cells followed by the continued expression of virulence genes until the wasp's offspring complete their development (Beck *et al.,* 2011).

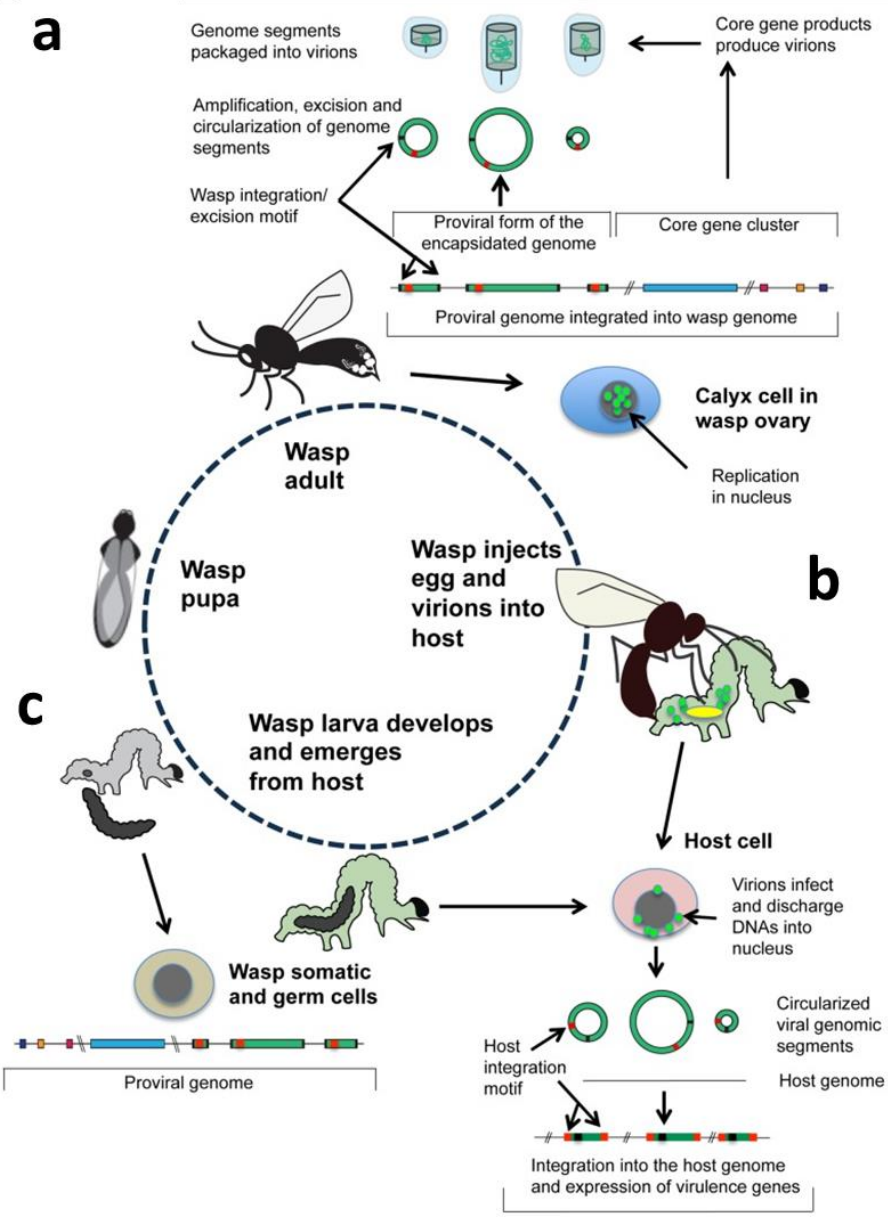


Figure 1. Life cycle and genome organization of BV. (a) Proviral genome in pupal and adult wasps. (b) Injection of virions and eggs into the host hemocoel (virions rapidly infect and discharge their DNAs). (c) The wasp completes development, while the host larva dies (from Strand and Burke, 2012).

1.3.2 PDV and host evolution

On rare occasions, parasitoid wasps can also oviposit in non-permissive species, and, in this case, the virus may not be able to fully interfere with host development (Beckage and Tan, 2002). Because the non-host regularly suppresses the parasitoid egg and/or juveniles, any surviving individual with a stable insertion of new genes in the germ line will represent an evolutionary novelty, with expanded functional capacities, if the resulting gene domestication event confers new physiological traits, that can be transferred over generations as proposed for the viral gene *BV2-5* (Gasmi *et al.,* 2015) (Fig. 2). This gene, expressed in the fat body and in the haemocytes of parasitized *Manduca sexta* larvae 24 h after oviposition by *Cotesia congregata*, plays an important role in protecting parasitized hosts, since it appears to limit the risk of accidental infection, which would be detrimental both for the host and the developing parasitoid progeny. This indicates that insects can paradoxically acquire selective advantages with the help of their natural enemies (Gasmi *et al.,* 2015; Di Lelio *et al.,* 2019).

This evolutionary pathway is supported by the finding that some insertions identified in lepidopteran species presented close to the 90% identities to the nucleotide sequences of BVs, flanked by specific sequences of lepidopteran. The presence of parts of BV circles, the organization of which is conserved, suggests that the direction of horizontal gene transfer (HGT) was from BVs to Lepidoptera. This hypothesis is confirmed by the identification of a regulatory signal involved in dsDNA circle production in the wasp, constituting a univocal signature of the BV origin of the sequence (Gasmi *et al.,* 2015).

BVs are therefore considered as a new source of sequences, contributing to insect genome evolution by HGT (Drezen *et al.,* 2017), which confer new functions that do not derive from the evolution of pre-existing genes (Di Lelio *et al.,* 2019).

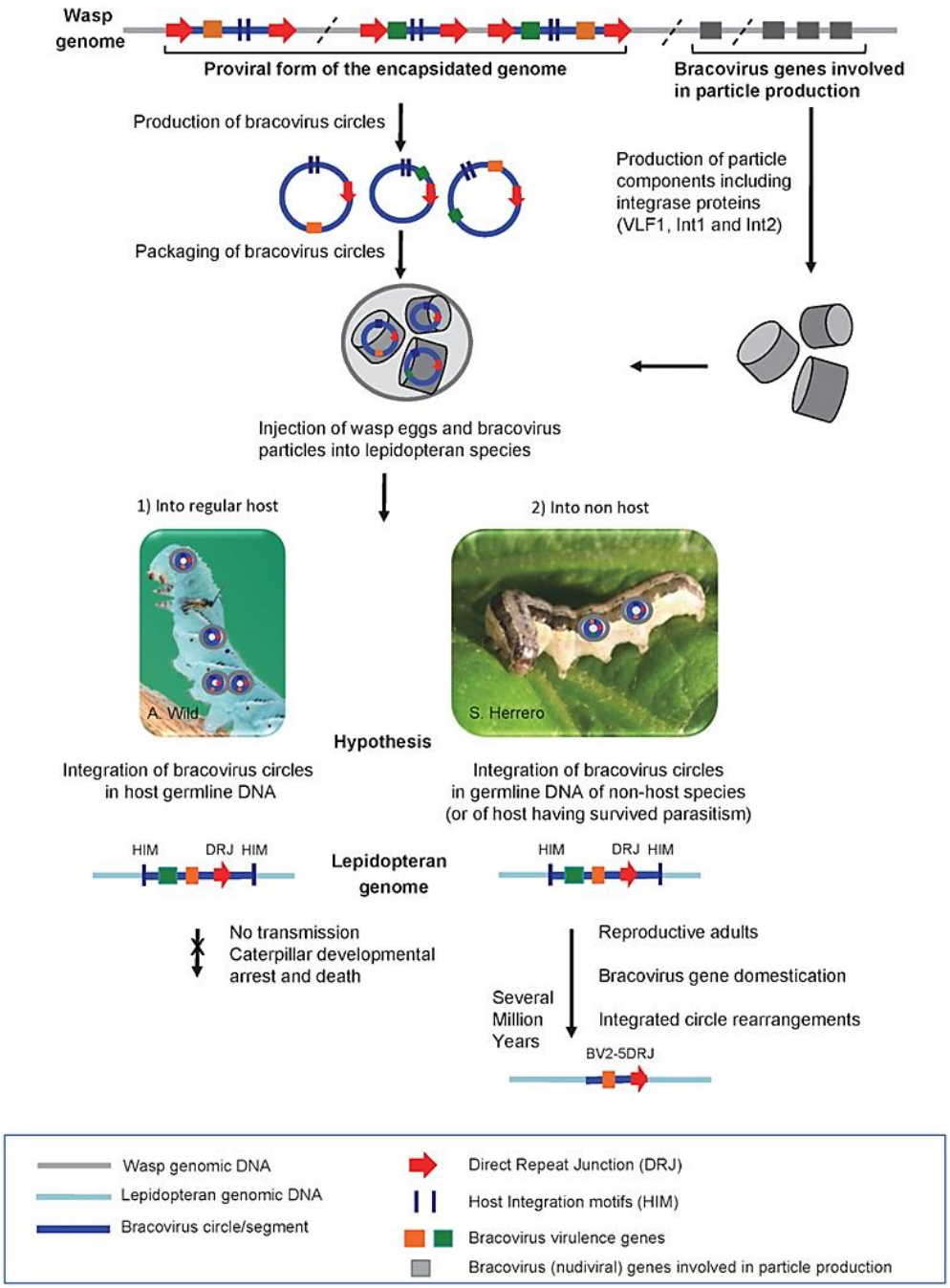


Figure 2. Horizontal Gene Transfer in Lepidoptera mediated *by Cotesia congregata* bracovirus (*Cc*BV) (from Gasmi *et al.,* 2015).

A homologue of the bracoviral gene *BV2-5* was recently found in the genome of *Spodoptera littoralis* (*Sl gasmin*) (Gasmi *et al*.,2015; Di Lelio *et al*., 2019), suggesting that *gasmin* was acquired by a basal ancestor of *Spodoptera* genus (as previously said about *Spodoptera exigua*) and maintained in distant species (Kergoat *et al.,* 2012). However, it was not found in the related species *Spodoptera frugiperda*, which likely lost the gene during evolution (Gasmi *et al.,* 2015).
Sl gasmin gene is highly expressed in larval stages exposed to an immune challenge and encodes an opsonizing factor triggering phagocytosis by the haemocytes (Di Lelio *et al.,* 2019). Phagocytosis is a rapid process and an effective barrier in response to intrusion of pathogens into the body cavity, mediated by immune cells (i.e. haemocytes) which recognize, bind, internalize and destroy the invading microorganisms (da Silva *et al.,* 2000; Hillyer, 2016; Di Lelio *et al.,* 2019). The first phase of phagocytic activity can be promoted by opsonins that label pathogens, and thus make them recognizable by haemocyte-surface phagocytic receptors (Browne *et al.,* 2013; Hillyer, 2016).
The increase in phagocytic activity to enhance the antimicrobial barrier is an effective strategy adopted by the host when other barriers are very rapidly disrupted by maternal secretions injected at the oviposition by the parasitic wasp. The proof of this concept was provided in a recent study where *S. littoralis* larvae exposed to RNAi-mediated silencing of *Sl gasmin in vivo* were unable to withstand *Bt*-induced septicaemia (Di Lelio *et al.,* 2019).
These results suggest that the acquired opsonizing factor is important in the modulation of phagocytosis efficiency *in vivo* and that, therefore, the horizontal gene transfer of viral symbiont genetic material reinforced the immune function in *S. littoralis* larvae (Di Lelio *et al.,* 2019).

1.3.3 PDV-inspired pest control strategies

The characterization of the virulence factors encoded by the bracovirus (*Tn*BV) associated with *Toxoneuron nigriceps* (Hymenoptera, Braconidae), an endophagous parasitoid of the tobacco budworm larvae, *Heliothis virescens* (Lepidoptera, Noctuidae), allowed the isolation of a host gene, designated *102*, highly expressed in host haemocytes which plays an important role in the cellular and humoral immune response (Falabella *et al.,* 2012). *102* gene codes for a protein essential in the encapsulation process which generates a scaffold of amyloid fibrils promoting the binding of toxic melanin precursors and

polymerization of melanin itself onto the target site, avoiding their harmful diffusion outside the capsule (Falabella *et al.,* 2012) that would be fatal for the insect (Lemaitre and Hoffmann, 2007, Cerenius *et al.,* 2008, 2010; Nappi *et al.,* 2009). The expression level of this gene in parasitized hosts is remarkably reduced from a few hours after wasp oviposition, suggesting that it represents an important target in the induction of the observed deep host immunosuppression, reproduced by *Tn*BV haemocoelic injections (Pennacchio *et al.,* unpublished).
A homologue of *102* gene has been isolated from a related noctuid moth species, *S. littoralis* (*Sl 102*), showing that this protein is quite conserved in insects, even at functional level, as demonstrated by the induction of a severe suppression of immune response by RNAi mediated silencing of the coding gene (Di Lelio *et al.,* 2014).
The *102* gene and the *Sl 102* are involved in the nodulation of microorganisms and in the encapsulation of large parasites (e.g. parasitoid eggs, nematodes) (Falabella *et al*., 2012; Di Lelio *et al*., 2014, Caccia *et al*., 2016), which are immune reactions sharing functional similarities (Lavine and Strand, 2002). The central importance of these genes in the modulation of immune response of lepidoptera and the strong suppression of its expression by *Tn*BV suggested the idea of assessing the impact of its silencing on the killing activity of natural antagonists of these important pests, trying to mimic the virulence strategy mediated by *Tn*BV. Interestingly, the silencing of this immune gene in *S. littoralis* was associated with a 5-6-fold increase of mortality induced by the entomopathogen *Bacillus thuringiensis* or by one of its toxins (Cry1A), as a consequence of a more intense septicaemia induced by gut bacteria entering the haemocoel through *Bt*-induced gut lesions (Caccia *et al.,* 2016).
These results pave the way towards the development of novel strategies of pest control based on RNAi-mediated silencing of immune genes to enhance the killing activity of *B. thuringiensis,* a widely used entomopathogen. However, there are several aspects to consider for achieving this goal, among which the development of efficient and safe delivery strategies is of central importance.

1.4 RNAi-based insect pest control

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 presence of complementary RNA (endogenous or exogenous), forms a very stable double-stranded structure. This leads to specific degradation of mature mRNA and, therefore, to the block of gene expression (Bartel, 2009). The discovery of RNAi constitutes an important milestone in the study of regulatory RNAs. The trigger for gene silencing is a double-stranded RNA (dsRNA) generated from an endogenous genomic locus or a foreign source, such as a transgene or a virus. The ability of exogenously supplied dsRNAs to silence the expression of a homologous target gene was first demonstrated in *Caenorhabditis elegans* (Fire *et al.,* 1998). Interestingly, RNAi is a sequence-specific method of suppressing a targeted gene's expression, and because each species is defined by the uniqueness of certain sequences of its genes, RNAi can potentially be designed in a species-specific way. In fact, RNAi strategies have been used to identify gene functions for insect development, physiology and reproduction (Schmitt-Engel *et al.,* 2015; Ulrich *et al.,* 2015) and could be used selectively to kill or to immunosuppress pest insects, by targeting essential genes, without adversely affecting non-target species (Gordon and Waterhouse, 2007; Price and Gatehouse, 2008; Whyard *et al.,* 2009; Gu and Knipple, 2013).

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; Martìn *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).

A particularly intriguing aspect of RNAi is that in highly susceptible insects the dsRNA is not only capable of entering gut cells but can spread to other tissues to induce systemic RNAi (Joga *et al.,* 2016). In insect different RNAi cellular internalization and export of dsRNA were described. In cell-autonomous mechanism the silencing process is limited in the cells that generate dsRNA or that are directly exposed to experimentally introduced dsRNA. The environmental (env) RNAi occurs in response to an environmental exposure to exogenous dsRNA (Whangbo and Hunter 2008). The clathrin-dependent endocytosis seems to play the primary role in the uptake of dsRNA in multiple insects, both in cultured cells and *in vivo,* and it is also known that the

uptake of naked dsRNA is length-dependent (Saleh *et al.,* 2006; Xiao *et al.,* 2015; Cappelle *et al.,* 2016; Pinheiro *et al.,* 2018).
An interesting aspect of the RNAi response in insects is its potential systemic character, also known as systemic (sys) RNAi. Specifically, in some insects, administration of dsRNA can result in the generation of an RNAi response throughout the entire insect's body. The systemic RNAi is robust in Coleoptera, absent in Diptera and not uniformly present in other insect orders (Joga *et al.,* 2016; Cooper *et al.,* 2019), such as in Lepidoptera, where occurs in noctuid moth species (e.g., *Helicoverpa* and *Spodoptera* spp.) (Tian *et al.,* 2009; Di Lelio *et al.,* 2014; Lim *et al.,* 2016; Cooper *et al.,* 2019), but the evidence of this response has only been indirectly determined by observing gene knockdown in tissues distant from the place of uptake (Bolognesi *et al.,* 2012; Li *et al.,* 2018). The systemic nature of RNAi is particularly useful in the development of a broader range of potential insecticidal dsRNAs that can target essential genes in many tissues of the pest insects (Huvenne and Smagghe, 2010). To maximize the potential of RNAi for insect control, it is important to define efficient dsRNA delivery methods for insecticidal applications. There are different techniques for the administration of dsRNA, such as microinjection (Kennerdell and Carthew 1998; Miller *et al.,* 2008; Tomoyasu *et al.,* 2008), ingestion (Timmon and Fire 1998; Timmons *et al.,* 2001), soaking (Tabara *et al.,* 1988; Yu *et al.,* 2013), electroporation (Osanai-Futahashi *et al.,* 2016) and virus-mediated delivery (Kontogiannatos *et al.,* 2013). The oral delivery is undoubtedly one of the most relevant for field application. The dsRNA used for ingestion experiments can either be expressed in microorganism or plants or can be synthesized *in vitro* and then fed to insects either by mixing with food or by supplying as solution droplets.

1.4.1 Bacteria-mediated RNA Interference

The use of bacteria as delivery vectors of dsRNA molecules was first demonstrated in the pioneering studies on RNAi in the bacteriophagous nematode *C. elegans* (Timmons and Fire 1998; Timmons *et al.,* 2001) and subsequently applied in various insect species, such as *Spodoptera exigua*, *Diabrotica virgifera*, and *Epiphyas postvittana* (Baum *et al.,* 2007; Tian *et al.,* 2009; Kim *et al.,* 2015). The efficacy of bacterially expressed dsRNA in the induction of systemic RNAi in *S. exigua* was first reported by Tian *et al.,* (2009). Feeding insects with dsRNA-producing bacteria determined a more sustained release of the dsRNA in the insect and prevented rapid degradation in the

environment and in the digestive system (Whyard *et al.,* 2009; Christiaens *et al.,* 2020). The bacteria-produced dsRNA pesticides can be sprayed on crops at any time, because of the facility to produce them in large amounts. This, indeed, can be considered as one of the most cost-effective method for production of dsRNA, which can be further improved using a strain of *Escherichia coli* (HT115) deficient in ribonuclease III activity (RNaseIII enzyme) that degrades dsRNAs (Timmons *et al.,* 2001).
Moreover, symbionts of insect pests can be engineered to increase the RNAi effiency and its use for pest control. Symbionts of two insect pests, the Western flower thrips *Frankliniella occidentalis* and the kissing bug *Rhodnius prolixus* were engineered and a long-lasting RNAi silencing effect was observed in both cases (Whritten *et al.,* 2016).

1.4.2 Transgenic plant-mediated RNA Interference

The delivery method that has received the most attention so far in a crop protection context is the use of transgenic crops producing a pest-specific dsRNAs that silences a critical insect gene following ingestion (Gordon and Waterhouse, 2007; Rosa *et al.,* 2018). Transgenic delivery has proved to be successful in the protection of several crops that can be engineered to express hairpin dsRNAs targeting genes from insects to increase their resistance to herbivorous insects (Baum and Roberts, 2014). This strategy is called host-induced gene silencing (HIGS) (Nowara *et al.,* 2010). Transgenic corn crop developed by Monsanto (currently Bayer CropScience), expressing a hairpin dsRNA targeting the Sucrose-non-fermenting7 (snf7) gene in *D. virgifera*, was the first commercial RNAi product targeting an insect pest (Bolognesi *et al.,* 2012; Bachman *et al.,* 2013; Christaens *et al.,* 2020) combined with two *B. thuringiensis* Cry proteins (Cry3Bb1 and Cry34/35Ab), to avoid the evolution of resistance (Head *et al.,* 2017). Engineering crops that resist to an insect pest through multiple mechanisms is one strategy for reducing the probability that resistance will be overcome. Most studies developed so far reported the suppression of gene expression, via plant-mediated dsRNA delivery, to disrupt the development and survival of the moth pest. For example, Zhu *et al.,* 2012 demonstrated that *Helicoverpa armigera* larvae fed with leaves of transgenic plants expressing dsRNA targeting the ecdysone receptor (EcR) gene died with significant molting defects. The identification of suitable insect targets and the delivery of sufficient amounts of intact dsRNA

expressing in planta for uptake by the insect are the key to the success of this approach.
The use of transgenic plant is a promising strategy because it allows to use a wide range of potential targets for suppression of gene expression in insect, while the combined use of RNAi biotechnology with existing crop protection strategies could also elude protein degradation-based resistance to *Bt* toxin, which was observed in polyphagous insect pest (Price and Gatehouse, 2008).

1.5 *Bacillus thuringiensis*

The bacterium *B. thuringiensis* (*Bt*), a ubiquitous Gram-positive rod-shaped and sporulating bacterium that infects invertebrates, predominantly in the phylum Arthropoda, is the main microorganism used in biological control, characterized by a broad host spectrum (Bale *et al.,* 2008; Raymond *et al.,* 2010). *B. thuringiensis* was discovered by Shigetane Ishiwata in 1901 (Aizawa, 2001), from diseased larvae of the silkworm (*Bombyx mori*) and rediscovered by Berliner ten years later. *B. thuringiensis* synthesizes several invertebrate toxins and provides the best example of a very effective natural antagonist, due to its striking insecticidal activity. In fact, *Bt* is used either as a biopesticide or as a source of resistance genes for transgenic crops (Romeis *et al.,* 2009). Different *Bt* products have been developed for insect control in agriculture and most of these products are based on spore-crystal preparations derived from a few wild-type strains (Bravo *et al.,* 2007).
Bt is usually isolated from the environment in the form of spores, which, once they enter into the victim, thanks to their arsenal of virulence factors, transfer from the digestive organs to the haemolymph, where they transit to the vegetative phase and, when the nutrients in the insect host are exhausted, pass to sporulation (Raymond *et al.,* 2010, Ruan *et al.,* 2015); then, the septicaemia induced by the host midgut microbiota largely contributes to the multifaceted killing mechanism by *Bt* (Caccia *et al.,* 2016). The effects of *Bt* are commonly attributed to Crystal (Cry) and Cytolytic (Cyt) toxin, (also known as δ –endotoxins). The crystal inclusions that are produced during the sporulation phase of growth of the bacteria act as the primary pathogenic factor. Once ingested by insects, these crystals are solubilized in the midgut, the toxins are then proteolytically activated by midgut proteases and bind to specific receptors located on the insect cell membrane (Bravo *et al.,* 2007), leading to cell disruption and insect death. Additionally, *Bt* isolates can also synthesize other insecticidal proteins during the vegetative growth phase; these proteins, subsequently secreted into

the culture medium, have been designated as vegetative insecticidal proteins (Vip) (Estruch *et al.,* 1996; Warren *et al.,* 1998) and secreted insecticidal protein (Sip) (Donovan *et al.,* 2006). At the same time, numerous non-toxin virulence factors of *Bt* have been discovered, which are crucial for virulence establishment and successful infection, including metalloproteases, chitinases, aminopolyol antibiotics and nucleotide-mimicking moieties (Malovichko *et al.,* 2019).

Cry toxins have specific activity against insects of different orders, such as Lepidoptera, Coleoptera, Hymenoptera and Diptera, and nematodes. Those active against lepidoptera are the best known from a mechanistic point of view, showing a conserved structure, in spite of the different amino acid sequences they have (Vilchez *et al*., 2020). The parasporal crystal inclusions ingested by susceptible larvae dissolve in the alkaline environment of the gut, and the solubilized inactive protoxins are cleaved by midgut proteases yielding the active toxin (Bravo *et al.,* 2005, 2017; Malovichko *et al.,* 2019; Vilchez *et al*., 2020). Toxin activation involves the proteolytic removal of an N-terminal peptide and approximately half of the remaining protein from the C-terminus in the case of the long Cry protoxins. The activated toxin then, before inserting into the membrane, binds to specific receptors on the brush border of the midgut epithelium columnar cells, such as aminopeptidase N (APN), cadherin-like proteins (CADR), alkaline phosphatase (ALP), and ABC transporters (Sato *et al*., 2019; Jin et al., 2020; Zhang *et al*., 2020; Wang *et a*l., 2020; Li *et al*., 2020). After receptor binding, conformational changes of the active toxin occur, followed by polymerization, and, finally, by membrane insertion and formation of lytic pores (Aronson and Shai, 2001; Bravo et al., 2005, Vilchez *et al*., 2020) (Fig. 3). Subsequently, cell lysis and disruption of the midgut epithelium leads to a lethal septicaemia, caused by midgut bacteria invading the body cavity (haemocoel) (Raymond *et al.,* 2010; Caccia *et al.,* 2016).

