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ORIGINAL PAPER

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

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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

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"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: TAATACGACTCACTATAGGGA GAGTGGAGAGGGTGAAGGTG; T7-*GFP* R: TAATAC GACTCACTATAGGGAGGGGCAGATTGTGTCGACAG). 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: CGGAGT GCTGCTTCAGAATC). 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

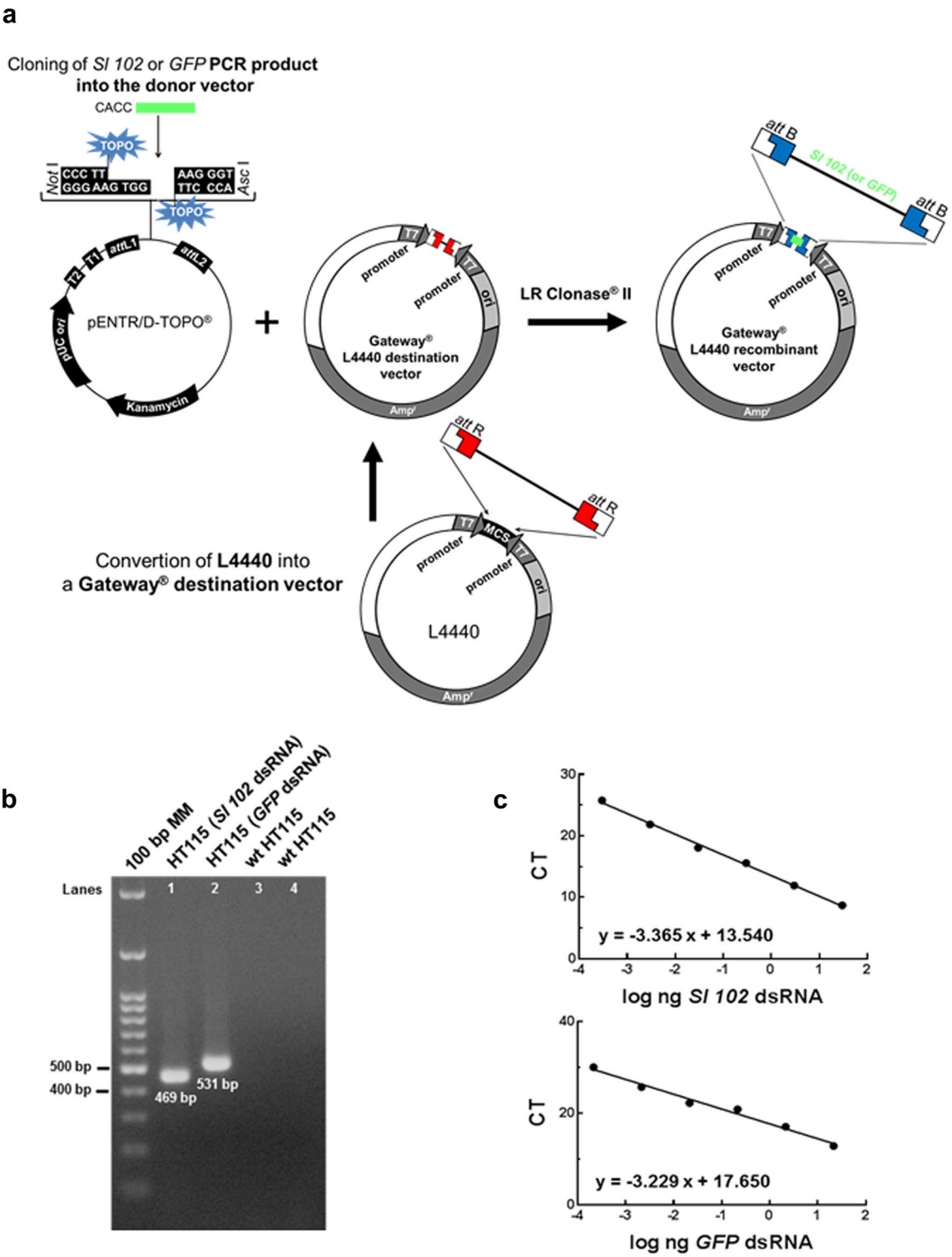

Fig. 1 Production of HT115 *Escherichia coli* cells expressing dsRNA. **a** Cloning and transformation protocol. **b** Expression of dsRNA by transformed HT115 *E. coli*; total RNA samples were subjected to RT-PCR, and amplicons were resolved on 1% agarose gel. Primers specific for *Sl 102* or *GFP* 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