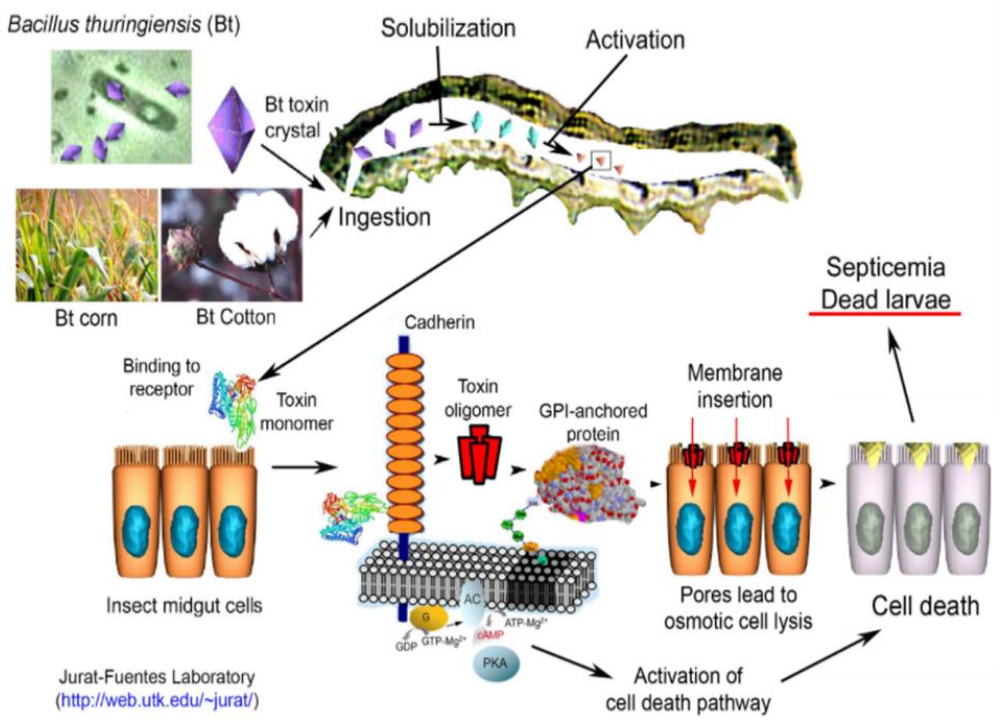


Figure 3. Mechanism of action by the *Bt* Cry toxin in Lepidoptera. The larvae ingest toxin crystals that are solubilized and the protoxins are processed and activated by midgut proteases in the insect digestive fluid. The solubilized and protease-activated toxin binds to receptors localized in the apical microvilli of insect midgut cells. The toxin inserts itself into the apical membrane forming pores which lead to osmotic cell lysis and cell death. (http://juratfuenteslab.utk.edu/Btresearchtable.html)

- **Cyt toxins** are mostly found in *Bt* strains active against Diptera and directly interact with membrane lipids for cytolytic action (Tharad et al., 2020). Cyt proteins are 27 kDa protoxins that after proteolytic activation give a 25 kDa protein that has pore-formation activity. The toxin interacts with lipids forming oligomers of more than 16 subunits (Bravo *et al.,* 2017).

- **Vip toxins** have insecticidal proprieties and are synthesized during the vegetative phase of growth; these are subsequently secreted into the culture medium and have been designated as vegetative insecticidal proteins. Vip toxin are classified into four families Vip1, Vip2, Vip3 and Vip4, according to their degree of amino acid similarity. They have a broad insecticidal spectrum. The binary toxin comprising Vip1 and Vip2 proteins inhibit actin

polymerization and exhibit insecticidal activity against some coleopterans (Palma *et al.,* 2014; Chakroun *et al.,* 2016), whereas Vip3 toxins are able to induce toxicity in lepidopteran species and are putative pore-formers (Estruch *et al.,* 1996; Donovan *et al.,* 2006; Crickmore *et al.,* 2016, Chakrabarty *et al*., 2020). Vip 3 proteins do not share the binding sites with the *Bt* Cry proteins, so pyramiding Vip3A proteins and Cry protein has been widely adopted in *Bt*-crops (Kurtz *et al*., 2010)

Cry toxins are widely used for controlling insect pests as spray products or expressed in transgenic *Bt*-plants. The development of plants genetically engineered that produce *Bt* Cry proteins is one of the first major biotechnological applications. In 1995, the commercial production and distribution of the *Bt* crops, such as corn, cotton, potato, and tobacco, was approved by the Environmental Protection Agency (EPA). The use of *Bt* transgenic plant, resistant to insect, has reduced considerably the use of chemical pesticides. Although the specificity of *Bt* Cry toxins toward target pest species is an advantage in agriculture, because effects on non-target insects and other organisms in the ecosystem are negligible, deployment of transgenic crops expressing a single specific *Bt* toxin can lead to problems in the field, where pest species not susceptible to that *Bt* toxin can proliferate and cause significant damage to the crop (Gatehouse, 2008). Moreover, one of the most critical issues associated with the use of *Bt* crops is the development of insect resistance, that can be due to different mechanism including alteration of activation of Cry toxins by midgut proteases (Li *et al.,* 2004), the development of an elevated immune response (Rahman *et al.,* 2004) and the alteration of toxin receptors that reduce the binding to insect gut membranes (Jurat-Fuentes *et al*.,2021). The spread of resistance and of the involved recessive alleles can be effectively limited by the so-called high dose/refuge strategy (Li *et al*., 2017). Indeed, the presence of susceptible insects in refuge areas and their breeding with resistant individuals will generate a susceptible heterozygous offspring (Fig.4). (Ferré *et al*., 2008; Li *et al* 2017).

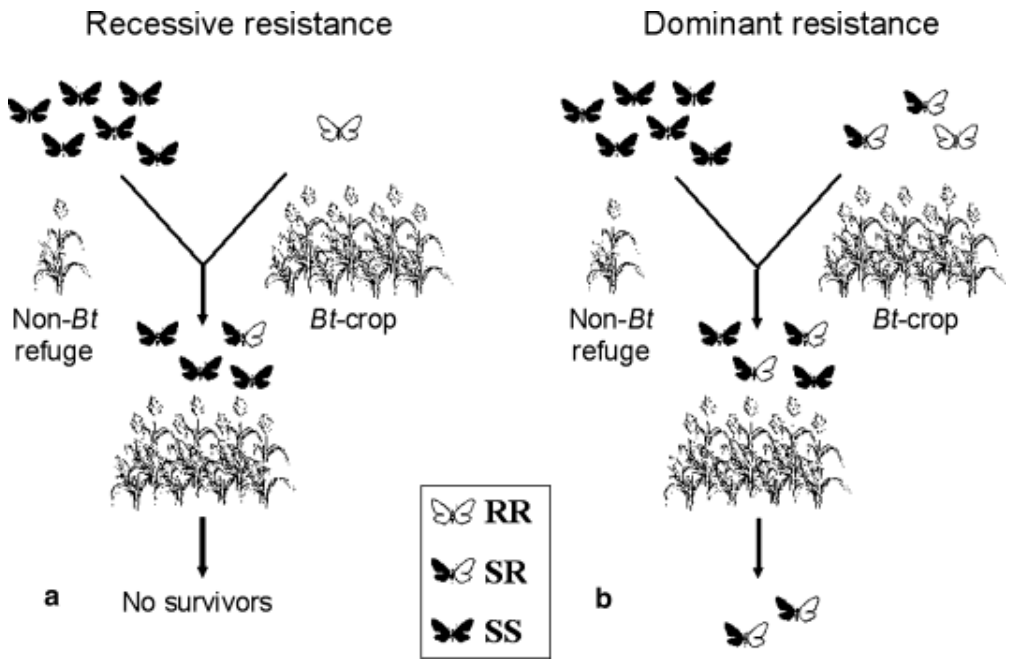


Figure 4 Schematic representation of the high dose/refuge strategy under two assumptions: resistance being recessive (a) or dominant (b). Butterflies represent adult survivors of different genotypes: RR, homozygous resistant; RS, heterozygous; SS, homozygous susceptible (from Ferré *et al*., 2008).

The use transgenic plant 'pyramids' producing two or more *Bt* toxins that kill the same pest is another strategy to delay the insect resistance (Bravo *et al.,* 2007). "Pyramided" *Bt* crops hold great promise and, in combination with the high dose/refuge strategy, will likely confer most protection to the *Bt* crop technology against insect resistance (Ferré *et al*., 2008).

The combination of *Bt* toxin and RNAi (to reduce expression of genes encoding proteins controlling essential functions of pests) in transgenic plant pyramids has a great potential, since it can have a synergic effect and can limit the selection of resistant populations (Ni *et al.,* 2017). More recently, a proof of concept has been provided of successful combination of RNAi-mediated silencing of immune genes and of *Bt* toxins, which enhances the lethality of the septicaemia due to the haemocoelic invasion of gut bacteria (Caccia *et al.,* 2020).

This latter study paves the way towards the development not only of new transgenic strategies, but also of new sprayable products, which require effective and safe delivery strategies of dsRNA.

1.6 Objectives

This PhD thesis aims to contribute to the definition of safe and efficient delivery strategies of dsRNAs targeting immune genes, as a tool for enhancing the killing activity of the entomopathogen *B. thuringiensis*.
The target genes are selected on the basis of studies on PDV associated with parasitic wasps. In particular, we will focus on an immune gene which is downregulated in parasitized hosts (*Sl 102*), and an immune gene which has been horizontally transferred from a PDV to a moth species (*Sl gasmin*). This is a new way of exploiting viral symbionts associated with natural antagonists as source of inspiration and genes to develop molecular biotechnologies mimicking and/or enhancing natural pest suppression mechanisms.

2 Enhancement of *Bacillus thuringiensis* toxicity by feeding *Spodoptera littoralis* larvae with bacteria expressing immune suppressive dsRNA

The aim of the first part of the present work was to develop a RNAi protocol for the delivery of dsRNA against *Sl 102* gene using recombinant bacteria as expression vector (Kim *et al.,* 2015).

2.1 MATERIALS AND METHODS

2.1.1 Insect rearing

Spodoptera littoralis strain is permanently lab-reared at the Department of Agricultural Sciences and derives from population collected on flower crops in Agro-Pontino (Latina, Italy). *S. littoralis* larvae were reared on artificial diet (41.4 g/l wheat germ, 59.2 g/l brewer's yeast, 165 g/l corn meal, 5.9 g/l ascorbic acid, 1.53 g/l benzoic acid, 1.8 g/l methyl 4-hydroxybenzoate and 29.6 g/l agar), at 25 ± 1 °C and 70 ± 5% RH, with 16:8 h light–dark period.

2.1.2 Hemocyte collection for 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 x g, at 4°C. After isolation, samples for RNA extraction were immediately put into TRIzol® reagent (Thermo Fisher Scientific) and kept at -80°C until total RNA extraction, that was performed according to manufacturer's instructions. The concentration of extracted RNA was assessed by measuring the absorbance at 260 nm, with a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific), and sample purity was evaluated assessing 260/280 nm absorbance ratio. RNA quality was checked by electrophoresis on 0.8% agarose gel.

2.1.3 *Sl 102* dsRNA *in vitro* synthesis

In order to synthesize *in vitro* a dsRNA targeting *Sl 102* gene (Accession Number KJ544881.1), total RNA was extracted from haemocytes of *S. littoralis* 6th instar larvae, retro-transcribed with the

Ambion ® RETROscript ® Kit (Thermo Fisher Scientific), and a 580 bp long *Sl 102* cDNA fragment was obtained by PCR (*Sl 102* F primer: TAC ATC CAA GTA AAT TTG CAA GGC; *Sl 102* R primer: GGC CCA GAA CAT TCT CAC CTC). 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-*Sl 102* F: TAA TAC GAC TCA CTA TAG GGA GAA CCT CCT GAG CGT GCC TGA; T7-*Sl 102* R: TAA TAC GAC TCA CTA TAG GGA GGG AGT GCT GCT TCA GAA TCA T). The resulting PCR product served as template to synthesize a dsRNA (469 bp long), using the Ambion ® MEGAscript ® RNAi Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Synthesized dsRNA was quantified by measuring its absorbance at 260 nm with a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific), and purity was evaluated by assessing 260/280 nm absorbance ratios. dsRNA was run on 1% agarose gels to check its integrity.
A *GFP* dsRNA, used in control experiments, was similarly produced starting from the cloning vector pcDNA® 3.1/CT-GFP TOPO ® (Thermo Fisher Scientific), which was used as template for a PCR reaction, performed with primers containing at their 5′ ends the T7 polymerase promoter sequence (T7-GFP F: TAA TAC GAC TCA CTA TAG GGA GAG TGG AGA GGG TGA AGGTG; T7-GFP R: TAA TAC GAC TCA CTA TAG GGA GGG GCA GAT TGT GTC GACAG). The resulting PCR product served as template to synthesize a dsRNA (531 bp long), as described above.

2.1.4 Cloning of *Sl 102* and transformation of bacteria for *Sl 102* dsRNA production

A L4440 recombinant vector, encoding *Sl 102* or *GFP* (negative control) dsRNA molecules, was produced with the Gateway® cloning technology and used to transform HT115 (Caccia *et al.,* 2020). The Gateway® cloning system enables the efficient transfer of DNA-fragments between plasmids, using a proprietary set of recombination sequences: the "Gateway® att" sites.
Total RNA extracted from *S. littoralis* haemocytes was subjected to retro-transcription (Ambion® RETROscript® Kit, Thermo Fisher Scientific) and, then, used for PCR amplification of *Sl 102*, with specific primers (*Sl 102* F: CAC CAA CCT CCT GAG CGT GCCT; *Sl 102* R: CGG AGT GCT GCT TCA GAA TC). A *GFP* fragment, used in control experiments, was amplified from the cloning vector pcDNA® 3.1/CT-

GFP TOPO® (Thermo Fisher Scientific), which served as template for a PCR reaction, using specific primers (*GFP* F: CAC CAG TGG AGA GGG TGA AGGTG; *GFP* R: GGG CAG ATT GTG TCG ACA G).
PCR products were ligated into the pENTR/D®-TOPO® vector (Thermo Fisher Scientific), compatible with the Gateway® technology, and the vector was introduced into chemically competent One Shot® TOP10 *E. coli* cells that were plated on LB agar (Lennox L Agar, Thermo Fisher Scientific). Plasmids from colonies grown overnight were extracted (Charge-Switch-Pro plasmid miniprep kit, Thermo Fisher) and sequenced. *Sl 102* and *GFP* fragments were cloned into a Gateway® - compatible L4440 vector, constructed by using the Gateway® vector conversion system, ligating a blunt-ended cassette containing *att*R sites flanking the *ccd*B gene and the chloramphenicol resistance gene. Cloning was performed using a transposition reaction catalyzed by the LR clonase ® enzyme (Thermo Fisher Scientific).
The resulting recombinant plasmids were introduced into competent *E. coli* HT115 cells for dsRNA overexpression. This strain is particularly suitable for the dsRNA overexpression because it lacks the RNase III gene, so that the expressed RNA molecules are less exposed to the risk of 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 (Newmark *et al.,* 2003; Timmons *et al.,* 2001; Timmons and Fire 1998).To produce dsRNA, the transformed bacteria were grown in the liquid LB (Luria Broth, Miller's LB Broth Base, Thermo Fisher Scientific) containing 100 µg/ml ampicillin and 12,5 µg/ml tetracycline at 37°C for 16 h under continuous shaking (250 rpm). Then, 5 ml of cultured broth was 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, for dsRNA overexpression, was induced by addition of 1 mM IPTG to transformed bacteria, whichwere incubated under continuous shaking at 37°C.
Bacteria producing dsRNA targeting *Sl 102* gene or producing *GFP* dsRNA are hereafter denoted as *Sl 102* dsRNA-Bac and *GFP* dsRNA-Bac, respectively. Bacterial cells were collected by centrifugation at 12,000×*g* for 1 min at 4 °C and suspended in phosphate buffered saline (PBS 1×, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4).

2.1.5 Pre-treatments of the transformed bacteria

To kill the bacteria to be used in all feeding bioassays and to facilitate the release of dsRNA, a sonication protocol was developed. Bacterial suspensions were subjected to an increasing number of sonication cycles on ice, in order to disrupt the cell wall and to facilitate the release of dsRNA in the insect gut, with an ultrasound homogeniser (Sonoplus, Bandelin), adopting decreasing time intervals between cycles. The bacteria viability after the treatments was evaluated by plating the resulting sonicated suspension on Petri dishes containing LB agar (supplied with 100 µg/ml ampicillin and 12.5 µg/ml tetracycline). Complete mortality was obtained with ten cycles of sonication (59 sec on/ 2 sec off, 95% amplitude).

2.1.6 qRT‑PCR absolute quantification of *Sl 102* dsRNA produced *in vivo*

The dsRNA produced by *E. coli* was extracted from cell pellets, using the protocol by Timmons *et al.,* (2001). The quantification was performed by quantitative real-time PCR using Applied Biosystems™ SYBR™ Green master mix (Thermo Fisher Scientific). The quantity of dsRNA was determined by relating its threshold value (*Ct*) values to an established standard curve, according to the absolute quantification method (Rutledge and Côté 2003).
The standard curve for *Sl 102* dsRNA was established by plotting the logarithm of six 10-fold serial dilutions of a starting solution containing 300 ng/ µl of L4440 Gateway® vector with insert, against the corresponding *Ct* value. The PCR efficiency (*E* = 98.274%) was calculated on the base of the slope and the coefficient of correlation (R^2) of the standard curve (slope = − 3.365, *y* intercept = 13.540, R^2 = 0.997), according to the following formula: $E = 10^{(-1/\text{slope})} - 1$. The standard curve for *GFP* dsRNA was similarly established, by plotting the logarithm of six 10-fold serial dilutions of a starting solution containing 200 ng/µl of L4440 Gateway® vector with insert, against the corresponding *Ct*. The PCR efficiency (*E* = 104.0477%) was calculated on the base of the slope and the correlation coefficient (R^2) of the standard curve (slope = − 3.229, *y* intercept = 17.650, R^2 = 0.984), according to the following formula: $E = 10^{(-1/\text{slope})} - 1$. All primer pairs were designed using Primer Express 3.0 software (Life Technologies), following the standard procedure. Negative controls (water) were included in each run of the qRT-PCR.

2.1.7 Oral administration of dsRNA to *S. littoralis* larvae

To assess the efficiency of dsRNA delivery through the use of sonicated bacteria, *S. littoralis* larvae were orally treated with *Sl 102* dsRNA-Bac, using two different protocols. In a first set of experiments, dsRNA-Bac was delivered by gavage with a microsyringe, as previously described (Di Lelio *et al.,* 2014; Caccia *et al.,* 2016). Briefly, newly molted *S. littoralis* 4^{th} instar larvae were anaesthetized on ice and 1 µl of *Sl 102* dsRNA-Bac (*GFP* dsRNA-Bac in controls) solution (corresponding to 45 ng of dsRNA) was poured into the lumen of the foregut by means of a Hamilton Microliter syringe (1701RNR 10 µl, gauge 26 s, length 55 mm, needle3). This treatment was repeated 3 times, at 24 h intervals. A group of larvae that received 1 µl of a solution of *Sl 102* dsRNA (45 ng/µl) synthesized *in vitro* (or *GFP* dsRNA in controls) acted as positive control, since this dose proved to be effective in the induction of gene silencing (Di Lelio *et al.,* 2014).
The second protocol was developed for feeding bioassays on artificial diet. Newly molted 4^{th} instar larvae were isolated in multi-well plastic trays (Bio-Rt-32, Frontier Agricultural Sciences), containing artificial diet, covered with perforated plastic lids (Bio-Cv-4, Frontier Agricultural Sciences), and maintained under the rearing conditions reported above. The experimental larvae, for 3 consecutive days, at 24 h intervals, were offered a small piece of diet with the upper surface (0.25 cm^2) uniformly overlaid with 1 µl of a solution of *Sl 102* dsRNA synthesized *in vitro* (45 ng/µl) or a *Sl 102* dsRNA-Bac suspension containing 45, 100 and 200 ng of dsRNA. Controls received *GFP* dsRNA synthesized *in vitro* or *GFP* dsRNA-Bac. Experimental larvae were maintained on artificial diet before and after the 3 administrations of dsRNA synthesized *in vitro* or of dsRNA-Bac suspension, which were overlaid on a small amount of the same diet, which was completely consumed in about 1 h. Silencing efficiency was evaluated by qRT-PCR 24 h after the last dsRNA administration, and the impact on immune competence was assessed by measuring the encapsulation index of injected chromatography beads (Di Lelio *et al.,* 2014 Becchimanzi *et al.,* 2020).

2.1.8 Expression profiles analysis of *Sl 102 gene by* qRT-PCR

Total RNA was extracted from haemocytes of *S. littoralis* larvae, using TRIzol® reagent (Thermo Fisher Scientific), according to

manufacturer's instructions. The concentration and purity of total RNA were determined using a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific).
The level of *Sl 102 gene* transcript was measured by one-step qRT-PCR using the Applied Biosystems™ SYBR™ Green master mix (Thermo Fisher Scientific), according to the manufacturer's instructions and as previously described (Di Lelio *et al*., 2014).
This was carried out using *Sl 102* gene-specific primers (*Sl 102* RT F: GGC GGT GTC GTC GTC GAT TAT G; *Sl 102* RT R: GAG CGA GGA AAT GTT CAA T), designed to detect a segment of the *Sl 102* mRNA external to the segment targeted by the dsRNA. The *S. littoralis β*-Actin gene (Accession Number Z46873) was used as an endogenous control for RNA loading (*β-actin* RT F: CGT CTT CCC ATC CAT CGT; *β-actin* RT R: CCT TCT GAC CCA TAC CAA CCA). All primers were designed using Primer Express, version 1.0 software (Applied Biosystems). The amount of target transcript relative to the endogenous control was determined using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001; Pfaffl 2001; Pfaffl *et al.,* 2002). For validation of the ΔΔ*Ct* method the difference between the *Ct* value of *Sl 102* and the Ct value of *β-Actin* transcripts [Δ*Ct* = *Ct* (*Sl 102*) - *Ct* (*β-actin*)] was plotted the log of 10 fold serial dilutions (5000, 500, 50, 5 and 0.5 ng) of the purified RNA samples. The plot of log total RNA input versus Δ*Ct* displayed a slope less than 0.1 (slope = 0.0154, R^2 = 0.0776), indicating that the efficiencies of the two amplicons were approximately equal.

2.1.9 Cellular immune response in *S. littoralis* larvae

The effect of gene silencing on the immunocompetence was assessed in 5th instars larvae by measuring the encapsulation index of injected chromatographic beads, a measure of immunosuppression induced by *Sl 102* silencing.
Encapsulation response was assessed as previously described (Di Lelio *et al.,* 2014; Becchimanzi *et al.,* 2020). Briefly, *S. littoralis* 5th instar 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, lenght 55 mm, needle 3) with 10 µl of PBS 1× 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 and placed in a climatic chamber. 24 hours after the injection the beads were recovered by dissection. Briefly, the larvae, anesthetized as described above, were 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 1× 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 E.I.) was expressed by using five levels in an arbitrary scale of encapsulation (Fig. 5), 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):

E.I. (%) = [Σ (encapsulation level x total beads of this level) total beads x 4] x 100

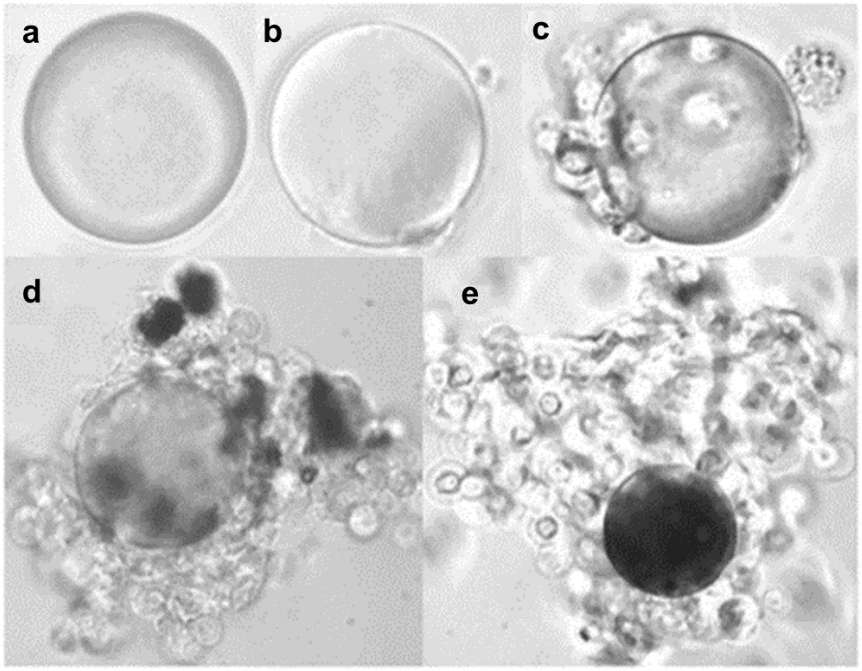


Figure 5. Encapsulation level: a) 0 (no cells adherent to the beads); b) 1 (up to 10 adherent cells); c) 2 (more than 10 adherent cells but with less than a complete layer); d) 3 (one or more complete layers without melanization); e) 4 (one or more complete layers with melanization).

2.1.10 Bioassays with Xentari™

The induction of effective immunosuppressive RNAi by *Sl 102* dsRNA-Bac prompted us to assess their potential in enhancing the efficacy of a Xentari™ (Valent BioSciences), a bioinsecticide based on *B. thuringiensis* subsp. *aizawai*, which contains several Cry toxins (Cry1Aa, Cry1Ab, Cry1Ca, Cry1Da, and Cry2Ab) and active on *Spodoptera* spp. To evaluate the impact of gene silencing on the virulence of the entomopathogen *B. thuringiensis*, three different bioassays were carried out to determine the impact of *Sl 102* gene silencing on the mortality of a commercial formulation of *Bt.*

Preliminary trials were performed in order to identify sublethal *Bt* doses (i.e., with no or very low effect on mortality and only moderately affecting the speed of larval development), which were 9 µg/cm^2 and 12 µg/cm^2 for 4th and 5th instar larvae, respectively (4 µg/cm^2, 9 µg/cm^2, 12 µg/cm^2 and 36 µg/cm^2 of Xentari™ were tested to identify the sublethal doses). The use of this *Bt* dose allowed the assessment of any increase in the mortality rate caused by the RNAi-induced immunosuppression. Xentari™ powder was dissolved in sterile distilled water.

In the first type of bioassay (sequential treatment), synchronous 4thinstar larvae were fed for 3 days with artificial diet overlaid with *Sl 102* dsRNA-Bac (or *GFP* dsRNA-Bac) (corresponding to 200 ng of dsRNA), as described above. Four hours after the administration of the last dsRNA dose, the experimental larvae, which in the meantime attained the 5th instar, were fed with a small piece of diet with the upper surface (1 cm^2) uniformly overlaid with a dose of 12 µg/cm^2 of Xentari™. This treatment with Xentari™ was repeated 3 times, at 24 h interval, and, since Xentari™ was suspended in water, control diet was overlaid with water.

A second bioassay was designed to evaluate the effect of the simultaneous administration of dsRNA and Xentari™, to better simulate field spraying with a product containing both components. Newly molted 4th instar larvae were fed with artificial diet overlaid with *Sl 102* dsRNA-Bac (or *GFP* dsRNA-Bac) (corresponding to 200 ng of dsRNA), as previously described, and, after 4 h, Xentari™ was administered at a dose of 9 µg/cm^2. This was done for 3 days. Controls were treated with water. The same experiment was performed with newly molted 5th instar larvae, using a dose of Xentari™ of 12 µg/cm^2. Mortality was daily recorded for 8 days, when the experimental larvae were weighed.

2.1.11 Statistical analysis

Data were analyzed using GraphPad Prism, version 6.0b. Encapsulation assay and *Sl 102* gene expression in gavage experiments were analyzed using the unpaired Student's *t* test, and larval weight was analyzed using One-Way ANOVA and Tukey's multiple-comparison *post hoc* test. When ANOVA assumptions were not fulfilled, non-parametric Kruskal–Wallis ANOVA followed by Dunn's multiple comparisons post-hoc test was used. *Sl 102* gene expression in feeding experiments was analyzed using Three-Way ANOVA to assess the effect of dsRNA treatment, production protocol and concentration. Levene's test was used to test the homogeneity of variance. When necessary, transformation of data was carried out to meet the assumptions of normality and homoscedasticity. When significant effects were observed ($P < 0.05$), the Bonferroni's *post hoc* test was used to compare mean values. Survival curves of *S. littoralis* larvae were compared using Kaplan–Meier and log-rank analyses. Normality of data was checked with Shapiro–Wilk test and Kolmogorov–Smirnov test, while homoscedasticity was tested with Levene's test and Bartlett's test.

2.2 RESULTS

2.2.1 Production of *Sl102*dsRNA-expressing bacteria

To produce bacteria expressing dsRNA, a partial sequence of *Sl 102* gene (or *GFP* in controls) was inserted into L4440 vector, using the rapid and highly efficient Gateway® recombinational cloning system (Landy 1989). Starting from the total haemocytes of *S. littoralis* larvae, where *Sl 102* gene is highly expressed, cDNA was prepared, performing a RT-PCR. The cDNA fragments (*Sl 102* and *GFP*), used for the dsRNA production, were amplified by PCR as reported above. To evaluate the success of the amplification and the quality of the PCR products, the fragments were loaded on agarose gel. The expected size of 469 bp (for *Sl 102*) and 531 bp (for *GFP*) were obtained.
The PCR product of the fragment of interest (*Sl 102* or *GFP* as control) was inserted in a donor vector to create the *att*L- containing entry clone. This latter has been used in a second recombination reaction with an *att*R-destination vector (L4440 vector properly converted into a Gateway® destination vector), to create an *att*B-containing expression clone used to transform HT115 *E. coli* cells (Fig. 6a). Production of dsRNA occurs thanks to *att*B site-specific attachment sites on *E. coli* chromosome, and dsRNA overexpression, under the T7 promoters, is induced by IPTG addition. The HT115 *E. coli* strain has been transformed with the recombinant vector containing the fragment of *Sl 102* gene to produce *Sl 102* dsRNA.
The selection of host cells containing the recombinant vector was carried out by plating 150 µl 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 above and, then, induced with isopropyl-β-D-1-tiogalattopiranoside (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 *Sl 102* sequence, and thus achieving the production of *Sl 102* dsRNA. At the end of the induction the OD_{600} was 1.2 (1×10^9 cells/ml). The amount of the dsRNA produced by bacteria (Fig. 6b) has been quantified by absolute qRT-PCR (Fig. 6c).

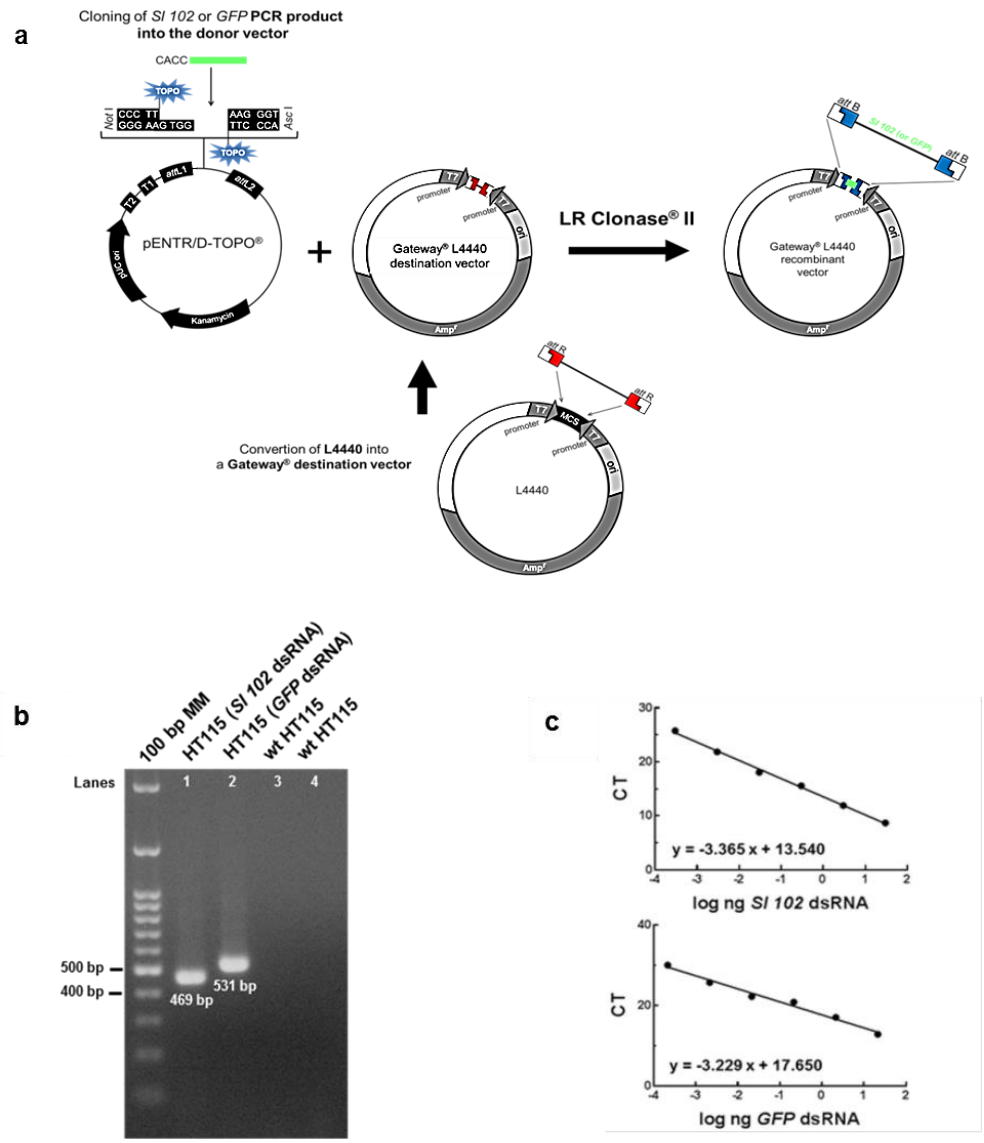


Figure 6. Production of HT115 *Escherichia coli* cells expressing dsRNA. a) Cloning protocol. b) Expression of dsRNA by transformed HT115 *E. coli*; total RNA samples were subjected to RT-PCR, and amplicons were resolved on agarose gel. Primers specific for *SI 102* or *GFP* genes produced amplicons of the expected size in HT115 *E. coli* expressing *SI 102* dsRNA or *GFP* dsRNA, respectively (lanes 1 and 2), whereas the same primers did not generate any amplicon when total RNA from non-transformed bacteria was used (wt HT115) (lanes 3 and 4). c) Calibration curves used for qRT-PCR absolute quantification of *SI 102* and *GFP* dsRNA present in *E. coli* suspensions used in the bioassays.

2.2.2 Bacteria inactivation

Sl 102 and *GFP* dsRNA-Bac produced were sonicated in order to disrupt the cell wall and to facilitate the release of dsRNA 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 (Fig. 7):

- 5 sonication cycles (10 sec on/ 15 sec off)
- 10 sonication cycles (10 sec on/15 sec off)
- 10 sonication cycles (59 sec on/2 sec off)

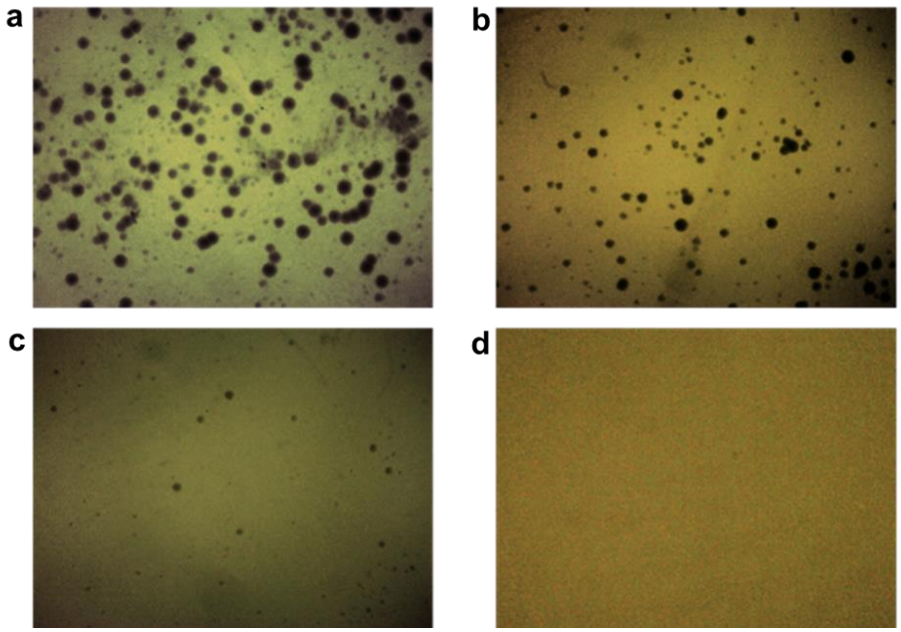


Figure 7. Survival of bacteria after different sonication cycle treatments. a) non-treated bacteria; b) 5 cycles (10 sec on/15 sec off); c)10 cycles (10 sec on/15 sec off); d)10 cycles (59 sec on/2 sec off).

The results showed lack of growth of bacteria treated with the 10 sonication cycles (59 sec on/2 sec off) (Fig. 7D) cycle. Prior to bioassay, transformed bacteria were thus inactivated applying this sonication

cycle. The use of killed bacteria is an essential requirement for their safe release in the environment.

2.2.3 Silencing efficiency of *Sl 102* dsRNA‑Bac

We first assessed RNAi efficiency and associated immunosuppression of *Sl 102* dsRNA-Bac by comparing their silencing effect with that induced by *Sl 102* dsRNA synthesized *in vitro*, adopting a protocol previously described (Di Lelio *et al.,* 2014). Thus, *Sl 102* dsRNA-Bac and *Sl 102* dsRNA produced *in vitro* (here after denoted as *Sl 102* dsRNA-synt) (*GFP* dsRNA-Bac and *GFP* dsRNA-synt were used as controls, respectively) were orally administered, for 3 days to 4th instar *S. littoralis* larvae, by gavage with a microsyringe. Since 45 ng/µl is the lowest dose of *Sl 102* dsRNA-synt inducing maximal down-regulation of *Sl 102* gene (Di Lelio *et al.,* 2014), an equal amount of dsRNA, measured by absolute qRT-PCR quantification was administered as *Sl 102* dsRNA-Bac. This experiment demonstrated that both dsRNA-synt and *Sl 102* dsRNA-Bac are associated with a significant level of silencing of the target gene compared to controls (Student's *t* test: for dsRNA-synt $t = 18.282$, $df = 28$, $P < 0.0001$, for dsRNA-Bac $t = 16.621$, $df = 28$, $P < 0.0001$) (Fig. 8), even though dsRNA-synt was by far more active than dsRNABac.

To explore whether the bacterial delivery of dsRNA confers protection against degradation, *Sl 102* dsRNA-Bac and *Sl 102* dsRNA-synt were overlaid on artificial diet and separately offered to *S. littoralis* larvae, in order to compare their silencing efficiency and immune suppressive activity, at different experimental doses. The transcription level of the target gene was significantly affected by the dsRNA treatment (Three-Way ANOVA: $F_{1,140} = 567.493$, $P < 0.0001$), exhibited a more pronounced down-regulation when dsRNA-Bac was used (Three-Way ANOVA: $F_{1,140} = 152.170$; $P < 0.0001$) and was positively correlated with the experimental dose used (Three-Way ANOVA: $F_{2,140} = 49.155$, $P < 0.0001$) (Fig. 8).

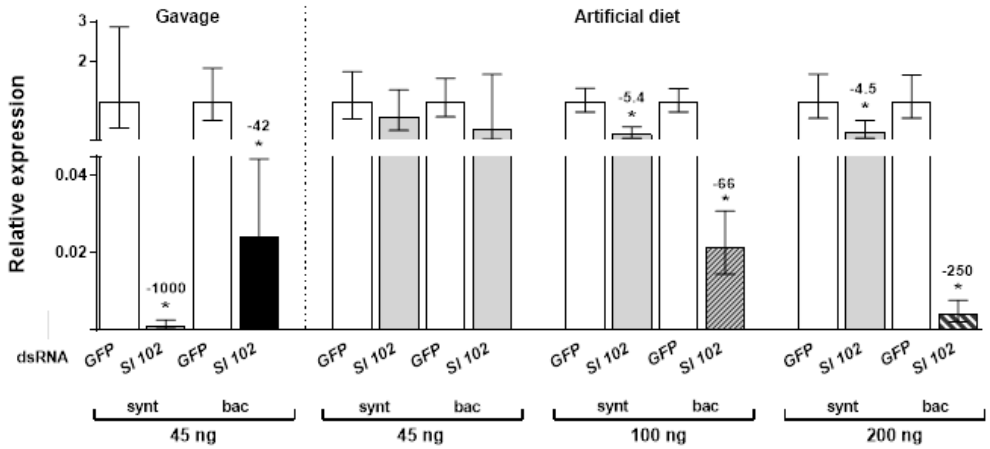


Figure 8. Transcript levels of *SI 102* gene in *S. littoralis* 4th instar larvae orally treated for 3 days with dsRNA. The *SI 102* gene was down-regulated upon ingestion of *SI 102* dsRNA administered by oral gavage, both in the case of dsRNA synthesized *in vitro* (*SI 102* dsRNA-synt) and suspensions of sonicated bacteria expressing *SI 102* dsRNA (*SI 102* dsRNA-bac). Delivery with artificial diet showed a silencing response that was dose-dependent and more pronounced when bacteria were used as delivery vectors. *GFP* dsRNA synthesized *in vitro* and bacteria expressing *GFP* dsRNA were used in control experiments. The values reported are the mean ± standard deviation (*$P < 0.0001$, Student's t test).

2.2.4 Immune suppressive effects of *SI 102* dsRNA-Bac

Since *SI 102* gene is involved both in the nodulation of microorganisms and in the encapsulation of large parasites, (e.g., parasitoid eggs, nematodes) (Falabella *et al.,* 2012; Di Lelio *et al.,* 2014; Caccia *et al.,* 2016), which are immune reactions sharing functional similarities (Lavine and Strand 2002), we used the encapsulation response against chromatography beads as a measure of immune suppression induced by *SI 102* silencing. After 12 h from the last administration of *SI 102* dsRNA-Bac and *SI 102* dsRNA produced *in vitro* by gavage with a microsyringe (*GFP* dsRNA-Bac and *GFP* dsRNA-synt were used as controls, respectively), the encapsulation assay was performed as described above. 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). Gene knockout was associated with a significant impairment of encapsulation response by

haemocytes of silenced larvae, for both types of dsRNAs (Student's *t* test: for dsRNA-synt $t = 118.64$, $df = 28$, $P < 0.0001$, for dsRNA-Bac $t = 63.508$, $df = 28$, $P < 0.0001$) (Fig. 9).
The encapsulation reaction showed a similar pattern of variation when *Sl 102* dsRNA-Bac and *Sl 102* dsRNA-synt were overlaid on artificial diet (Three-Way ANOVA: dsRNA treatment $F_{1,124} = 1350{,}724$, $P < 0.0001$; dsRNA production $F_{1,124} = 27.604$, $P < 0.0001$; dsRNA dose $F_{2,124} = 26.472$, $P < 0.0001$) (Fig. 9).

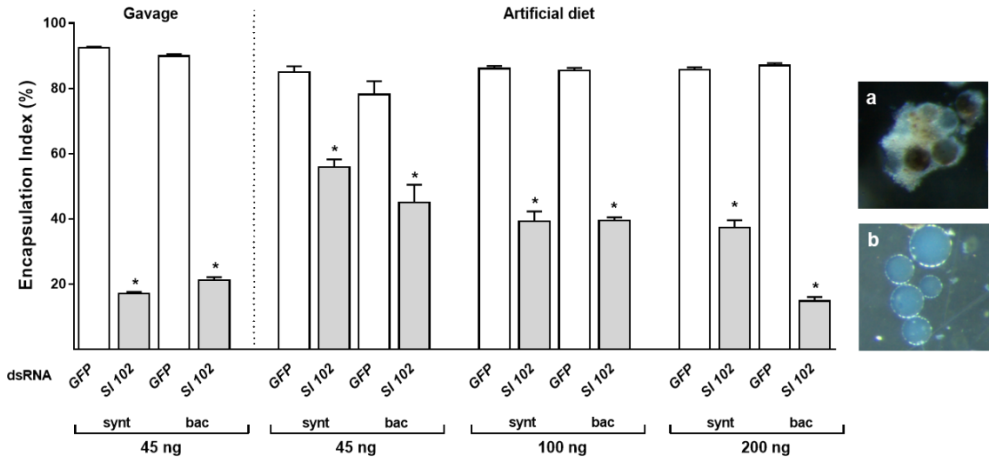


Figure 9. Encapsulation assay in *S. littoralis* 4th larvae treated for 3 days with *Sl 102* dsRNA synthesized *in vitro* (*Sl 102* dsRNA-synt) or transformed HT115 *E. coli* expressing *Sl 102* dsRNA (*Sl 102* dsRNA-bac). Chromatography beads injected into the body cavity of control larvae were encapsulated and melanized (a). On the contrary, the efficiency of encapsulation was lower in silenced larvae, independently from the dsRNA administration method (gavage or with artificial diet) (b). The encapsulation index was affected by oral delivery method and, in the case of oral administration on artificial diet, by dsRNA quantity. *GFP* dsRNA synthesized *in vitro* and bacteria expressing *GFP* dsRNA were used in control experiments. The values reported are the mean ± standard errors (*$P < 0.0001$, Student's t test).

2.2.5 *Sl 102* dsRNA-Bac enhance the killing activity of *B. thuringiensis*

The induction of effective immune suppressive RNAi by *Sl 102* dsRNA-Bac prompted us to assess their potential in enhancing the efficacy of a *Bt*-based biopesticide (Xentari™).
In a first set of experiments (sequential treatments), 4th instar *S. littoralis* larvae were fed with artificial diet overlaid with *Sl 102* dsRNA-Bac for 3 days, as described above for gavage experiments. Four hours after the last dsRNA treatment, Xentari™ was administered to larvae with the artificial diet for 3 subsequent days. Xentari™ induced a significantly higher mortality only in larvae fed with *Sl 102* dsRNA-Bac (log-rank test: $\chi^2 = 172.3$, $P < 0.0001$, $df = 3$) (Fig. 10a) and determined a significant weight reduction in the surviving larvae (Kruskal–Wallis: KW = 95.08; $P < 0.0001$) (Fig. 10b), which completely failed to pupate.
A second set of experiments was performed to test the efficacy of the simultaneous administration of *Sl 102* dsRNA-Bac and Xentari™. This experiment was designed to reproduce more closely the possible effects of a field application of both active ingredients (dsRNA and *Bt*). The results obtained, both with 4th and 5th instars larvae, clearly showed that simultaneous administration of *Sl 102* dsRNA-Bac and Xentari™ caused a significantly higher mortality in *Sl 102*-silenced larvae compared to controls (Figs. 11a, 12a) (log-rank test 4th instar larvae: $\chi^2 = 49.02$; $df = 3$; $P < 0.0001$; log-rank test 5th instar larvae: $\chi^2 = 156.6$; $df = 3$; $P < 0.0001$) and had a significant impact on body weight both of 4th instar (Kruskal–Wallis: KW = 65.96; $P < 0.0001$) and 5th instar larvae (Kruskal–Wallis: KW = 135.1; $P < 0.0001$) (Figs. 11b, 12b), which completely failed to pupate.

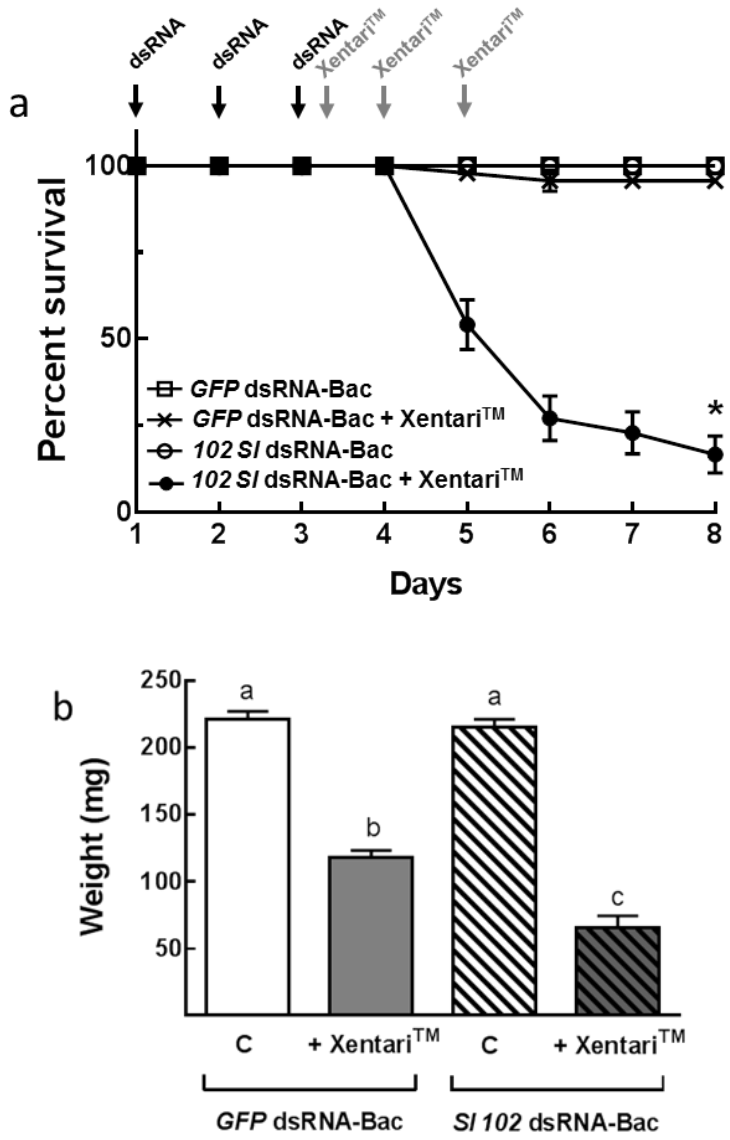


Figure 10 Bioassay with *S. littoralis* 4th instar larvae exposed to dsRNA before *Bt* treatment. Newly molted larvae were treated for 3 days with artificial diet layered with transformed HT115 *E. coli* expressing *SI 102* dsRNA (*SI 102* dsRNA-Bac, corresponding to 200 ng of dsRNA) and then with 12 µg/cm^2 of Xentari™ for 3 more days. Survival was monitored until day 8 (a), when the weight was assessed on the surviving experimental larvae (b). Bacteria expressing *GFP* dsRNA were used in control experiments. The timing of the treatments is indicated with arrows. The values reported are the mean ± standard errors (in a *P < 0.0001 based on log-rank test; in b different letters denote statistical difference based on Kruskal–Wallis test).

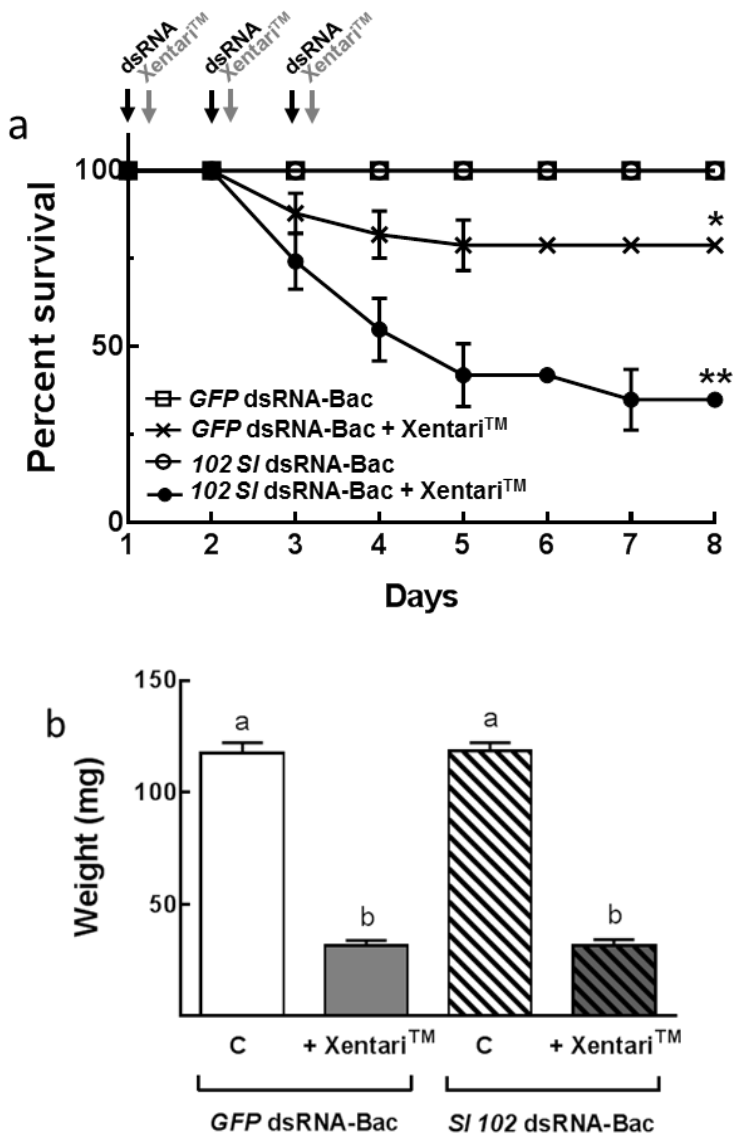


Figure 11. Bioassay with *S. littoralis* 4th instar larvae simultaneously exposed to dsRNA and *Bt*. Newly molted larvae were treated for 3 days with artificial diet layered with transformed HT115 *E. coli* expressing *SI 102* dsRNA (*SI 102* dsRNA-Bac, corresponding to 200 ng of dsRNA) and with 9 µg/cm^2 of Xentari™. Survival was monitored until day 8 (a) when the weight was assessed on the surviving experimental larvae (b). Bacteria expressing *GFP* dsRNA were used in control experiments. The timing of the treatments is indicated by arrows. The values reported are the mean ± standard errors (in a $^{**}P < 0.0001$ and $^{*}P < 0.0046$ based on log-rank test; in b different letters denote statistical difference based on Kruskal–Wallis).

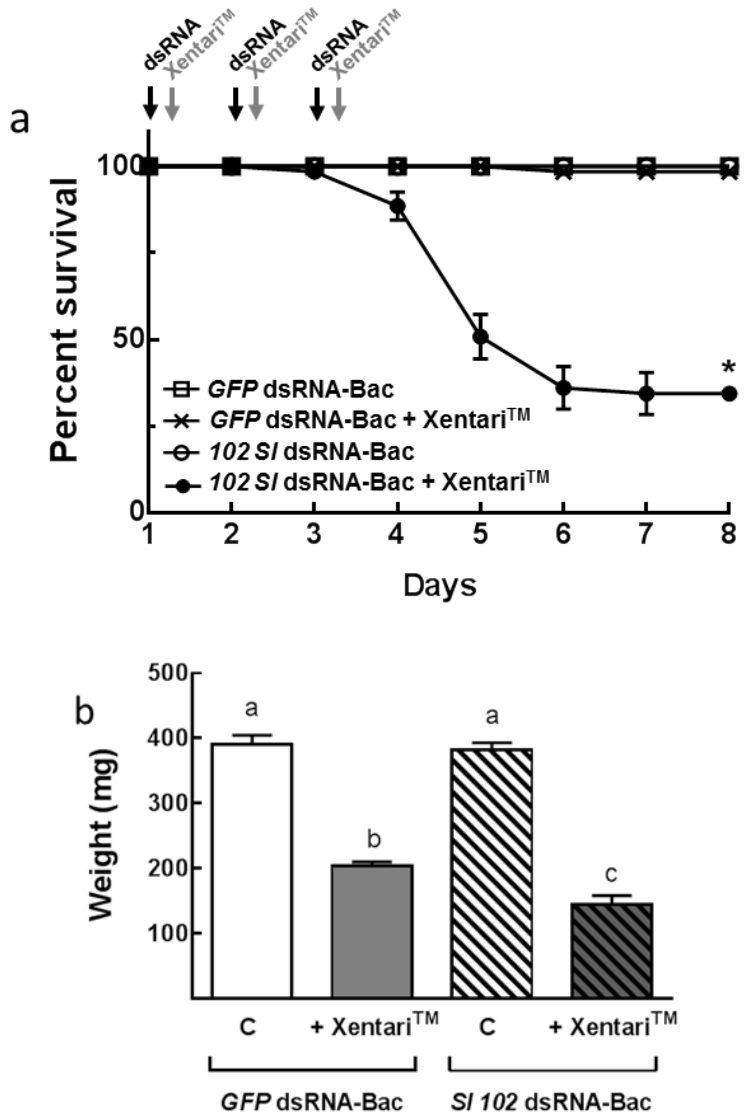


Figure 12. Bioassays with *S. littoralis* 5th instar larvae simultaneously exposed to dsRNA and *Bt*. Newly molted larvae were treated for 3 days with artificial diet layered with transformed HT115 *E. coli* expressing *SI 102* dsRNA (*SI 102* dsRNA-Bac, corresponding to 200 ng of dsRNA) and with 12 µg/cm^2 of Xentari™. Survival was monitored until day 8 (a), when the weight was assessed on the surviving experimental larvae (b). Bacteria expressing *GFP* dsRNA were used in control experiments. The timing of the treatments is indicated by arrows. The values reported are the mean ± standard errors (in a *P < 0.0001 based on log-rank test; in b different letters denote statistical difference based on Kruskal–Wallis).

3 Enhancement of *Bacillus thuringiensis* toxicity by feeding *Spodoptera littoralis* larvae with transgenic tobacco plants expressing dsRNA

Because the feeding substrate can influence the gut profile of digestive enzymes and the resulting degradation to which ingested dsRNAs are exposed, we wanted to evaluate the efficacy of *Sl* 102 dsRNA when produced by transgenic plants, which represent a natural food source.

3.1 MATERIALS AND METHODS

3.1.1 Production of transgenic tobacco plants expressing *Sl 102* dsRNA

Tobacco (*Nicotiana tabacum* L., 'Samsun' NN) provided by La Semiorto Sementi srl© (Sarno, Italy) was transformed as previously described (Corrado *et al.,* 2016). The binary vector carrying *Sl 102 dsRNA* was produced using the Gateway® tecnology. Explants of *Nicotiana tabacum,* from healthy fully expanded leaves (4-5 week-old tissue), were co-cultivated with a suspension of *Agrobacterium tumefaciens* cells. A total of 100 explants from 25 leaves were co-cultivated and selected on Murashige Skoog medium supplemented with 30 g/l of sucrose and 50 mg/l kanamycin. From putatively transformed explants, a total of 90 green calli were obtained, followed by 70 regenerated shoots. The emerging shoots showed root formation in one month. Putative transgenic plants were transferred in sterile soil and grown in controlled conditions (T0 generation). Second generation plants (T1) were identified by successive rounds of selection in the same medium and molecular analysis.

Molecular selection of transgenic plants was performed by PCR followed by qRT-PCR, using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001), to check *Sl 102* dsRNA expression level on T1 generation, using as calibrator a genotype with a low level of transgene expression. The housekeeping gene *EF-1α* was used as an endogenous reference gene for the normalization of the expression levels of the target genes. The genotype that showed, in a preliminary analysis, the lowest level of expression of the transgene was used as calibrator.

3.1.2 Insect rearing and preparation of experimental larvae for feeding bioassays

Spodoptera littoralis larvae were reared on artificial diet, as previously described, at 25 ± 1°C, 70 ± 5% R.H., and under a 16:8 h light/dark period.
All feeding bioassays on plant tissues described below were carried out in triplicate under the same environmental conditions, using *S. littoralis* larvae fasted during molting previously maintained on sub-apical leaves of 4 weeks-old wild-type (WT) tobacco plants in plastic boxes (30x40x15), bottom lined with 50 ml of 1,5% (w/v) agar agar in water to create a moist environment required to keep turgid the experimental tomato leaf disks.
The experimental larvae were daily checked and those molting within 4 h formed synchronized groups that were fed with leaf disks from the WT tomato plants and from two transformed lines showing high expression levels of the transgene.

3.1.3 Time course of *Sl 102* gene silencing and immune suppression

Different time course experiments were carried out on *S. littoralis* larvae fed with transgenic plant leaves expressing *Sl 102* dsRNA, in order to assess the level of silencing of the targeted gene and to evaluate if it was possible to reproduce the immunosuppressed phenotype induced by *Sl 102* dsRNA synthesized *in vitro* (Di Lelio *et al.,* 2014) and *Sl 102* dsRNA-Bac (Caccia *et al.,* 2020). All the described experiments were carried out in plastic boxes, prepared as described above. Briefly, newly hatched *S. littoralis* larvae were fed on WT tobacco leaves for 12 h. Then, 1st instar larvae were grouped (100 larvae per box) and fed with tobacco leaves and kept under the same environmental conditions reported above. Larvae attaining the end of the 3rd instar (just before moulting) were daily selected, transferred into additional boxes (25 larvae per box), in order to prevent cannibalism, and reared until pupation on experimental tobacco leaves.
In the first time-course analysis, leaf material from transgenic plants was offered throughout the development, from 1st instar larvae until pupation. Fully grown 2nd, 3rd, 4th and 5th instars, ready to moult, first day 6th instars and prepupae, i.e., 24 h after feeding cessation, alimented with WT and transgenic tobacco plants, were collected into

TRIzol® reagent (Thermo Fisher Scientific) and the whole body processed for RNA extraction, according to manufacturer's protocol.
In the second time course experiment, feeding on transgenic leaves started only when the experimental larvae attained the 4th instar, in order to assess the impact of the feeding duration on gene silencing. In this case, RNA was extracted from haemocytes, as described elsewhere (Di Lelio *et al*., 2019), obtained from the same developmental stages indicated above, starting from fully grown 4th instars.
For a finer evaluation of the minimal duration of feeding required to induce gene silencing, the same experiment was performed again and the samples were collected 14 h, 24 h, 38 h, 48 h, 62 h, 72 h after moulting into the 4th instar (Di Lelio *et al.,* 2019).
Silencing efficiency and its impact on cellular immune response were assessed as described above.

3.1.4 *Sl 102* silencing by transgenic tobacco and effects on *Bt* killing activity

The impact of *Sl 102* gene silencing on *B. thuringiensis* killing activity was assessed by feeding bioassays on *S. littoralis* larvae, as described below.
For the bioassays with *Bt* bioinsecticide, synchronous 4th and 5th larvae, reared on WT tobacco plants or on the 2 transgenic lines, were obtained as described above, and singly transferred into multi-well plastic rearing trays (RT32W, Frontier Agricultural Sciences, United States), bottom-lined with 1 ml of 1.5 % (w/v) agar in water, to keep turgid the leaf disks. The rearing wells, each containing a leaf disk and a larva, were closed by perforated plastic lids (RTCV4, Frontier Agricultural Sciences, United States). The leaf disks were uniformly sprayed with Xentari™ (Valent BioSciences), a bioinsecticide based on *Bt* subsp. aizawai, which contains several Cry toxins (Cry1Aa, Cry1Ab, Cry1Ca, Cry1Da, and Cry2Ab), active on different *Spodoptera* species. Experimental tobacco leaves sprayed with distilled water were used as controls. Preliminary bioassays to determine the sub-lethal doses of Xentari™ in controls (larvae fed on WT tobacco) were performed. Sub-lethal doses were 1 µg/cm^2 and 3 µg/cm^2, for 4th and 5th instar larvae, respectively.
In the first bioassay, newly moulted 4th instar larvae were fed *ad libitum* on WT and transgenic leaves for 3 days. Then, experimental larvae were singly transferred in the multi-well plastic rearing trays prepared as described above and fed *ad libitum* for 3 days with leaves of each

tobacco plant line treated with Xentari™ or water in controls. Mortality was daily recorded from the beginning of the bioassay for 8 days and the weight of the surviving larvae recorded on day 8.
Two additional bioassays were carried out on 4th and 5th instars larvae respectively, to mimic the effect of a *Bt* spray on incipient populations under field conditions. These bioassays were carried out by feeding with 4th instar or 5th instar larvae on leaves pieces from each experimental tobacco plant lines for 24 h (the minimum time interval of feeding required to induce a significant level of gene silencing) and then treated with *Bt* for the following 3 days. Mortality was daily recorded from the beginning of the bioassay for 6 days and the weight of the surviving larvae recorded on day 6.

3.1.5 Statistical analysis

Sl 102 dsRNA expression in tobacco transgenic lines was analyzed using the unpaired Student's *t* test. *Sl 102* gene expression in larvae and the encapsulation assay data were analyzed using One-Way ANOVA followed, by Tukey's multiple-comparison *post hoc* test. Normality of data was checked by using the Shapiro-Wilk test and the D'Agostino-Pearson test, while homoscedasticity was checked with Bartlett's test. When ANOVA assumptions were not fulfilled (as for larval weight), nonparametric Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons post-hoc test was used. Survival curves of *S. littoralis* larvae were compared using Kaplan-Meier log-rank analysis. All data were analyzed using GraphPad Prism, version 6.0b.

3.2 RESULTS

3.2.1 Expression level of *Sl 102* dsRNA in transgenic tobacco plants

The two selected transgenic lines, used for the experiment on *S. littoralis* larvae, showed a significant increase in the level of transgene expression, which resulted 14 (Student's *t*-test: $t = 8.156$, $df = 10$, $P < 0.001$), and 11-fold higher (Student's *t*-test: $t = 12.20$, $df = 10$, $P < 0.001$), in the line 1 and 2, respectively, compared with the line showing the lowest level of transgene expression (calibrator) (Fig.13).

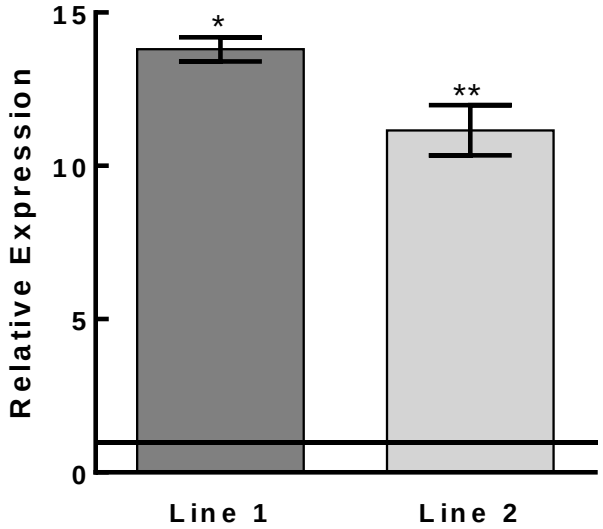


Figure 13. Transcriptional analysis of *Sl 102* dsRNA by qRT-PCR on transgenic lines. The two transgenic plant lines showing the highest expression of *Sl 102* dsRNA were used in feeding bioassays (*P = 0.0013; **P = 0.000265, Student's *t* test). The broken line represents the calibrator (i.e. the line showing the lowest level of transgene expression).

3.2.2 Silencing effects of *Sl 102* dsRNA transgenic plants

The time-course analysis experiments demonstrate that a significant reduction of the *Sl 102* transcript levels can be achieved in *S. littoralis* larvae fed with the two tested transgenic tobacco plants expressing dsRNA *Sl 102* (Fig. 14a and 14b).

When the larvae were fed precociously with leaves of tobacco transgenic plants (from 1st instar larvae), a significant level of gene silencing was observed only when the experimental larvae attained the 4th instar (last day) (One Way ANOVA: $F_{2, 23} = 659.3$, $P < 0{,}0001$) (Fig. 14a). This reduction of *102 Sl* transcription rate was consistently observed throughout the remaining part of the bioassay, up to the prepupal stage (One Way ANOVA: 5th instar - $F_{2, 26} = 488.5$, $P < 0{,}0001$; 6th instar - $F_{2, 23} = 1114$, $P < 0{,}0001$; prepupal stage - $F_{2, 24} = 89.00$, $P < 0.0001$) (Fig. 14a).

The obtained results suggest that the level of gene silencing may be influenced by the amount of leaf tissue consumed, which is very low until the start of the more intense feeding activity with the onset of the 4th instar.

To check this hypothesis, we performed the feeding bioassay starting with 4th instar larvae. The occurrence of a significant level of gene silencing was already observed at the end of the 4th instar and was recorded for all the following time points considered (One Way ANOVA: 4th instar- $F_{2, 82} = 124.8$, $P < 0.0001$; 5th instar - $F_{2, 81} = 240.6$, $P < 0.0001$; 6th instar - $F_{2, 56} = 665.6$, $P < 0.0001$; prepupal stage - $F_{2, 44} = 187.6$, $P < 0.0001$) (Fig. 14b).

Moreover, in the third experiment, gene silencing was measured in detail during the three days of 4th instar, demonstrating that, despite larvae do not feed during the previous instars (1st, 2nd and 3rd instars larvae) the silencing effect was persistent (Fig. 15). The reduction of gene transcript level increased over time during the 3 days of 4th instar and was significant already after 24 h of exposure to tobacco transgenic plants. (14h-Kruskall-wallis: KW = 10.24, $P = 0.006$; 24h-One-Way ANOVA: $F_{2,52} = 229.7$; $P < 0.0001$; 38h-Kruskall-wallis: KW = 42.94; $P < 0.0001$; 48h-Kruskall-wallis: KW = 42.34; $P < 0.0001$; One-Way ANOVA: 62h-One-Way ANOVA $F_{2, 56} = 1733$; $P < 0.0001$; One-Way ANOVA: 72 h- $F_{2, 57} = 645.5$; $P < 0.0001$)

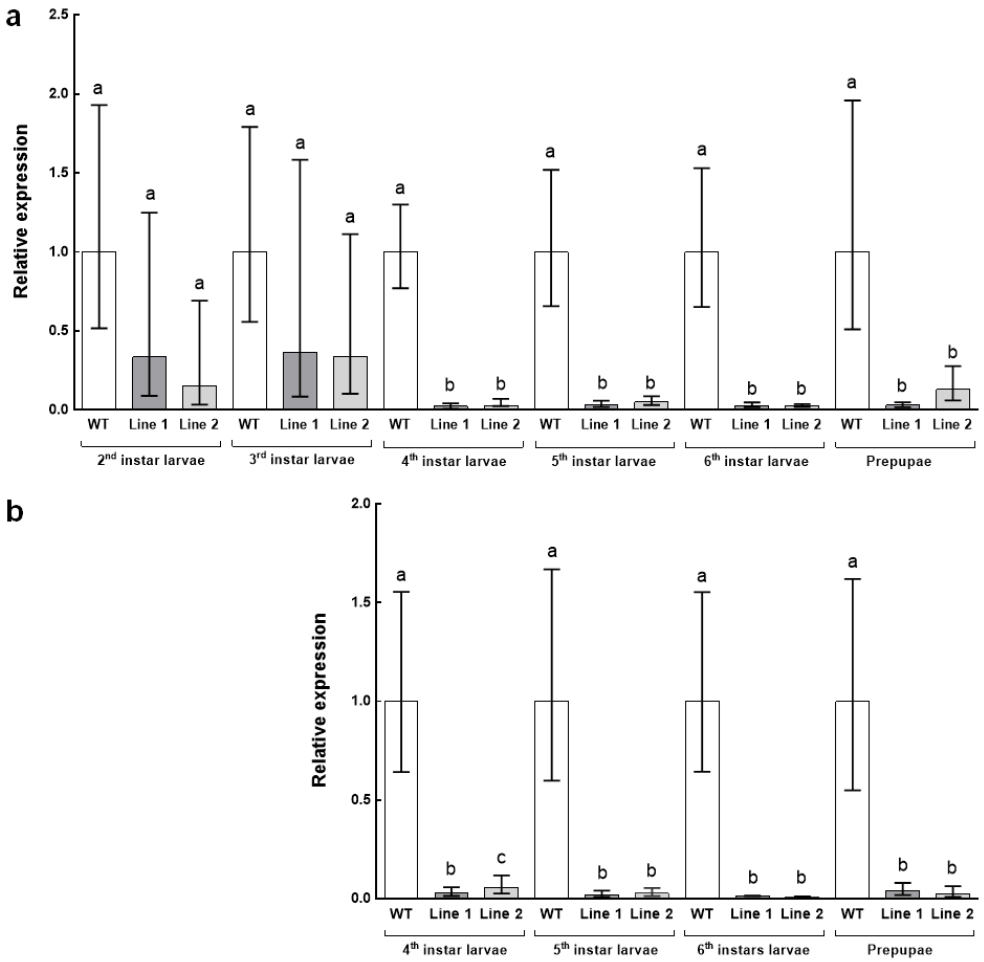


Figure 14. Gene silencing in *S. littoralis* larvae reared on wild type (WT) (controls) or *Sl 102* dsRNA-expressing (Line 1 and Line 2) tobacco plant leaves. Larvae were maintained on wild type tobacco (WT) and fed throughout the development to pupal stage with transgenic plant leaves, starting from 1st (a) or 4th instar (b). Control larvae were reared on WT tobacco leaves. The comparison of the mean values is performed within each developmental instar, and values statistically different are denoted with different letters ($P < 0.0001$, One Way ANOVA). Error bars represent the standard deviation of the mean.

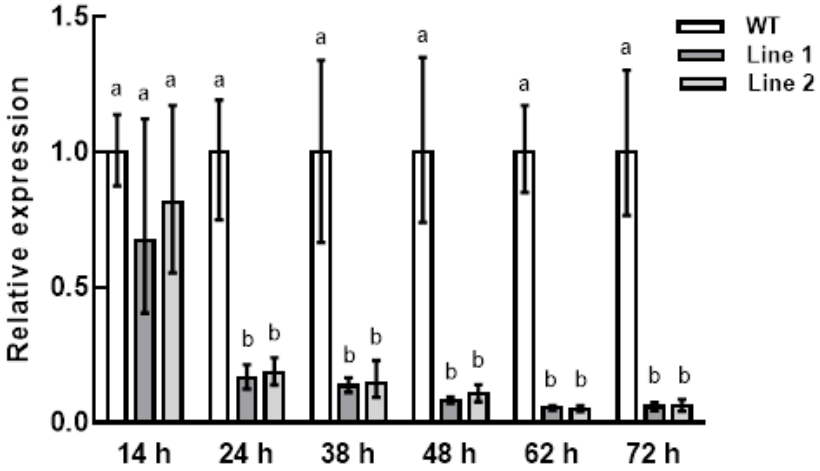


Figure 15. Gene silencing in *S. littoralis* 4th instar larvae reared on wild type (WT) (controls) tobacco plant leaves or expressing *Sl 102* dsRNA (Line 1 and Line 2). Larvae were maintained on wild type tobacco (WT) and fed with transgenic plant leaves immediately after the moult into 4th instar. Control larvae were reared on WT tobacco leaves. The comparison of the mean values is performed within each time, and values statistically different are denoted with different letters ($P < 0.0001$, One Way ANOVA). Error bars represent the standard deviation of the mean.

3.2.3 Immunosuppressive effect of *Sl 102* dsRNA-expressing transgenic tobacco plants

The level of *Sl 102* silencing observed was expected to induce an immune suppressed phenotype, characterized by the impairment of cellular immune response by haemocytes (Falabella *et al.,* 2012; Di Lelio *et al.,* 2014; Caccia *et al.,* 2016; 2020).
Indeed, encapsulation and melanization reactions in silenced 5th instar larvae were significantly affected compared with those observed in control larvae fed with WT tobacco (One Way ANOVA: $F_{2, 112} = 4568$; $P < 0.0001$) (Fig.16). In particular, 24 hours after their haemocoelic injection, chromatography beads recovered from the haemocoel of control larvae were completely encapsulated and melanized by haemocytes (E.I. = 87.0 %), while encapsulation response totally failed in larvae fed with the transgenic plant Line 1 (E.I. = 16.7 %,) and Line 2 (E.I. = 18.0 %) leaves (Fig. 16).

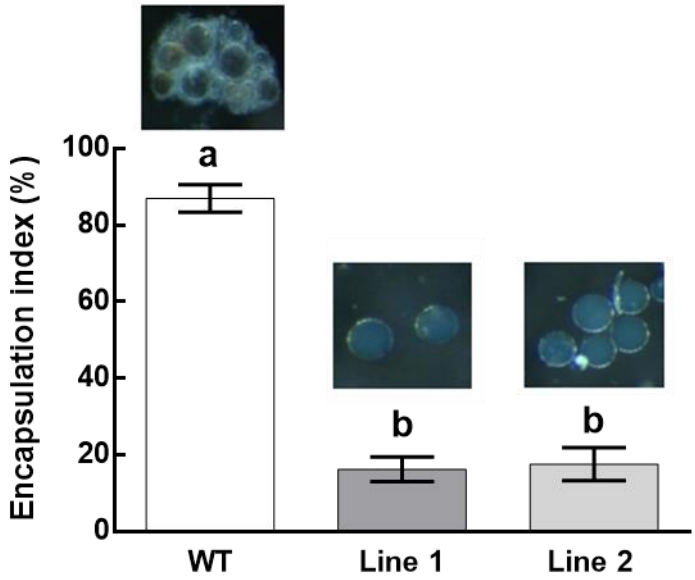


Figure 16. Encapsulation index in *S. littoralis* larvae reared on wild type (WT) (controls) or *Sl 102* dsRNA-expressing (Line 1 and Line 2) tobacco plant leaves. Encapsulation response was significantly inhibited in *S. littoralis* larvae showing *Sl 102* silencing. Chromatography beads collected from the haemolymph of control larvae (WT) were completely encapsulated while haemocyte capsule formation was not observed in larvae fed with transgenic plant lines. The values reported are the mean ± standard errors. Mean values denoted with different letters are significantly different ($P < 0.0001$, One Way ANOVA) .

3.2.4 Sl 102 dsRNA-transgenic plants enhance the killing activity of *B. thuringiensis*

The high efficiency of transgenic plants in the induction of *Sl 102* silencing and immune suppression prompted us to assess if silenced *S. littoralis* larvae were more susceptible to a treatment with a *Bt*-based biopesticide (Xentari™), as previously reported for different delivery methods of *Sl 102* dsRNA (Caccia *et al.,* 2016; 2020).
In a first set of experiments (sequential treatments), 4th instar *S. littoralis* larvae were alimented on leaf disks of transgenic plants expressing *Sl 102* dsRNA and on day 4 were exposed with Xentari™ for three subsequent days and maintained on transgenic leaf tissues until day 8. Xentari™ induced significantly higher levels of mortality in larvae fed with *Sl 102* dsRNA-transgenic plants (Log-rank test: $\chi^2 = 271.5$, $df = 5$, $P < 0.0001$) compared to controls (Fig. 17a). Surviving larvae showed a significant reduction of weight increase (One-Way ANOVA, $F_{5,199} = 448.7$, $P < 0.0001$;) (Fig. 17b).
Based on the results obtained above, showing that after 24 hours of feeding on transgenic plants a significant level of gene silencing is observed in 4th instar larvae, we started the *Bt* treatment on leaf disks for 3 consecutive day, starting 24 h after the onset of the bioassay. Both with 4th and 5th instar larvae, the administration of *Sl 102* dsRNA-transgenic plants and Xentari™ caused significantly higher mortality in *Sl 102*-silenced larvae compared to controls (Figures 18a and 19a) (Log-rank test - 4th instar larvae: $\chi^2 = 235$, $df = 5$, $P < 0.0001$; 5th instar larvae: $\chi^2 = 230.2$, $df = 5$, $P < 0.0001$). This was accompanied by a significant development impairment both for 4th instar (Kruskall-Wallis: KW = 119.0, $P < 0.0001$) (Fig. 18b) and 5th instar larvae (Kruskall-Wallis: KW = 135.1, $P < 0.0001$) (Fig. 19b).

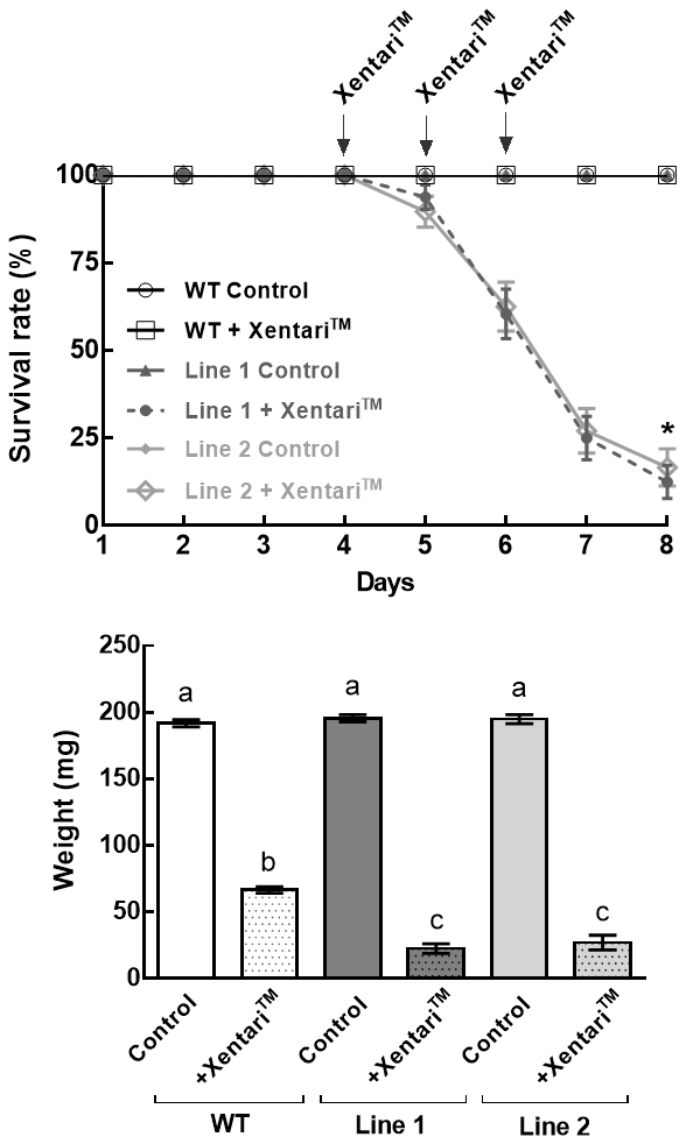


Figure 17. Bioassay with *S. littoralis* 4th instar larvae fed with *Sl 102* dsRNA-transgenic plants before *Bt* treatment. Newly molted 4th instar larvae were alimented for 3 days with leaf disks of the experimental tobacco plants and then, soon after molting in 5th instar, with 3 µg/cm^2 of Xentari™ for 3 additional days. Survival was monitored until day 8 (a), when the weight was assessed on the surviving experimental larvae (b). Experimental lines treated only with water were used in control experiments. The timing of the treatments is indicated with arrows. The values reported are the mean ± standard errors: in (a) the asterisk denote a statistical difference ($P < 0.0001$, Log-rank Mantel-Cox test); in (b) different letters indicate a statistical difference ($P < 0.0001$, Kruskal–Wallis test)

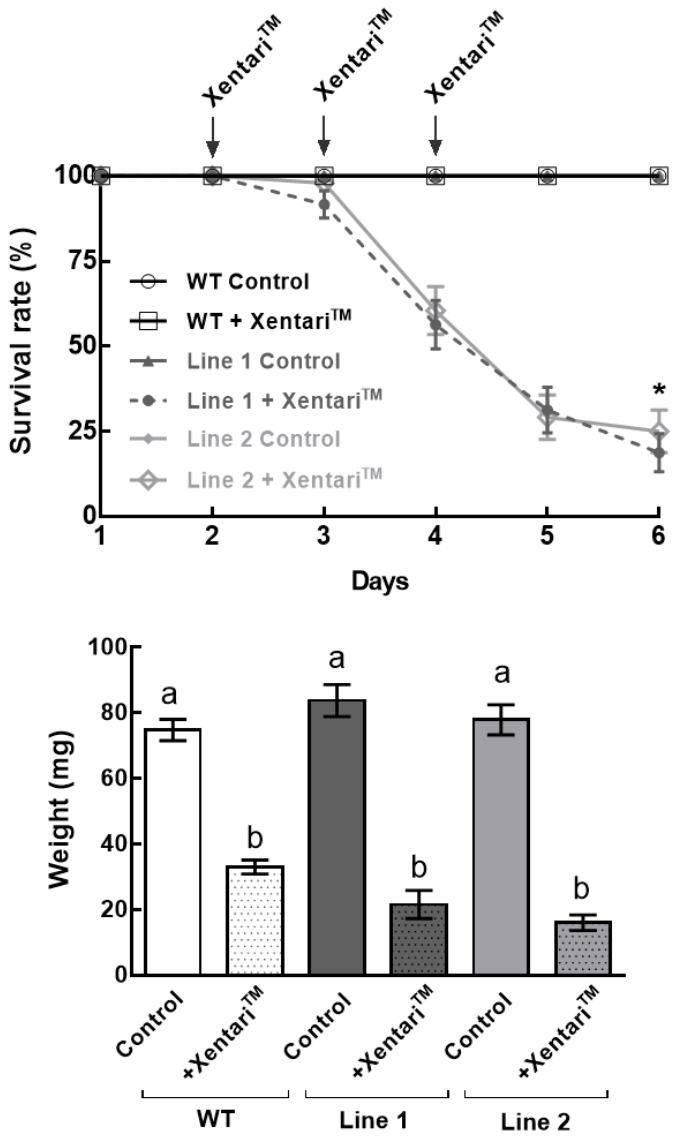


Figure 18. Bioassay with *S. littoralis* 4th instar larvae concurrently exposed to dsRNA and *Bt*. Newly moulted larvae were alimented for 24 h with no treated experimental plant leaves and, for following 3 days, with leaf disks sprayed with 1 µg/cm^2 of Xentari™. Survival was monitored until day 6 (a) when the weight was assessed on the surviving experimental larvae (b). The experimental leaves sprayed with water were used as control. The timing of the treatments is indicated by arrows. The values reported are the mean ± standard errors: in (a) the asterisk denote a statistical difference ($P < 0.0001$, Log-rank Mantel-Cox test); in (b) different letters indicate a statistical difference ($P < 0.0001$, Kruskal–Wallis test).

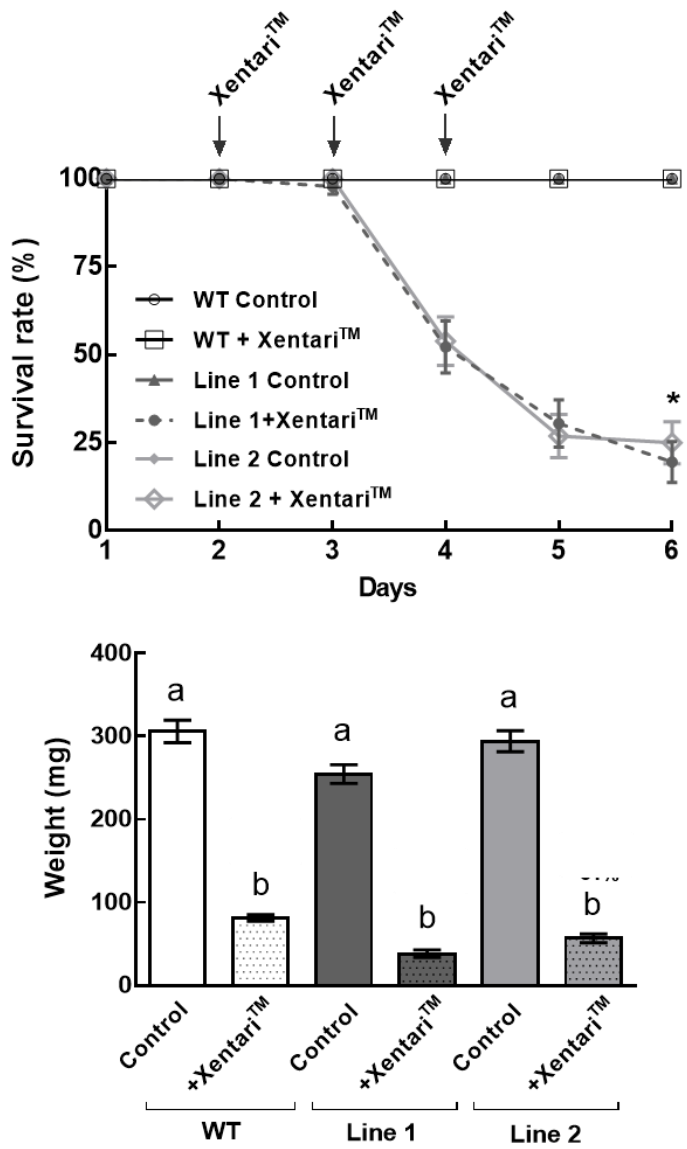


Figure 19. Bioassays with *S. littoralis* 5t^{h} instar larvae concurrently exposed to dsRNA and *Bt*. Newly moulted larvae were alimented for 24 h with no treated experimental plant leaves and, for following 3 days, with leaf disks sprayed with 3 µg/cm^2 of Xentari™. Survival was monitored until day 6 (a) when the weight was assessed on the surviving experimental larvae (b). The experimental leaves sprayed with water were used as control. The timing of the treatments is indicated by arrows. The timing of the treatments is indicated by arrows. in (a) the asterisk denote a statistical difference (P < 0.0001, Log-rank Mantel-Cox test); in (b) different letters indicate a statistical difference (P < 0.0001, Kruskal–Wallis test).

4 Multiple gene silencing (*Sl 102* and *Sl gasmin* genes) to enhance immunosuppression and pathogen sensitivity

This chapter is to assess if a gene silencing strategy concurrently impairing two complementary arms of the cellular immune response in *Spodoptera littoralis* can result in a more pronounced enhancement of the killing activity by *B. thuringiensis*. The experimental design was based on RNAi-mediated silencing of two immune genes: *Sl 102* (controlling encapsulation and nodulation) and *gasmin* (controlling phagocytosis).

4.1 MATERIALS AND METHODS

4.1.1 *In vitro* synthesis of *Sl 102* dsRNA and *Sl gasmin* dsRNA

Total RNA extracted from haemocytes of *S. littoralis* 6th instar larvae was retro-transcribed (AmbionRETROscript kit, Life Technologies). *Sl 102* dsRNA was obtained as described above.

A 789 bp long cDNA fragment of *Sl gasmin* gene (Accession Number MG880078*)* was obtained by PCR, using the *Sl gasmin* dsRNA forward primer (GCC GGC ATG TTG TCT ATT ACC) in combination with the *Sl gasmin* dsRNA reverse primer (TCC TTC CAG CTT CTG AGT CA). 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-*Sl gasmin* forward TAA TAC GAC TCA CTA TAG GGA GTT CGA GGA TAC AAG CAG AG; T7-*Sl gasmin* reverse TAA TAC GAC TCA CTA TAG GGA GGG ATG CTC AGG ATA TCT GTT AC). The resulting PCR product was used as template to synthesize *Sl gasmin* dsRNA (522 bp long), using the AmbionMEGAscript™ RNAi Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Control dsRNA, 500 bp long, was obtained from a control template supplied by the kit used. dsRNA preparations were quantified by measuring their absorbance at 260 nm with a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific), and purity was evaluated by assessing 260/280 nm absorbance ratios. Products were run on 1% agarose gels to confirm their integrity.

4.1.2 Oral administration of dsRNA to *S. littoralis* larvae

Spodoptera littoralis larvae were reared on artificial diet, as previously described, at 25 ± 1°C, 70 ± 5% R.H., and under a 16:8 h light/dark period.
RNAi experiments targeting both *Sl 102* (controlling encapsulation and nodulation) and *Sl gasmin* (controlling phagocytosis) were carried out to assess the possible occurrence of a synergistic effect of a gene silencing strategy concurrently impairing two complementary arms of the cellular immune response in *S. littoralis.*
Newly molted *S*. *littoralis* 4th instar larvae (1st day) were anaesthetized on ice and 1 µl (containing 80 ng of dsRNA) of *Sl 102* dsRNA, *Sl gasmin* dsRNA or a mix of these two dsRNAs synthesized *in vitro* (using GFP dsRNAs control) was poured into the lumen of the foregut by means of a Hamilton Microliter 1701RN syringe (10 µl, gauge 26s, length 51 mm, needle 2). dsRNA treatments consisted of one oral administration of 80 ng per day, for 3 days (from 4th to 5th instar). After the last dsRNA administration and prior to any experiment, haemocytes from treated larvae were used for qRT-PCR analysis, to confirm the occurrence of gene silencing.

4.1.3 *Sl gasmin* and *Sl 102* expression analysis by qRT-PCR

Total RNA used for transcriptional analysis was extracted as described above, according to manufacturer's instructions**.** The concentration and purity of total RNA were determined using a Varioskan™ Flash Multimode Reader (Thermo Frisher Scientific). The RNA was used for transcriptional analysis of the relative expression of *Sl 102 gene* and *Sl gasmin* transcripts, which was measured by one-step qRT-PCR, using the SYBR Green PCR Kit (Applied Biosystems), according to the manufacturer's instructions, as previously described. The level of gene transcription was assessed by relative qRT-PCR. The *S. littoralis β-actin* gene (Accession Number Z46873) was used as an endogenous control for RNA loading for both genes.
Expression profiles analysis of *Sl 102 gene by* qRT-PCR was carried out as described above. PrimerExpress 1.0 software (Applied Biosystems) was used to design the primers. Relative gene expression data were analyzed using the $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen 2001; Pfaffl 2001; Pfaffl *et al.,* 2002).

qRT-PCR for measurement of *Sl gasmin* expression was carried out using specific primers (*Sl gasmin* RT *fw*: AGT CGT TCA GAA TGG TAA CA; *Sl gasmin* RT *rev*: GAC GCA TTG AAG CCA ATC AT), designed to detect a region of *Sl gasmin* mRNA not included in the sequence targeted by the dsRNA. For validation of the ΔΔ*Ct* method, the difference between the Ct value of *Sl gasmin* and the *Ct* value of *β-actin* transcripts [Δ*Ct* = *Ct* (*Sl gasmin*)-*Ct* (*β-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 Δ*Ct* displayed a slope less than 0.1 (slope= 0.0133, R^2 = 0.0493), indicating that the efficiencies of the two amplicons were approximately equal.

4.1.4 Cellular immune assays

The impact of gene silencing on cellular immune responses was assessed by scoring its effect on encapsulation and phagocytosis. Encapsulation response was assessed as previously described (Di Lelio *et al.,* 2014; Becchimanzi *et al.,* 2020).
Briefly, CM Sepharose fast flow chromatography beads (Pharmacia), suspended in PBS 1×, were injected into the haemocoel of *S*. *littoralis* larvae using a Hamilton Microliter 1702 RN syringe (25 µl, gauge 22s, length 55 mm, needle 3). After 24 h, beads were recovered upon larval dissection and scored to evaluate their encapsulation rate, which was expressed with the encapsulation index (E.I. = [∑ (encapsulation degree × total beads of this degree)/ total beads × 4] × 100), that takes into account both the encapsulation degree of each recovered bead (0—no cells adherent to the beads, 1—up to 10 adherent cells,2—more than 10 adherent cells but no complete layer around the bead, 3—one or more complete layers without melanization, 4—one or more complete layers with melanization) and the relative abundance of beads with a given encapsulation degree (for details see section 2.1.9).
To measure phagocytosis competence of *S*. *littoralis* haemocytes, an *in vitro* assay was performed at 12 h after the last dsRNA administration. Briefly, haemolymph samples were collected from a cut of the leg into ice-cold PBS 1× (1:1 v/v) and added with an equal volume of a PBS 1× suspension of 2 × 10^6 fluorescein conjugated *E*. *coli* cells (K-12 strain BioParticles, fluorescein conjugate, Invitrogen). After incubation with *E*. *coli* (10 min), samples were loaded into a Burker chamber, where total and fluorescent haemocytes were counted under a fluorescence microscope (Axioskop 20; Carl Zeiss Microscopy).

Prior starting incubation experiments, vital staining with trypan blue was used to routinely check the viability of collected haemocytes. A haemolymph aliquot was mixed with 0.4% (w/v) trypan blue (Sigma-Aldrich) (2:1 v/v), prior to count viable and dead cells under a light transmitted microscope (Axioskop 20), using a Burker chamber.

4.1.5 Bioassays with Xentari™

Oral delivery of the dsRNAs indicated above was performed to 4thinstar larvae of *S. littoralis* (that in the meantime attained the 5th instar) for 3 consecutive days; 24 hours after the administration of the last dsRNA dose, the experimental larvae were fed with artificial diet overlaid with a dose of 12 µg/cm^2 of Xentari™, diluted in 50 µl of distilled water, while the same volume of water was used for controls. This treatment was repeated 3 times, at 24 h intervals. Mortality was daily recorded for 9 days, and the weight of the surviving larvae was recorded at day 7, to have larger number of larvae for statistical analysis.

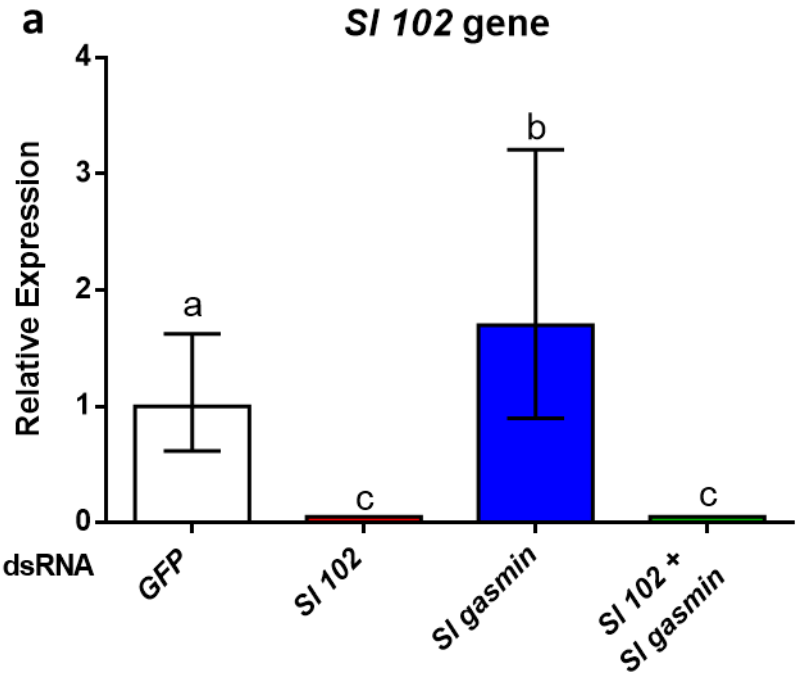
4.1.6 Statistical analysis

Encapsulation assay, phagocytosis assay and gene expression were analyzed using One-WayANOVA and Tukey's multiple-comparison *post hoc* test. Normality of data was checked by using the Shapiro-Wilk test and the D'Agostino-Pearson test, while homoscedasticity was checked with Bartlett's test. Survival curves of *S. littoralis* larvae were compared using Kaplan-Meier log-rank analysis. All data were analyzed using GraphPad Prism, version 6.0b.

4.2 RESULTS

4.2.1 Silencing of genes controlling different immune barriers

In order to assess the possible occurrence of a synergistic effect of a gene silencing strategy concurrently impairing two complementary arms of the cellular immune response in *S. littoralis*, we carried out RNAi experiments targeting both *Sl 102* (controlling encapsulation and nodulation) and *Sl gasmin* (controlling phagocytosis).
The results obtained show that the transcriptional level of both genes, when treated with a combined administration of *Sl 102* dsRNA and *Sl gasmin* dsRNA, was significantly reduced compared to control larvae and was comparable to that obtained with the administration of the individual dsRNA for each gene (*Sl 102* gene expression: One-Way ANOVA: $F_{3,110} = 2465$, $P < 0.0001$); *Sl gasmin* gene expression: One-Way ANOVA: $F_{3,122} = 1680$, $P < 0.0001$) (Fig. 20). Moreover, it is interesting to note that the combined administration of both dsRNAs is able to concurrently down-regulate the transcription of both genes.



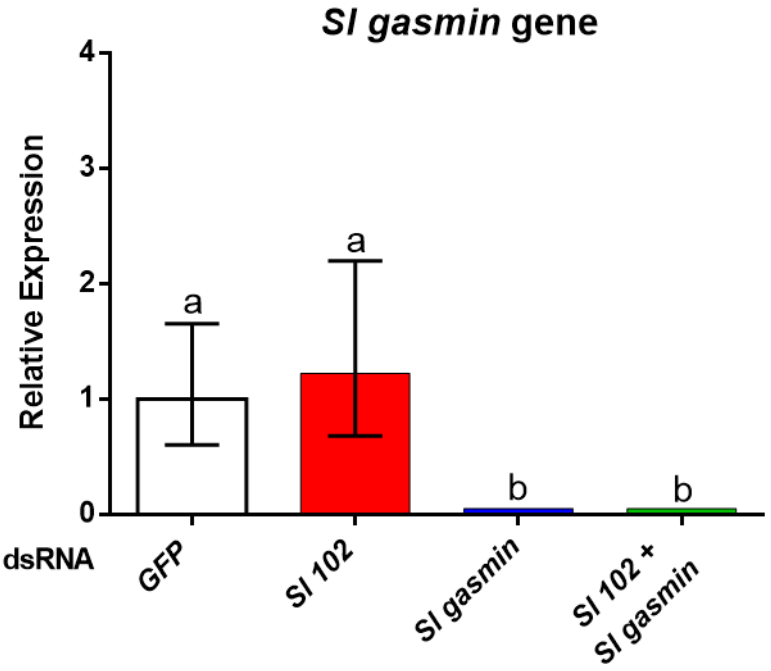
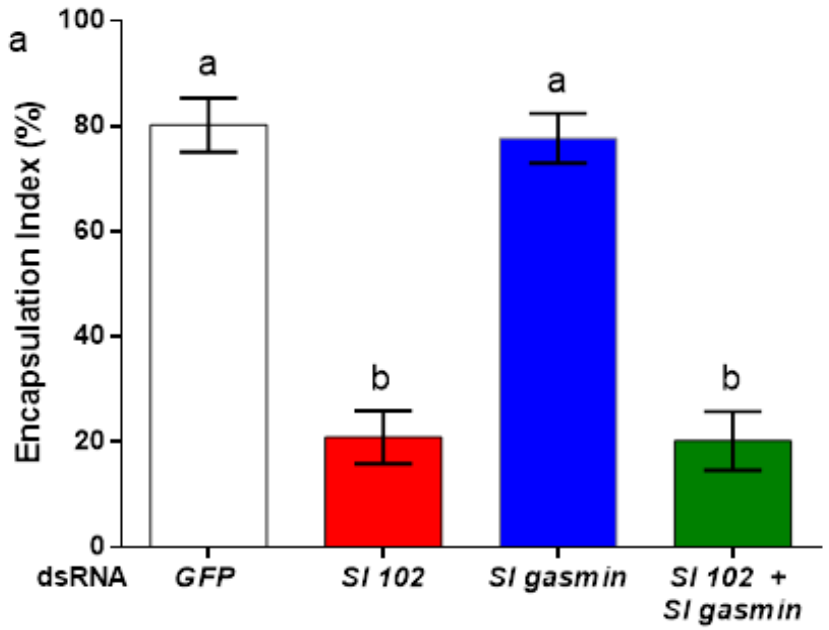


Figure 20. Relative expression in *S. littoralis* larvae of *Sl 102* (a) and *Sl gasmin* (b) as affected by oral administration of specific dsRNAs or a mixture of both of them. The transcriptional level of both genes, when treated with combined administration of *Sl 102* and *Sl gasmin* dsRNA, was significantly reduced compared to control larvae and was comparable to that obtained with the administration of the individual dsRNA for each gene. Values denoted with different letters are significantly different (One-Way ANOVA: $P < 0.0001$). Error bars represent the standard deviation of the mean.

4.2.2 Immune suppressive effect of *Sl 102, Sl gasmin* and of both dsRNAs

Encapsulation and melanisation reactions in silenced 5th instar larvae, treated with *Sl 102* gene and with both gene dsRNA, were significantly affected, compared with those observed in control larvae. Encapsulation capacity in larvae treated with *Sl gasmin* dsRNA was not altered by gene silencing (One Way ANOVA: $F_{3, 77} = 887.1$; $P < 0.0001$ (Fig 21a)). In particular, 24 h after their haemocoelic injection, chromatography beads recovered from the haemocoel of control larvae and of experimental larvae treated with *Sl gasmin* dsRNA were completely encapsulated and melanized by haemocytes (*GFP* – E.I. = 80.2 %; *Sl gasmin* – E.I. = 77.7 %), while encapsulation response totally failed in larvae treated with *Sl 102* dsRNA (E.I. = 20.9%,) and with both dsRNAs (E.I. = 20.2).

In contrast, phagocytosis of bacteria was strongly inhibited in experimental larvae treated with *Sl gasmin* dsRNAand with both gene dsRNA, as their haemocytes were almost completely unable to internalize Gram-negative (*E. coli*); as expected *Sl 102* gene silencing did not interfere with the nodulation response (One Way ANOVA: $F_{3,56} = 326.8$, $P < 0.0001$) (Fig 21b).



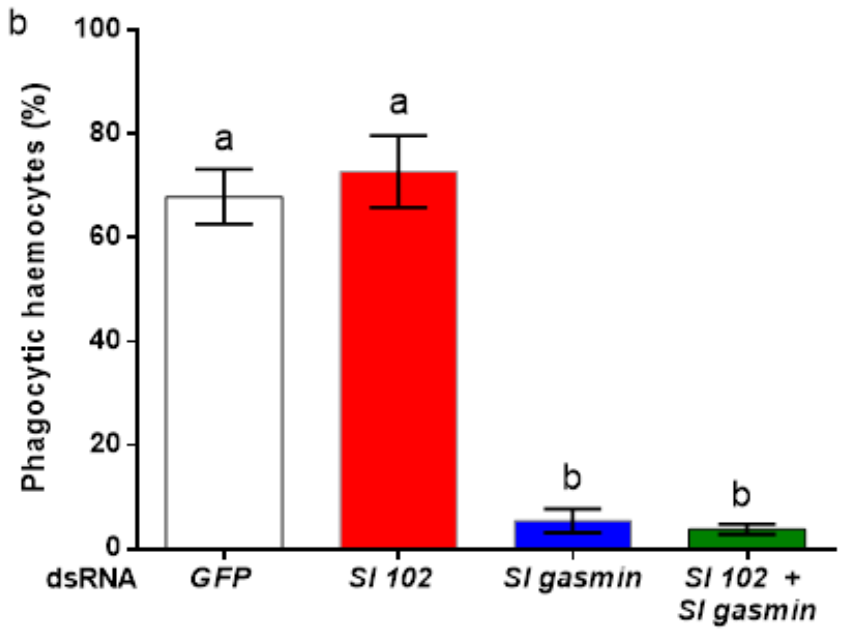
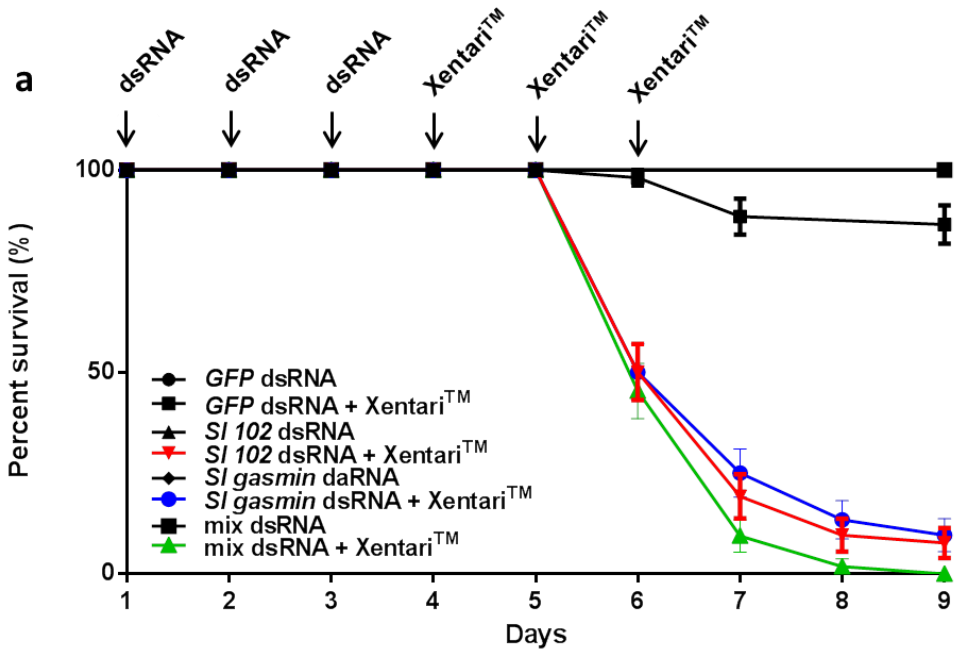


Figure 21. Cellular immune responses by *S. littoralis* larvae as affected by RNAi mediated single and double silencing. a) Chromatography beads injected into *S. littoralis* larvae orally treated with *Sl gasmin* dsRNA or double dsRNA were regularly encapsulated and melanized as in control, while the gene silencing of *Sl gasmin* gene did not influence encapsulation of chromatography beads. b) Conversely, the phagocytic capacity of haemocytes against Gram-negative *(E. coli*) was significantly reduced by RNAi mediated silencing of *Sl gasmin* and of the combined two gene. The values reported are the mean ± standard errors. Different letters denote mean values that are statistically different (**P* < 0.0001, One Way ANOVA Test).

4.2.3 Susceptibility of immunosuppressed *S. littoralis* larvae to *Bt*

These results prompted us to assess if the concurrent silencing of two genes controlling different arms of the immune response may have any synergistic effect on enhancing the killing activity of *B. thuringiensis*. The results showed that Xentari™ induced a significantly higher mortality in *S. littoralis* 4th instar larvae alimented with *Sl 102* dsRNA, *Sl gasmin* and a mixture of these 2 dsRNAs compared to controls (Log-rank test: χ^2 = 436.1, *df* = 7, *P* < 0.0001) (Fig. 22a) and determined a significant weight reduction in the surviving larvae (One Way ANOVA: $F_{2, 277}$ = 45.85; *P* < 0.0001) (Fig. 22b).

However, the survival larvae of *Sl 102* silenced larvae resulted not significantly different when compared with *Sl gasmin* silenced larvae (Log-rank test: $\chi^2 = 0.1829$, $df = 1$, $P < 0.6706$) and with larvae silenced for both genes (Log-rank test: $\chi^2 = 2.514$, $df = 1$, $P < 0.1128$), after treatment with Xentari™. Moreover, statistical difference was observed between larvae silenced with *Sl gasmin* and combined dsRNAs (Log-rank test: $\chi^2 = 4.218$, $df = 1$, $P < 0.0400$). Collectively, these results indicate that the separate silencing of *Sl 102* gene and of *Sl gasmin* is sufficient to induce a significant enhancement of the killing activity of *Bt* treatment and their concurrent silencing produces a more pronounced effect only when compared to *Sl gasmin* alone, showing, therefore, a very limited synergic interaction.



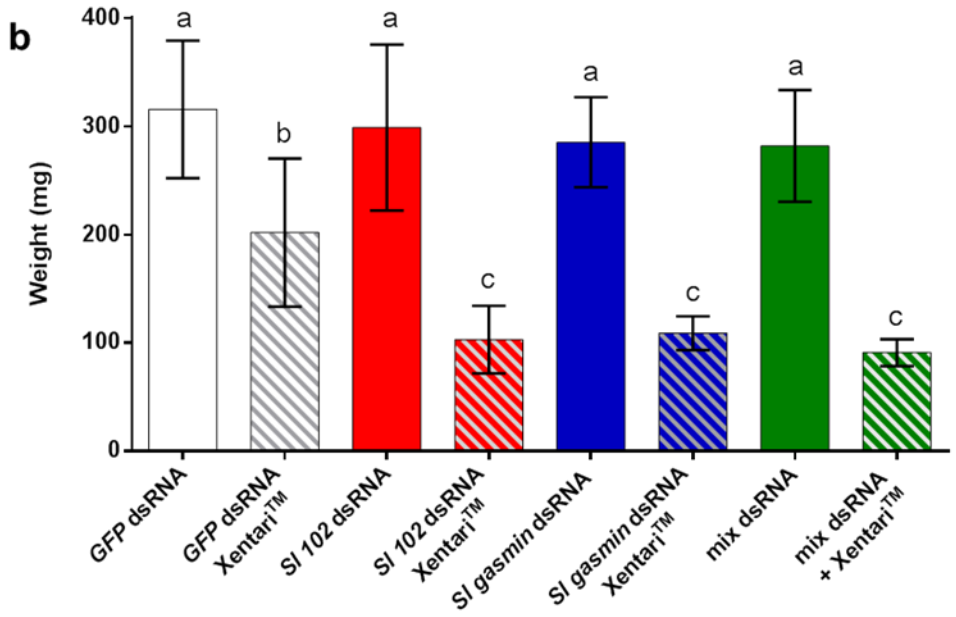


Figure 22. Survival of *S. littoralis* larvae as affected by silencing of different immune genes and the concurrent treatment with *Bt*. Bioassay with *S. littoralis* 4th instar larvae exposed to dsRNA before *Bt* treatment. Newly molted larvae were treated for 3 days with artificial diet layered with dsRNA synthesized *in vitro* (*Sl 102* dsRNA, *Sl gasmin* dsRNA or a mix of these two dsRNAs, corresponding to 80 ng of dsRNA) and then with 12 µg/cm^2 of Xentari™ for 3 more days (a). Survival was monitored until day 9 (a), when the weight was assessed on the surviving experimental larvae (b). The values reported are the mean ± standard errors. Different letters denote mean values that are statistically different (*P< 0.0001, One Way ANOVA Test).

5 DISCUSSION AND CONCLUSION

The study of the molecular mechanisms mediated by novel bioinsecticides derived from biocontrol agents and the identification of their cognate receptors offer the opportunity to develop bioinspired strategies of pest control mimicking the negative effects on host insects by natural antagonists. This can be accomplished by using molecular tools, such as RNA interference. The rationale behind this approach is to modulate the expression of insect genes targeted by virulence factors encoded by natural antagonists or their associated symbionts, so that the pathological alterations induced by them can be reproduced, determining a fitness reduction. The use of “RNAi mediated crop protection” technologies has been already proposed, and a wealth of different approaches have been pursued (Gu and Knipple, 2013; Younis *et al.,* 2014; Rodrigues and Figueira, 2016). In most cases, what has been done so far is the silencing of genes controlling important physiological functions, in order to induce lethal phenotypes (Joga *et al.,* 2016; Cooper *et al.,* 2018). This approach generates a direct killing effect which, in terms of population dynamics of target pests and associated natural enemies, is not different from that generated by an insecticide treatment. Here we propose a completely different approach, which is based on an indirect killing activity due to an enhanced sensitivity to natural antagonists, induced by reduced immunocompetence, which is achieved by RNAi mediated down-regulation of immune genes targeted by natural virulence factors. In other words, we try to reproduce the inactivation of insect defence barriers through molecular strategies adopted by their pathogens and parasites.

The proof of concept supporting the validity of this approach was provided by us in a relatively recent paper (Caccia *et al.,* 2016), where it was shown that the effective silencing of an immune gene in Lepidoptera is associated with a nearly 6-fold increase of mortality induced by *Bacillus thuringiensis* or by one of its toxins (Cry1A). Indeed, the *Bt*-induced gut lesions and the subsequent haemocoelic septicaemia play a key-role in the killing mechanism of this entomopathogen, which can be enhanced by RNAi-modulated immunosuppression (Caccia *et al.,* 2016). Unlike direct killing, this type of pest suppression, enhancing the impact of ecosystem service providers (i.e. insect natural antagonists), has no negative impact on beneficials, which can survive and continue to develop on natural hosts that are not drastically reduced in number by a strong insecticidal activity, whatever the origin. Moreover, 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 integrated pest management (IPM) plans.

The development of RNAi-based plant protection tools has been undoubtedly favoured by the high RNAi efficiency in Coleoptera but was tested in other insect orders of remarkable economic importance, such as Lepidoptera. In particular *Helicoverpa* and *Spodoptera* spp. have proved to be quite susceptible to orally administered dsRNA, which appear to induce a systemic RNAi response (Tian *et al.,* 2009; Di Lelio *et al.,* 2014; Lim *et al.,* 2016; Cooper *et al.,* 2019). However, the efficiency of RNAi response is also largely dependent on the efficiency of the delivery method, an essential pre-requisite to develop RNAi based pest control strategies for field applications, which are efficient, safe and economically sustainable.

In the present study, we have explored the use of bacteria and transgenic plants as potential delivery vectors of dsRNA targeting the immune gene *Sl 102* in *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae, one of the most damaging insects in agriculture (Hill, 1987), in order to evaluate their impact on the efficacy of a *Bt*-based commercial product (Xentari™) used for *Spodoptera* spp. control. *Sl 102* immune gene, downregulated by a virulence factor encoded by a BV associated with a parasitic wasp, encodes the protein precursor of amyloid fibers mediating both humoral and cellular immune responses in Lepidoptera (Falabella *et al.,* 2012; Di Lelio *et al.,* 2014; Caccia *et al.,* 2016). First of all, we produced dsRNA- expressing *E. coli* bacterial cells, using the Gateway® recombinational cloning system (Hartley *et al.,* 2000; Walhout *et al.,* 2000; Reboul *et al.,* 2001). The Gateway® technology allowed us the transformation of *E. coli* cells by a simple two-step method that exploits specific vectors and recombination enzymes. This standardized and high-fidelity method proved to be time-saving and convenient for our purposes and may represent the approach of choice for the production of large amounts of dsRNA and large-scale screenings of RNAi targets.

The transformed bacteria expressing *Sl 102* dsRNA were killed by sonication, to meet environmental safety requirements for any use under field conditions. Moreover, the sonication is 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. Bacteria expressing *Sl 102* dsRNA, when injected directly into the oral cavity, were effective in silencing the target gene, even thought to

a reduced extent compared to dsRNA synthesized *in vitro.* In contrast *Sl 102* dsRNA-Bac showed a higher efficacy, compared to *Sl 102* dsRNA-synt, when orally administered with artificial diet.
The level of RNAi-induced gene silencing by *Sl 102* dsRNA-Bac, along with the alteration of the encapsulation response by haemocytes, showed a clear dose-dependent response. Comparatively, naked dsRNA synthesized *in vitro* was less effective when administered with the feeding substrate. Indeed, at all experimental doses considered, the decrease of the transcript level and the encapsulation index induced by dsRNA-synt were always less evident than those observed upon ingestion of *Sl 102* dsRNA-Bac. This evidence further corroborates previous reports indicating that the bacterial envelope can protect dsRNA molecules against degradation (both environmental and inside the insect gut) and likely allows a more prolonged presence/release of dsRNA (Yang and Han 2014; Kim *et al.,* 2015; Lim *et al.,* 2016; Vatanparast and Kim 2017).
The oral efficiency of bacterial-delivered dsRNA targeting *Sl 102 gene* prompted us to assess their use for enhancing the virulence of entomopathogens. Our results clearly demonstrate that the immunosuppression induced by *Sl 102* dsRNA-Bac strongly synergizes *Bt*-based bioinsecticides. Indeed, these bacterial cells administered with the feeding substrate to *S. littoralis* larvae were able to enhance the mortality induced by *Bt*, regardless of previous or simultaneous administration of dsRNA and of the experimental larval stage treated. However, *Bt* exposure of larvae already showing gene silencing seems to have an impact on mortality slightly higher than that observed in response to concurrent administration of dsRNA and *Bt*, whichever is the instar treated. Indeed, the already-established immunosuppression likely favors a more rapid spread of bacterial septicaemia.
Bt sprays used to control lepidopteran larvae contain mixtures of Cry1 and Cry2 toxins, since they are based on spores and crystals produced by the *kurstaki* and *aizawaii* strains (Lacey *et al.,* 2015). The toxin miscellaneous in these formulations retards but cannot avoid the development of resistance under strong selective pressure in the field (Lacey *et al.,* 2015; Peralta and Palma 2017). Moreover, a major concern threatening their use is generated by the decrease in the efficacy of *Bt* sprays on mature larvae and as a consequence of reiterated exposure to *Bt* toxins of species with multiple generations across the growing season (Navon, 2000; Janmaat and Myers 2003; Cory, 2017). To alleviate these problems, several molecules able to improve *Bt* efficacy have been found (e.g., proteins that improve toxin production by the bacteria and agents that enhance permeability of the

peritrophic matrix and facilitate toxin accumulation near the binding sites) (Xu *et al.,* 2001; Mohan *et al.,* 2008; Fang *et al.,* 2009) and included in *Bt* formulations to enhance their efficacy. Our results can further contribute to the goal of enhancing the impact and the long-term efficacy of *Bt* spray formulations, by impairing the immune response of the insect, which is essential in counteracting the septicaemia induced by *Bt* toxins.

In order to evaluate if it was possible to reproduce on a natural food substrate the immunosuppressed phenotype induced by *Sl 102* dsRNA synthesized *in vitro* (Di Lelio *et al.,* 2014) or produced by bacteria (Caccia *et al.,* 2019), *S. littoralis* larvae were fed on two different lines of tobacco plants expressing the *Sl 102* dsRNA at different levels. Both transgenic tobacco lines induced a significant level of gene silencing when fed to *S. littoralis* larvae. When the larvae were fed precociously with leaves of tobacco transgenic plants (from 2nd instar), the reduction of *Sl 102* gene transcription rate started from 4th instar larvae and was consistently observed throughout the remaining part of the bioassay, up to the prepupal stage. The gene silencing measured in 2nd and 3rd instar larvae was not observed because it was influenced by the low amount of plant tissue ingested. This hypothesis was confirmed by the feeding bioassay starting with 4th instar larvae where the occurrence of gene silencing was already observed at the end of the 4th instar and was statistically significant for all the following time points considered. The level of *Sl 102* silencing observed was expected to induce an immune suppressed phenotype, characterized by the impairment of both nodulation and encapsulation responses by haemocytes. So, we have verified that the use of transgenic plants expressing dsRNA produces similar results to that obtained with dsRNA-Bac (Caccia *et al.,* 2020).

The high efficiency of transgenic plants in the induction of *Sl 102* silencing and the resulting immune suppression prompted us to assess if silenced *S. littoralis* larvae were more susceptible to a treatment with a Xentari^{TM}, as previously reported for different delivery methods of *Sl 102* dsRNA (Caccia *et al.,* 2016; 2020). Also in this case, our results clearly demonstrates that the immunosuppression induced by *Sl 102* dsRNA-transgenic plants strongly synergizes *Bt*-based bioinsecticides and were broadly in line with those obtained with dsRNA-Bac (Caccia *et al.,* 2020). These results further reinforce the potential of plant-mediated RNAi silencing of insect genes for crop protection (Gordon and Waterhouse, 2006; Huvenne and Smagghe, 2010), which has been already unlocked by the recent introduction on the market in North

America of genetically manipulated maize plants, which express dsRNA targeting the coleopteran species *D. virgifera* (Zotti *et al.,* 2018). The study of host-parasitoid interactions allowed the identification of a new immune gene in *S. littoralis*, named *gasmin,* acquired through horizontal gene transfer from a bracovirus associated with wasps attacking noctuid moths, which encodes an opsonizing protein promoting phagocytosis of invading pathogens. Similarly, to what observed for *Sl 102*, the RNAi mediated silencing of *gasmin* enhances the killing activity of *B. thuringiensis*, even though to a lower extent (Di Lelio *et al.,* 2019). RNAi experiments targeting both *Sl 102* (controlling encapsulation and nodulation) and *gasmin* (controlling phagocytosis) were carried out using dsRNA synthesized *in vitro*, to assess the possible occurrence of a synergic effect of a gene silencing strategy concurrently impairing two complementary arms of the cellular immune response in *S. littoralis*. We obtained good results, observing a level of silencing of the two immune genes, either when the experimental larvae were exposed to a combined administration of *Sl 102* and *Sl gasmin* dsRNAs or when the dsRNA were separately offered. It is interesting to note that the combined administration of both dsRNA is able to concurrently down-regulate the transcription of both genes and the immune functions they control. However, the concurrent disruption of two complementary immune barriers did not result in a stronger killing activity by *Bt*. Indeed, the significantly mortality increase observed in *S. littoralis* treated with *Sl 102* dsRNA was comparable with that obtained with the mixture of the two dsRNA. While the mortality of larvae treated only with *gasmin* dsRNA was comparatively lower than that induced by the mixture of the two dsRNA. This latter result can be likely due to the fact that in Lepidoptera several opsonins are present and may replace *Sl gasmin* in providing immune protection (Kim *et al.,* 2006; Tian *et al.,* 2009; Zhan *et al.,* 2016; Di Lelio *et al*., 2019). Collectively, the experimental evidence gathered indicates that the silencing of *Sl 102* gene is sufficient to induce a significant enhancement of the killing activity of *Bt* treatment and that the concurrent silencing of *gasmin* does not produce any synergic interaction.

In conclusion, the results obtained show that the ingestion of *Sl 102* dsRNA-bac and of transgenic plant tissues expressing dsRNA to *S. littoralis* larvae triggers a systemic RNAi response and a consistent immune suppression. Thus, immune suppressive dsRNAs vectored by bacteria may be exploited as synergic factors in novel *Bt* sprays, allowing the use of lower field doses, then contributing to the prevention of resistance insurgence. Moreover, novel transgenic plant pyramids

can be designed based on our results, with the aim of enhancing the insecticide activity of *Bt* toxins and its durability.

The sustainability of the “indirect killing” approach we propose is further corroborated by its potential positive effects on insect natural antagonists. From a theoretical point of view, the induction of a reduced immune competence in the target pest, compared to other RNAi pest control strategies, appears to be ecologically more sustainable as it enhances the ecological services provided by natural antagonists. Indeed, such an approach can promote the establishment and proliferation of biological control agents, rather than favoring their dispersal as a consequence of a treatment directly killing the target pest and reducing its density. Further theoretical studies, using a modeling approach, and a field validation of their results are required to corroborate this hypothesis.

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APPENDIX

In these 3 years, I had the opportunity to work in the entomology lab "Ermenegildo Tremblay" (Dipartimento di Agraria, Università degli Studi di Napoli "Federico II", Portici), under the guidance of Prof. Francesco Pennacchio. Due to Covid pandemic, smart-working activities (from March to September 2020) replaced the planned visit to the laboratory of Professor Angharad Gatehouse at the School of Biology, Newcastle University, UK

- Papers:

Caccia S, Astarita F, **Barra E**, Di Lelio I, Varricchio P and Pennacchio F (2019). Enhancement of *Bacillus thuringiensis* toxicity by feeding *Spodoptera littoralis* larvae with bacteria expressing immune suppressive dsRNA. *Journal of pest science*. https://doi.org/10.1007/s10340-019-01140-6

Rizzo D, Taddei A, Da Lio D, Nugnes F, **Barra E**, Stefani L, Bartolini L, Griffo RV, Spigno P, Cozzolino L, Rossi and Garonna AP (2020). Identification of the Red-Necked Longhorn Beetle Aromia bungii (Faldermann, 1835) (Coleoptera: Cerambycidae) with Real-Time PCR on Frass. *Sustainability*. 12(15), 6041; doi:10.3390/su12156041

Transgenic plants expressing immune suppressive dsRNA enhance *Bacillus thuringiensis* efficacy against *Spodoptera littoralis* larvae (in preparation)

- Oral Presentations:

"RNAi-mediated immune suppression of pest insects to enhance the impact of their natural antagonists. 9th PhD Insect Science meeting". Florence, Italy. November 2018.

"Immune suppressive dsRNA in *Spodoptera littoralis* enhance the impact of the entomopathogen *Bacillus thuringiensis*". European PhD Network "Insect Science" X Annual Meeting. Genova, Italy. December 2019.

“Induce immunosuppression as a strategy to enhance insect biocontrol”. European PhD Network "Insect Science” XI Annual Meet. Web Workshop**.** December 2020.

- Posters:

“RNAi-mediated immune suppression of pest insects to enhance the impact of their natural antagonists”. XI European Congress of Entomology. Naples, Italy. July 2018

“Induced immunosuppression as a strategy to enhance insect biocontrol”. Young Scientists for Plant Health. Web Workshop. December 2020

Journal of Pest Science
https://doi.org/10.1007/s10340-019-01140-6

ORIGINAL PAPER

Enhancement of *Bacillus thuringiensis* toxicity by feeding *Spodoptera littoralis* larvae with bacteria expressing immune suppressive dsRNA

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Received: 12 February 2019 / Revised: 19 June 2019 / Accepted: 8 July 2019

Abstract
RNAi interference (RNAi) for insect pest control is often used to silence genes controlling vital functions, thus generating lethal phenotypes. Here, we propose a novel approach, based on the knockout of an immune gene by dsRNA-expressing bacteria as a strategy to enhance the impact of spray applications of the entomopathogen *Bacillus thuringiensis* (*Bt*). The target gene, *Sl 102,* controls the encapsulation and nodulation responses in the noctuid moth *Spodoptera littoralis* (Lepidoptera, Noctuidae). To deliver *Sl 102* dsRNA, we have developed a bacterial expression system, using HT115 *Escherichia coli*. This allows a much cheaper production of dsRNA and its protection against degradation. Transformed bacteria (dsRNA-Bac) administered through artificial diet proved to be more effective than dsRNA synthesized in vitro, both in terms of gene silencing and immunosuppression. This is a likely consequence of reduced dsRNA environmental degradation and of its protected release in the harsh conditions of the gut. The combined oral administration with artificial diet of dsRNA-Bac and of a *Bt*-based biopesticide (Xentari™) resulted in a remarkable enhancement of *Bt* killing activity, both on 4th and 5th instar larvae of *S. littoralis*, either when the two components were simultaneously administered or when gene silencing was obtained before *Bt* exposure. These results pave the way toward the development of novel *Bt* spray formulations containing killed dsRNA-Bac, which synergize *Bt* toxins by suppressing the insect immune response. This strategy will preserve the long-term efficacy of *Bt*-based products and can, in principle, enhance the ecological services provided by insect natural antagonists.

Keywords RNA interference · Insect control · Systemic RNAi · dsRNA delivery · Gene silencing · Entomopathogen

Key message

- RNAi for insect control requires the development of effective delivery strategies of dsRNA.
- Bacteria expressing a dsRNA targeting an immune gene induce its silencing when ingested by larvae of the noctuid moth *Spodoptera littoralis*.
- The resulting immunosuppression enhances the killing activity of a *Bt*-based biopesticide.
- These immune suppressive bacteria can be used as synergistic factors to develop more effective *Bt* sprays, and to preserve *Bt* efficacy.

Introduction

RNAi interference (RNAi), the sequence specific gene silencing mediated by short non-coding dsRNA, that promotes mRNA cleavage or repression of mRNA translation was first discovered by Fire et al. (1998) in their pioneering study on the nematode *Caenorhabditis elegans*. Since then, RNAi has been reported in almost all eukaryotes as a fine-tuned mechanism of gene regulation (Carthew and Sontheimer 2009; Gebert and MacRae 2019) and as an important component of antiviral defense barriers (Ding 2010; Bronkhorst and van Rij 2014; Ding et al. 2018). More recent studies have revealed an unexpected and intense movement of regulative dsRNAs even between organisms (Knip et al. 2014). This fascinating phenomenon, called

Communicated by E. Roditakis.

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Published online: 15 July 2019

"cross-kingdom RNAi," in some cases contributes to the communication between plant or animal hosts and associated pathogens, parasites or symbiotic microorganisms (Knip et al. 2014; Wang et al. 2015, 2017; Weiberg et al. 2015).

The RNAi pathway has been largely exploited as a potent loss-of-function tool to unravel gene functions in animals (Housden et al. 2017), including insects (Di Lelio et al. 2014; Sugahara et al. 2015; Li et al. 2017, 2018; Jia et al. 2018; Pan et al. 2018). Interestingly, in insects the oral ingestion of dsRNA can trigger a silencing response in most body tissues (i.e., systemic RNAi), which can be profitably exploited for the development of RNAi-based control strategies against agricultural pests and pathogen vectors, by selectively targeting genes controlling physiological and developmental pathways of vital importance (Joga et al. 2016; Cooper et al. 2019). RNAi-plants to control coleopteran pests have recently reached the market (Zotti et al. 2018) and, along with other RNAi-based biopesticides, are expected to become an effective alternative to chemical products.

Systemic RNAi is robust in Coleoptera, absent in Diptera and unevenly present in other insect orders (Joga et al. 2016; Cooper et al. 2019), such as in Lepidoptera, where occurs in several noctuid species (e.g., *Helicoverpa* and *Spodoptera* spp.) (Tian et al. 2009; Di Lelio et al. 2014; Lim et al. 2016; Cooper et al. 2019). This paves the way toward the development of RNAi-based pest control strategies, which, however, can be profitably pursued if effective oral delivery methods, to overcome environmental and insect gut degradation of dsRNA molecules, are developed (Yu et al. 2013; Joga et al. 2016; Cooper et al. 2019). Polymers currently being used as carriers for oral delivery of dsRNA molecules in Lepidoptera (He et al. 2013; Christiaens et al. 2018) are comparatively less effective than plants and bacteria (Zhang et al. 2017; Zotti et al. 2018). The idea of using bacteria as delivery vectors of dsRNA molecules was first proposed in the pioneering studies on RNAi in the bacteriophagous nematode *C. elegans* (Timmons and Fire 1998; Timmons et al. 2001). This proof of concept prompted studies on the exploitation of the bacterial delivery strategy for pest control purposes, in order to overcome the technical and economic problems associated with the use of dsRNA synthesized in vitro. Tian et al. (2009) first reported the efficacy of bacterially expressed dsRNA in the induction of systemic RNAi in insects, in particular in the lepidopteran pest *Spodoptera exigua*. Several other studies have clearly shown that bacterial delivery (1) is cost-effective, (2) protects dsRNA molecules against degradation and (3) allows the development of new plant protection products/tools (Kim et al. 2015; Lim et al. 2016; Zhu et al. 2016; Ganbaatar et al. 2017; Israni and Rajam 2017; Vatanparast and Kim 2017; Wang et al. 2018).

We have recently shown that RNAi-mediated silencing of an immune gene in *S. littoralis* larvae, obtained by oral microinjection of dsRNA synthesized in vitro, results in a significant enhancement of insect mortality triggered by *Bacillus thuringiensis* (*Bt*) (Caccia et al. 2016; Di Lelio et al. 2019). This evidence sheds light on *Bt* killing mechanism (Caccia et al. 2016; Di Lelio et al. 2019) and paves the way toward the development of novel pest control strategies based on immunosuppression as a tool to enhance the impact of entomopathogens. Here, we contribute to this goal by exploring the use of bacteria as delivery vectors of dsRNAs targeting the immune system, in order to enhance the insecticidal activity of commercially available *Bt*-based biopesticides.

Materials and methods

Insect rearing

Spodoptera littoralis larvae were reared on artificial diet (41.4 g/l wheat germ, 59.2 g/l brewer's yeast, 165 g/l corn meal, 5.9 g/l ascorbic acid, 1.53 g/l benzoic acid, 1.8 g/l methyl 4-hydroxybenzoate and 29.6 g/l agar), at 25 ± 1 °C and 70 ± 5% RH, with 16:8 h light–dark period.

In vitro synthesis of *Sl 102* dsRNA

Total RNA was extracted from haemocytes of *S. littoralis* 6th instar larvae, retro-transcribed with the Ambion® RETROscript® Kit (Thermo Fisher Scientific), and a 580 bp long *Sl 102* cDNA fragment was obtained by PCR (*Sl 102* F primer: TACATCCAAGTAAATTTGCAAGGC; *Sl 102* R primer: GGCCCAGAACATTCTCACCTC). 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-*Sl 102* F: TAATACGACTCACTATAGGGAGAACCTCCTGAGCGTGCCTGA; T7-*Sl 102* R: TAATACGACTCACTATAGGGAGGGAGTGCTGCTTCAGAATCAT). The resulting PCR product served as template to synthesize a dsRNA (469 bp long), using the Ambion® MEGAscript® RNAi Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Synthesized dsRNA was quantified by measuring its absorbance at 260 nm with a Varioskan Flash Multimode Reader (Thermo Fisher Scientific), and purity was evaluated by assessing 260/280 nm absorbance ratios. dsRNA was run on 1% agarose gels to check its integrity.

A *GFP* dsRNA, used in control experiments, was similarly produced starting from the cloning vector pcDNA® 3.1/CT-GFP TOPO® (Thermo Fisher Scientific), which was used as template for a PCR reaction, performed with primers containing at their 5′ ends the T7 polymerase promoter

sequence (T7-*GFP* F: TAATACGACTCACTATAGGGAGAGTGGAGAGGGTGAAGGTG; T7-*GFP* R: TAATACGACTCACTATAGGGAGGGGCAGATTGTGTCGACAG). The resulting PCR product served as template to synthesize a dsRNA (531 bp long), as described above.

Production of transformed HT115 *Escherichia coli* expressing *Sl 102* dsRNA

A L4440 recombinant vector, encoding *Sl 102* or *GFP* (negative control) dsRNA molecules, was produced with the Gateway® cloning technology and used to transform HT115 *E. coli* cells.

Cloning of *Sl 102* and transformation of bacteria for *Sl 102* dsRNA production

Total RNA extracted from *S. littoralis* haemocytes was subjected to retro-transcription (Ambion® RETROscript® Kit, Thermo Fisher Scientific) and, then, used for PCR amplification of *Sl 102*, with specific primers (*Sl 102* F: CACCAACCTCCTGAGCGTGCCT; *Sl 102* R: CGGAGTGCTGCTTCAGAATC). A *GFP* fragment, used in control experiments, was amplified from the cloning vector pcDNA® 3.1/CT-GFP TOPO® (Thermo Fisher Scientific), which served as template for a PCR reaction, using specific primers (*GFP* F: CACCAGTGGAGAGGGTGAAGGTG; *GFP* R: GGGCAGATTGTGTCGACAG).

PCR products were ligated into the pENTR/D®-TOPO® vector (Thermo Fisher Scientific), compatible with the Gateway® technology, and the vector was introduced into chemically competent One Shot® TOP10 *E. coli* cells that were plated on LB agar. Plasmids from colonies grown overnight were extracted (Charge-Switch-Pro plasmid miniprep kit, Thermo Fisher) and sequenced. *Sl 102* and *GFP* fragments were cloned into a Gateway®-compatible L4440 vector, constructed by using the Gateway® vector conversion system, ligating a blunt-ended cassette containing *att*R sites flanking the *ccd*B gene and the chloramphenicol resistance gene. Cloning was performed using a transposition reaction catalyzed by the LR clonase® enzyme (Thermo Fisher Scientific).

The resulting recombinant plasmids were introduced into competent *E. coli* HT115 cells that lack RNase III and can be induced to express T7 polymerase in the presence of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Newmark et al. 2003; Timmons et al. 2001; Timmons and Fire 1998).

To produce dsRNA, the transformed bacteria were grown in the liquid broth Luria–Bertani (LB), containing 100 µg/ml ampicillin and 12.5 µg/ml tetracycline, at 37 °C for 16 h, under continuous shaking (250 rpm). Then, 5 ml of cultured broth was 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, for dsRNA overexpression, was induced by the addition of 1 mM IPTG to transformed bacteria, which were incubated overnight at 37 °C, under continuous shaking. Bacteria producing dsRNA targeting *Sl 102* gene or producing *GFP* dsRNA are hereafter denoted as *Sl 102* dsRNA-Bac and *GFP* dsRNA-Bac, respectively.

Bacterial cells were collected by centrifugation at 12,000×*g* for 1 min at 4 °C and suspended in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4). To kill the bacteria to be used in all feeding bioassays and to facilitate the release of dsRNA, a sonication protocol was developed. Bacterial suspensions were subjected to an increasing number of sonication cycles on ice with an ultrasound homogeniser (Sonoplus, Bandelin), adopting decreasing time intervals between cycles. The bacteria viability after the treatments was evaluated by plating the resulting sonicated suspension on Petri dishes containing LB agar (supplied with 100 µg/ml ampicillin and 12.5 µg/ml tetracycline). Complete mortality was obtained with ten cycles of sonication (59 s on/2 s off, 95% amplitude).

qRT-PCR absolute quantification of *Sl 102* dsRNA produced by bacteria

dsRNA produced by *E. coli* was extracted from cell pellets, using the protocol by Timmons et al. (2001). The quantification was performed by quantitative real-time PCR using Applied Biosystems™ SYBR™ Green master mix (Thermo Fisher Scientific). The quantity of dsRNA was determined by relating its threshold value (CT) values to an established standard curve, according to the absolute quantification method (Rutledge and Côté 2003).The standard curve for *Sl 102* dsRNA was established by plotting the logarithm of 6 10-fold dilutions of a starting solution containing 300 ng/µl of L4440 Gateway® vector with insert, against the corresponding CT value. The PCR efficiency ($E = 98.274\%$) was calculated on the base of the slope and the coefficient of correlation (R^2) of the standard curve (slope = − 3.365, y intercept = 13.540, $R^2 = 0.997$), according to the following formula: $E = 10^{(-1/\text{slope})} - 1$. The standard curve for *GFP* dsRNA was similarly established, by plotting the logarithm of 6 10-fold dilutions of a starting solution containing 200 ng/µl of L4440 Gateway® vector with insert, against the corresponding CT. The PCR efficiency ($E = 104.0477\%$) was calculated on the base of the slope and the correlation coefficient (R^2) of the standard curve (slope = − 3.229, y intercept = 17.650, $R^2 = 0.984$), according to the following formula: $E = 10^{(-1/\text{slope})} - 1$.

All primer pairs were designed using Primer Express 3.0 software (Life Technologies), following the standard

procedure. Negative controls (water) were included in each run of the qRT-PCR.

Oral administration of dsRNA to *Spodoptera littoralis* larvae

To assess the efficiency of dsRNA delivery through the use of sonicated bacteria, *S. littoralis* larvae were orally treated with *Sl 102* dsRNA-Bac, using two different protocols. In a first set of experiments, dsRNA-Bac was delivered by gavage with a microsyringe, as previously described (Di Lelio et al. 2014; Caccia et al. 2016). Briefly, newly molted *S. littoralis* 4th instar larvae were anaesthetized on ice and 1 µl of *Sl 102* dsRNA-Bac (*GFP* dsRNA-Bac in controls) solution (corresponding to 45 ng of dsRNA) was poured into the lumen of the foregut by means of a Hamilton Microliter syringe (1701RNR 10 µl, gauge 26 s, length 55 mm, needle 3). This treatment was repeated three times, at 24 h intervals. A group of larvae that received 1 µl of a solution of *Sl 102* dsRNA (45 ng/µl) synthesized in vitro (or *GFP* dsRNA in controls) acted as positive control, since this dose proved to be effective in the induction of gene silencing (Di Lelio et al. 2014).

The second protocol was developed for feeding bioassays on artificial diet. Newly molted 4th instar larvae were isolated in multi-well plastic trays (Bio-Rt-32, Frontier Agricultural Sciences), containing artificial diet, covered with perforated plastic lids (Bio-Cv-4, Frontier Agricultural Sciences), and maintained under the rearing conditions reported above. The experimental larvae, for 3 consecutive days, at 24 h intervals, were offered a small piece of diet with the upper surface (0.25 cm^2) uniformly overlaid with 1 µl of a solution of *Sl 102* dsRNA synthesized in vitro (45 ng/µl) or a *Sl 102* dsRNA-Bac suspension containing 45, 100 and 200 ng of dsRNA. Controls received *GFP* dsRNA synthesized in vitro or *GFP* dsRNA-Bac. Experimental larvae were maintained on artificial diet before and after the 3 administrations of dsRNA synthesized in vitro or of dsRNA-Bac suspension, which were overlaid on a small amount of the same diet, which was completely consumed in about 1 h.

Silencing efficiency was evaluated by qRT-PCR, as described below, 24 h after the last dsRNA administration, and the impact on immune competence was assessed by measuring the encapsulation index of injected chromatography beads, as previously described (Di Lelio et al. 2014).

qRT-PCR relative quantification of *Sl 102* transcription

Total RNA was extracted from haemocytes of *S. littoralis* larvae, using TRIzol® reagent (Thermo Fisher Scientific), according to manufacturer's instructions. Gene transcription level was assessed by qRT-PCR, which was carried out by using *Sl 102* gene-specific primers (*Sl 102* RT F: GGCGGT GTCGTCGTCGATTATG; *Sl 102* RT R: GAGCGAGGA AATGTTCAAT), designed to detect a segment of the *Sl 102* mRNA external to the segment targeted by the dsRNA. *S. littoralis β-actin* gene (Accession Number Z46873) was used as endogenous control for RNA loading (*β-actin* RT F: CGTCTTCCCATCCATCGT; *β-actin* RT R: CCTTCT GACCCATACCAACCA). All primers were designed using Primer Express, version 1.0 software (Applied Biosystems). The level of mRNA was measured by one-step qRT-PCR using the Applied Biosystems™ SYBR™ Green master mix (Thermo Fisher Scientific), according to the manufacturer's instructions. The amount of the target transcript relative to the endogenous control was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001; Pfaffl 2001; Pfaffl et al. 2002). For validation of the ΔΔCT method, the difference between the CT value of *Sl 102* and the CT value of *β-Actin* transcripts [ΔCT = CT (*Sl 102*) − CT (*β-actin*)] was plotted versus the log of 10-fold serial dilutions (5000, 500, 50, 5 and 0.5 ng) of the purified RNA samples. The plot of log total RNA input versus ΔCT displayed a slope less than 0.1 (slope = 0.0154, $R^2 = 0.0776$), indicating that the efficiencies of the 2 amplicons were approximately equal.

Bioassays with Xentari™

Three different feeding bioassays on *S. littoralis* larvae were carried out, in order to evaluate the impact of *Sl 102* gene silencing on the killing activity of the entomopathogen *Bacillus thuringiensis* (*Bt*). Preliminary trials were performed in order to identify sublethal *Bt* doses (i.e., with no or very low effect on mortality and only moderately affecting the speed of larval development), which were 9 µg/cm^2 and 12 µg/cm^2 for 4th and 5th instar larvae, respectively. The use of this *Bt* dose allowed the assessment of any increase in the mortality rate caused by the RNAi-induced immunosuppression.

In the first type of bioassay (sequential treatment), 4th instar larvae were fed for 3 days with artificial diet overlaid with *Sl 102* dsRNA-Bac (or *GFP* dsRNA-Bac) (corresponding to 200 ng of dsRNA), as described above. Four hours after the administration of the last dsRNA dose, the experimental larvae, which in the meantime attained the 5th instar, were fed with artificial diet overlaid with a dose of 12 µg/cm^2 of Xentari™ (Valent BioSciences), a bioinsecticide based on *Bt* subsp. *aizawaii*, containing several Cry toxins (Cry1Aa, Cry1Ab, Cry1Ca, Cry1 Da and Cry2Ab). This treatment with Xentari™ was repeated 3 times, at 24 h interval, and, since Xentari™ was suspended in water, control diet was overlaid with water.

A second bioassay was designed to evaluate the effect of the simultaneous administration of dsRNA and Xentari™, to better simulate field spraying with a product containing

both components. Newly molted 4th instar larvae were fed with artificial diet overlaid with *Sl 102* dsRNA-Bac (or *GFP* dsRNA-Bac) (corresponding to 200 ng of dsRNA), as previously described, and, after 4 h, Xentari™ was administered at a dose of 9 µg/cm^2. This was done for 3 days. Controls were treated with water. The same experiment was performed with newly molted 5th instar larvae, using a dose of Xentari™ of 12 µg/cm^2. Mortality was daily recorded for 8 days, when the experimental larvae were weighed.

Statistical analysis

Data were analyzed using GraphPad Prism, version 6.0b. Encapsulation assay and *Sl 102* gene expression in gavage experiments were analyzed using the unpaired Student's *t* test, and larval weight was analyzed using One-Way ANOVA and Tukey's multiple-comparison post hoc test. When ANOVA assumptions were not fulfilled, nonparametric Kruskal–Wallis ANOVA followed by Dunn's multiple comparisons post hoc test was used. *Sl 102* gene expression in feeding experiments was analyzed using Three-Way ANOVA to assess the effect of dsRNA treatment, production protocol and concentration. Levene's test was used to test the homogeneity of variance. When necessary, transformation of data was carried out to meet the assumptions of normality and homoscedasticity. When significant effects were observed ($P < 0.05$), the Bonferroni's post hoc test was used to compare mean values. Survival curves of *S. littoralis* larvae were compared using Kaplan–Meier and log-rank analyses. Normality of data was checked with Shapiro–Wilk test and Kolmogorov–Smirnov test, while homoscedasticity was tested with Levene's test and Bartlett's test.

Results

Production of bacteria expressing *Sl 102* dsRNA

To produce bacteria expressing dsRNA, a partial sequence of *Sl 102* gene (or *GFP* in controls) was inserted into L4440 vector, using the rapid and highly efficient Gateway® recombinational cloning system (Landy 1989). Briefly, the PCR product of the fragment of interest (*Sl 102* or *GFP* as control) was inserted in a donor vector to create the *att*L-containing entry clone. This latter has been used in a second recombination reaction with an *att*R-destination vector (L4440 vector properly converted into a Gateway® destination vector), to create an *att*B-containing expression clone used to transform HT115 *E. coli* cells (see Fig. 1a). Production of dsRNA occurs thanks to *att*B site-specific attachment sites on *E. coli* chromosome, and dsRNA overexpression, under the T7 promoters, is induced by IPTG addition. The amount of the dsRNA produced by bacteria (Fig. 1b) has been quantified by absolute qRT-PCR (Fig. 1c).

Sl 102 dsRNA-Bac produced were sonicated in order to disrupt the cell wall and to facilitate the release of dsRNA in the insect gut. Moreover, the use of killed bacteria is an essential requirement for their safe release in the environment.

Silencing efficiency and immune suppressive effects of *Sl 102* dsRNA-Bac

We first assessed RNAi efficiency and associated immunosuppression of *Sl 102* dsRNA-Bac by comparing their silencing effect with that induced by *Sl 102* dsRNA synthesized in vitro, adopting a protocol previously described (Di Lelio et al. 2014). Thus, *Sl 102* dsRNA-Bac and *Sl 102* dsRNA produced in vitro (hereafter denoted as *Sl 102* dsRNA-synt) (*GFP* dsRNA-Bac and *GFP* dsRNA-synt were used as controls, respectively) were orally administered, for 3 days to 4th instar *S. littoralis* larvae, by gavage with a microsyringe. Since 45 ng/µl is the lowest dose of *Sl 102* dsRNA-synt inducing maximal down-regulation of *Sl 102* gene (Di Lelio et al. 2014), an equal amount of dsRNA, measured by absolute qRT-PCR quantification (Fig. 1c), was administered as *Sl 102* dsRNA-Bac.

This experiment demonstrated that both dsRNA-synt and *Sl 102* dsRNA-Bac are associated with a significant level of silencing of the target gene compared to controls (Student's *t* test: for dsRNA-synt $t = 18.282$, $df = 28$, $P < 0.0001$, for dsRNA-Bac $t = 16.621$, $df = 28$, $P < 0.0001$) (Fig. 2), even though dsRNA-synt was by far more active than dsRNA-Bac. Since *Sl 102* gene is involved both in the nodulation of microorganisms and in the encapsulation of large parasites (e.g., parasitoid eggs, nematodes) (Falabella et al. 2012; Di Lelio et al. 2014; Caccia et al. 2016), which are immune reactions sharing functional similarities (Lavine and Strand 2002), we used the encapsulation response against chromatography beads as a measure of immune suppression induced by *Sl 102* silencing. Indeed, gene knockout was associated with a significant impairment of encapsulation response by haemocytes of silenced larvae, for both types of dsRNAs (Student's *t* test: for dsRNA-synt $t = 118.64$, $df = 28$, $P < 0.0001$, for dsRNA-Bac $t = 63.508$, $df = 28$, $P < 0.0001$) (Fig. 3).

To explore whether the bacterial delivery of dsRNA confers protection against degradation, *Sl 102* dsRNA-Bac and *Sl 102* dsRNA-synt were overlaid on artificial diet and separately offered to *S. littoralis* larvae, in order to compare their silencing efficiency and immune suppressive activity, at different experimental doses. The transcription level of the target gene was significantly affected by the dsRNA treatment (Three-Way ANOVA: $F_{1,140} = 567.493$; $P < 0.0001$), exhibited a more pronounced down-regulation

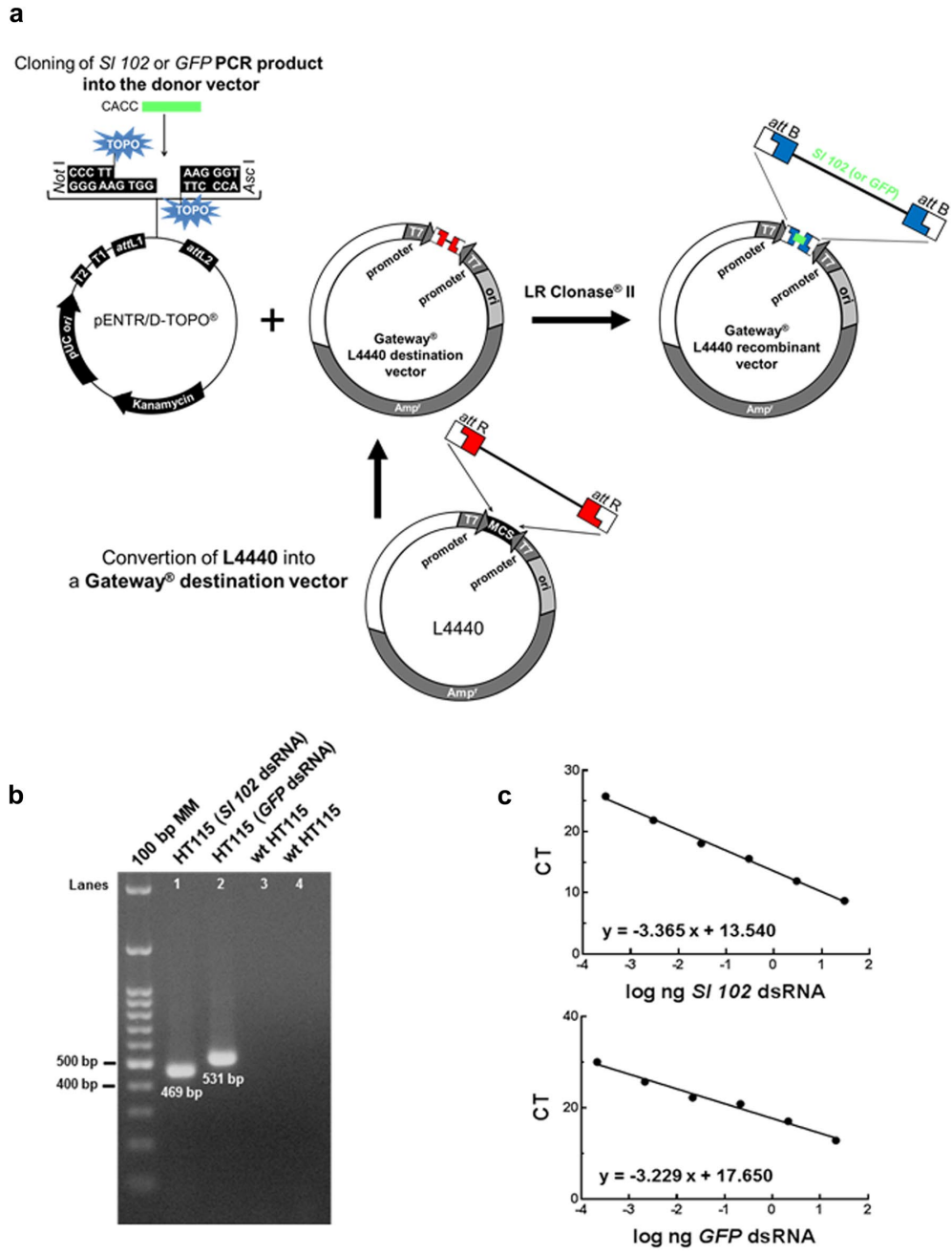

Fig. 1 Production of HT115 *Escherichia coli* cells expressing dsRNA. **a** Cloning and transformation protocol. **b** Expression of dsRNA by transformed HT115 *E. coli*; total RNA samples were subjected to RT-PCR, and amplicons were resolved on 1% agarose gel. Primers specific for *Sl 102* or *GFP* genes produced amplicons of the expected size in HT115 *E. coli* expressing *Sl 102* dsRNA or *GFP* dsRNA, respectively (lanes 1 and 2), whereas the same primers did not generate any amplicon when total RNA from non transformed bacteria was used (wt HT115) (lanes 3 and 4). **c** Calibration curves used for qRT-PCR absolute quantification of *Sl 102* and *GFP* dsRNA present in *E. coli* suspensions used in the bioassays

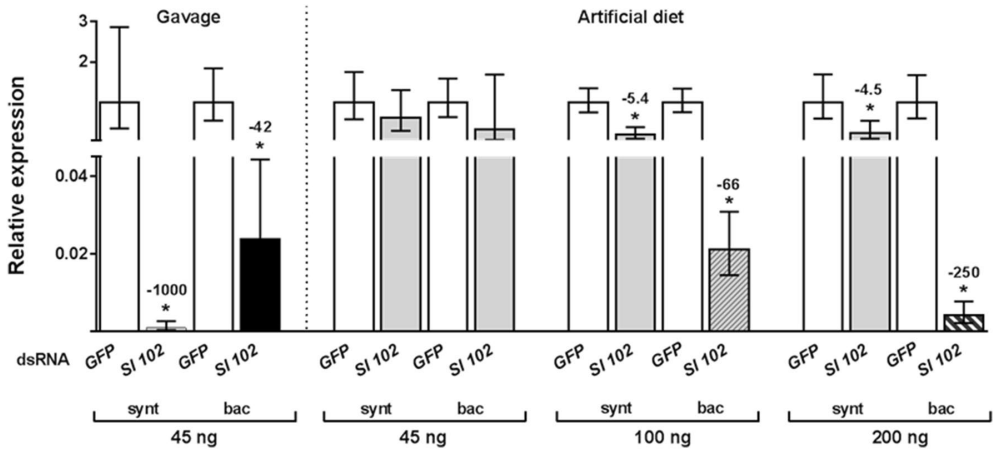

Fig. 2 Transcript levels of *Sl 102* gene in *S. littoralis* 4th instar larvae orally treated for 3 days with dsRNA. The *Sl 102* gene was down-regulated upon ingestion of *Sl 102* dsRNA administered by oral gavage, both in the case of dsRNA synthesized in vitro (*Sl 102* dsRNA-synt) and suspensions of sonicated bacteria expressing *Sl 102* dsRNA (*Sl 102* dsRNA-bac). Delivery with artificial diet showed a silencing response that was dose-dependent and more pronounced when bacteria were used as delivery vectors. *GFP* dsRNA synthesized in vitro and bacteria expressing *GFP* dsRNA were used in control experiments. The values reported are the mean ± standard errors (*$P < 0.0001$, Student's *t* test)

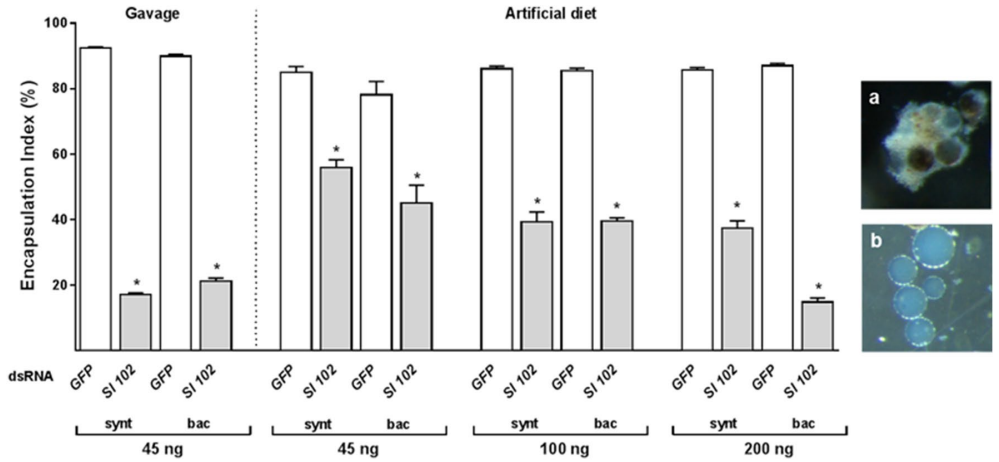

Fig. 3 Encapsulation assay in *S. littoralis* 4th larvae treated for 3 days with *Sl 102* dsRNA synthesized in vitro (*Sl 102* dsRNA-synt) or transformed HT115 *E. coli* expressing *Sl 102* dsRNA (*Sl 102* dsRNA-bac). Chromatography beads injected into the body cavity of control larvae were encapsulated and melanized (**a**). On the contrary, the efficiency of encapsulation was lower in silenced larvae, independently from the dsRNA administration method (gavage or with artificial diet) (**b**). The encapsulation index was affected by oral delivery method and, in the case of oral administration on artificial diet, by dsRNA quantity. *GFP* dsRNA synthesized in vitro and bacteria expressing *GFP* dsRNA were used in control experiments. The values reported are the mean ± standard errors (*$P < 0.0001$, Student's *t* test)

when dsRNA-Bac was used (Three-Way ANOVA: $F_{1,140} = 152.170$; $P < 0.0001$) and was positively correlated with the experimental dose used (Three-Way ANOVA: $F_{2,140} = 49{,}155$; $P < 0.0001$) (Fig. 2). The encapsulation reaction showed a similar pattern of variation (Three-Way ANOVA: dsRNA treatment $F_{1,124} = 1350{,}724$, $P < 0.0001$; dsRNA production $F_{1,124} = 27.604$, $P < 0.0001$; dsRNA dose $F_{2,124} = 26.472$, $P < 0.0001$) (Fig. 3).

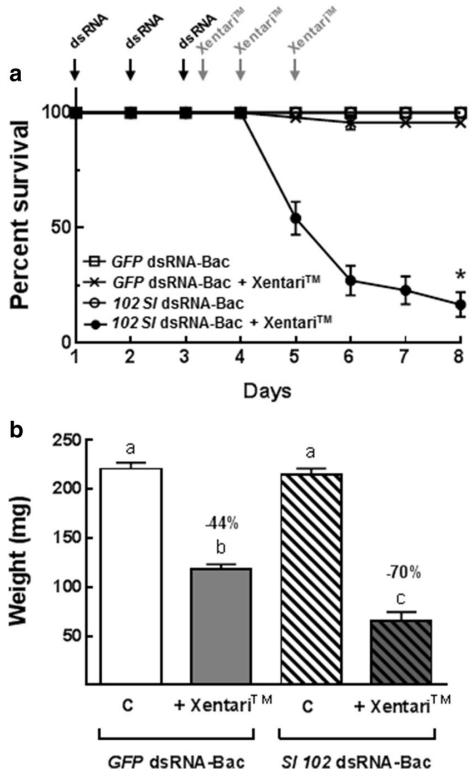

Fig. 4 Bioassay with *S. littoralis* 4th instar larvae exposed to dsRNA before *Bt* treatment. Newly molted larvae were treated for 3 days with artificial diet layered with transformed HT115 *E. coli* expressing *Sl 102* dsRNA (*Sl 102* dsRNA-Bac, corresponding to 200 ng of dsRNA) and then with 12 µg/cm^2 of Xentari™ for 3 more days (see "Materials and methods" section for experimental details). Survival was monitored until day 8 (**a**), when the weight was assessed on the surviving experimental larvae (**b**). Bacteria expressing *GFP* dsRNA were used in control experiments. The timing of the treatments is indicated with arrows. The values reported are the mean ± standard errors (in **a** **P* < 0.0001 based on log-rank test; in **b** different letters denote statistical difference based on Kruskal–Wallis test, followed by Dunn's multiple-comparison post hoc test)

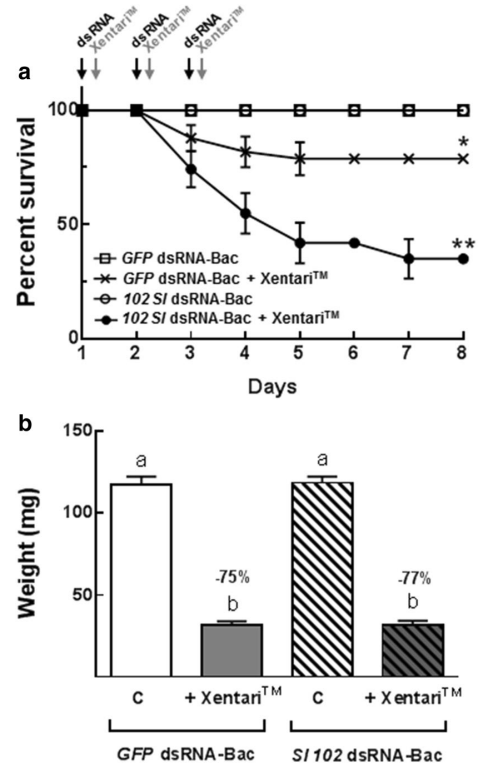

Fig. 5 Bioassay with *S. littoralis* 4th instar larvae simultaneously exposed to dsRNA and *Bt*. Newly molted larvae were treated for 3 days with artificial diet layered with transformed HT115 *E. coli* expressing *Sl 102* dsRNA (*Sl 102* dsRNA-Bac, corresponding to 200 ng of dsRNA) and with 9 µg/cm^2 of Xentari (see "Materials and methods" section for experimental details). Survival was monitored until day 8 (**a**) when the weight was assessed on the surviving experimental larvae (**b**). Bacteria expressing *GFP* dsRNA were used in control experiments. The timing of the treatments is indicated by arrows The values reported are the mean ± standard errors (in **a** ***P* < 0.0001 and **P* < 0.0046 based on log-rank test; in **b** different letters denote statistical difference based on Kruskal–Wallis, followed by Dunn's multiple comparisons post hoc test)

Sl 102 dsRNA-Bac enhance the killing activity of *Bacillus thuringiensis*

The induction of effective immune suppressive RNAi by *Sl 102* dsRNA-Bac prompted us to assess their potential in enhancing the efficacy of a *Bt*-based biopesticide (Xentari™).

In a first set of experiments (sequential treatments), 4th instar *S. littoralis* larvae were fed with artificial diet overlaid with *Sl 102* dsRNA-Bac for 3 days, as described above for gavage experiments. Four hours after the last dsRNA treatment, Xentari™ was administered to larvae with the artificial diet for 3 subsequent days. Xentari™ induced a significantly higher mortality only in larvae fed with *Sl 102* dsRNA-Bac (log-rank test: Chi-square = 172.3, *df* = 3, *P* < 0.0001) (Fig. 4a) and determined a significant weight reduction in the surviving larvae (Kruskal–Wallis: KW = 95.08; *P* < 0.0001) (Fig. 4b), which completely failed to pupate.

A second set of experiments was performed to test the efficacy of the simultaneous administration of *Sl 102* dsRNA-Bac and Xentari™. This experiment was designed to reproduce more closely the possible effects of a field application of both active ingredients (dsRNA and *Bt*). The results obtained, both with 4th and 5th instar larvae, clearly showed that simultaneous administration of

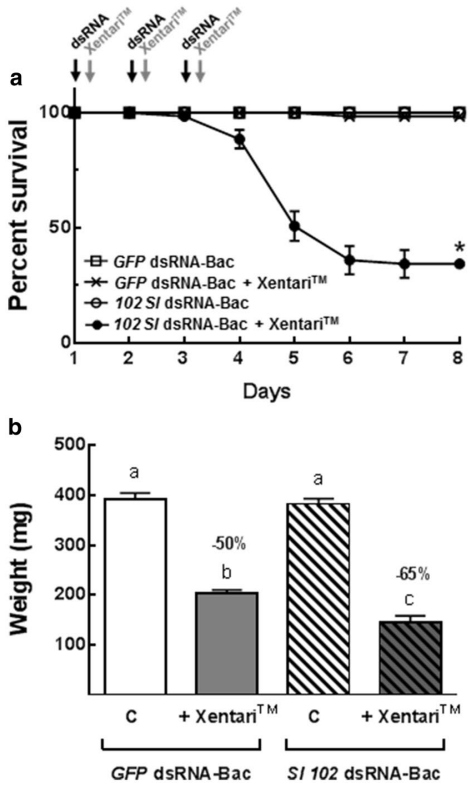

Fig. 6 Bioassays with *S. littoralis* 5th instar larvae simultaneously exposed to dsRNA and *Bt*. Newly molted larvae were treated for 3 days with artificial diet layered with transformed HT115 *E. coli* expressing *Sl 102* dsRNA (*Sl 102* dsRNA-Bac, corresponding to 200 ng of dsRNA) and with 12 µg/cm^2 of Xentari (see "Materials and methods" section for experimental details). Survival was monitored until day 8 (**a**), when the weight was assessed on the surviving experimental larvae (**b**). Bacteria expressing *GFP* dsRNA were used in control experiments. The timing of the treatments is indicated by arrows. The values reported are the mean ± standard errors (in **a** **P* < 0.0001 based on log-rank test; in **b** different letters denote statistical difference based on Kruskal–Wallis test followed by Dunn's multiple-comparison post hoc test)

Sl 102 dsRNA-Bac and Xentari™ caused a significantly higher mortality in *Sl 102*-silenced larvae compared to controls (Figs. 5a, 6a) (log-rank test 4th instar larvae: Chi-square = 49.02; *df* = 3; *P* < 0.0001; log-rank test 5th instar larvae: Chi-square = 156.6; *df* = 3; *P* < 0.0001) and had a significant impact on body weight both of 4th instar (Kruskal–Wallis: KW = 65.96; *P* < 0.0001) and 5th instar larvae (Kruskal–Wallis: KW = 135.1; *P* < 0.0001) (Figs. 5b, 6b), which completely failed to pupate.

Discussion

RNAi-based control strategies of insect pests offer new opportunities for the development of sustainable Integrated Pest Management plans, due to their specificity and reduced or null effect on nontarget species. This potential has been already unlocked by the recent introduction on the market in North America of genetically manipulated maize plants, which express dsRNA targeting the coleopteran species *Diabrotica virgifera* (Zotti et al. 2018). The development of this novel plant protection tool has been undoubtedly favoured by the high RNAi efficiency in Coleoptera. It would be desirable to further expand the reach of this insect control strategy by hitting pest species in other insect orders of remarkable economic importance, such as Lepidoptera. Although efficiency of RNAi response in Lepidoptera varies among species and depends on the efficiency of the delivery method, *Helicoverpa* and *Spodoptera* spp. have proved to be quite susceptible to orally administered dsRNA, which may trigger a systemic RNAi response (Tian et al. 2009; Di Lelio et al. 2014; Lim et al. 2016; Cooper et al. 2019).

In a previous study, we have shown that immune impairment of *S. littoralis* larvae, induced by oral administration of dsRNA molecules, causes an increase of susceptibility to the entomopathogen *B. thuringiensis* and accounts for the key importance of septicaemia in the killing activity of this biocontrol agent (Caccia et al. 2016). This proof of concept allows the development of novel pest control strategies aiming to enhance the impact of entomopathogens by RNAi-mediated silencing of immune genes. However, to pursue this goal, it is essential to develop RNAi delivery strategies for field applications, which are efficient, safe and economically sustainable. In the present study, we have explored the use of bacteria as potential delivery vectors of dsRNA targeting insect immune genes and evaluated their impact on the efficacy of a *Bt*-based commercial product (Xentari™) used for *Spodoptera* spp. control.

We produced dsRNA-expressing *E. coli* bacteria, taking advantage of the Gateway® recombinational cloning system (Hartley et al. 2000; Walhout et al. 2000; Reboul et al. 2001).

The Gateway® technology allowed us the transformation of *E. coli* cells by a simple two-step method that exploits specific vectors and recombination enzymes. This standardized and high-fidelity method proved to be time-saving and convenient for our purposes and may represent the approach of choice for the production of large amounts of dsRNA and large-scale screenings of RNAi targets.

Bacteria expressing *Sl 102* dsRNA were effective in silencing the target gene, even though to a reduced extent compared to dsRNA-synt, and in the induction of immunosuppression when injected directly into the oral cavity

of *S. littoralis* larvae (gavage); in contrast, it is of interest to note that *Sl 102* dsRNA-Bac showed a higher efficacy, compared to *Sl 102* dsRNA-synt, when orally administered with artificial diet (Figs. 2, 3). The level of RNAi-induced gene silencing by *Sl 102* dsRNA-Bac, along with the alteration of the encapsulation response by haemocytes, showed a clear dose-dependent response. Comparatively, naked dsRNA synthesized in vitro was less effective when administered with the feeding substrate. Indeed, at all experimental doses considered, the decrease of the transcript level and the encapsulation index induced by dsRNA-synt were always less evident than those observed upon ingestion of *Sl 102* dsRNA-Bac. This evidence further corroborates previous reports indicating that the bacterial envelope protects dsRNA molecules against degradation (both environmental and inside the insect gut) and likely allows a more prolonged presence/release of dsRNA (Yang and Han 2014; Kim et al. 2015; Lim et al. 2016; Vatanparast and Kim 2017).

The oral efficiency of bacterial-delivered dsRNA targeting *Sl 102 gene* prompted us to assess their use for enhancing the virulence of entomopathogens. Our results clearly demonstrate that the immunosuppression induced by *Sl 102* dsRNA-Bac strongly synergizes *Bt*-based bioinsecticides. Indeed, these bacterial cells administered with the feeding substrate to *S. littoralis* larvae were able to enhance the mortality induced by *Bt*, regardless of previous or simultaneous administration of dsRNA and of the experimental larval stage treated. However, *Bt* exposure of larvae already showing gene silencing seems to have an impact on mortality slightly higher than that observed in response to concurrent administration of dsRNA and *Bt*, whichever is the instar treated. Indeed, the already-established immunosuppression likely favors a more rapid spread of bacterial septicaemia.

Bt sprays used to control lepidopteran larvae contain mixtures of Cry1A and Cry2A toxins, since they are based on spores and crystals produced by the *kurstaki* and *aizawaii* strains (Lacey et al. 2015). The toxin miscellaneous in these formulations retards but cannot avoid the development of resistance under strong selective pressure in the field (Lacey et al. 2015; Peralta and Palma 2017). Moreover, a major concern threatening their use is generated by the decrease in the efficacy of *Bt* sprays on mature larvae and as a consequence of reiterated exposure to *Bt* toxins of species with multiple generations across the growing season (Navon 2000; Janmaat and Myers 2003; Cory 2017). To alleviate these problems, several molecules able to improve *Bt* efficacy have been found (e.g., proteins that improve toxin production by the bacteria and agents that enhance permeability of the peritrophic matrix and facilitate toxin accumulation near the binding sites) (Xu et al. 2001; Mohan et al. 2008; Fang et al. 2009) and included in *Bt* formulations to enhance their efficacy. Our results further contribute to the goal of enhancing the impact and the long-term efficacy of *Bt* spray formulations, by impairing the immune response of the insect, which is essential in counteracting the septicaemia induced by *Bt* toxins.

Here, we demonstrate that the insecticide activity of *B. thuringiensis,* one of the most widely used biopesticides, can be enhanced modulating the immune competence of the target pest. From a theoretical point of view, the induction of a reduced immune competence in the target pest appears to be ecologically more sustainable as it can enhance the ecological services provided by natural antagonists. Indeed, such an approach will promote the establishment and proliferation of biological control agents, rather than favoring their dispersal as a consequence of a treatment directly killing the target pest and reducing its density.

In conclusion, the oral delivery of *Sl 102* dsRNA-bac to *S. littoralis* larvae along with the food triggers a systemic RNAi response and a consistent immune suppression. Thus, immune suppressive dsRNAs vectored by bacteria may be exploited as synergistic factors in novel *Bt* sprays and to preserve the insecticidal activity of *B. thuringiensis*.

Author contributions

SC and FP conceived and designed research. FA, EB, SC, IDL and PV performed experiments. EB, IDL and SC analyzed data. SC and FP wrote the manuscript. All authors read and approved the manuscript.

Acknowledgements L4440 HT115 *E. coli* were kindly donated by Elia Di Schiavi (Consiglio Nazionale delle Ricerche, Naples, Italy). This work was supported by the Ministero dell'Istruzione, dell'Università e della Ricerca, Futuro in Ricerca 2013 (RBFR13PMT1) (to SC) and by the European Union's Horizon 2020 research and innovation program, under Grant Agreement No. 773554 (EcoStack) (to FP).

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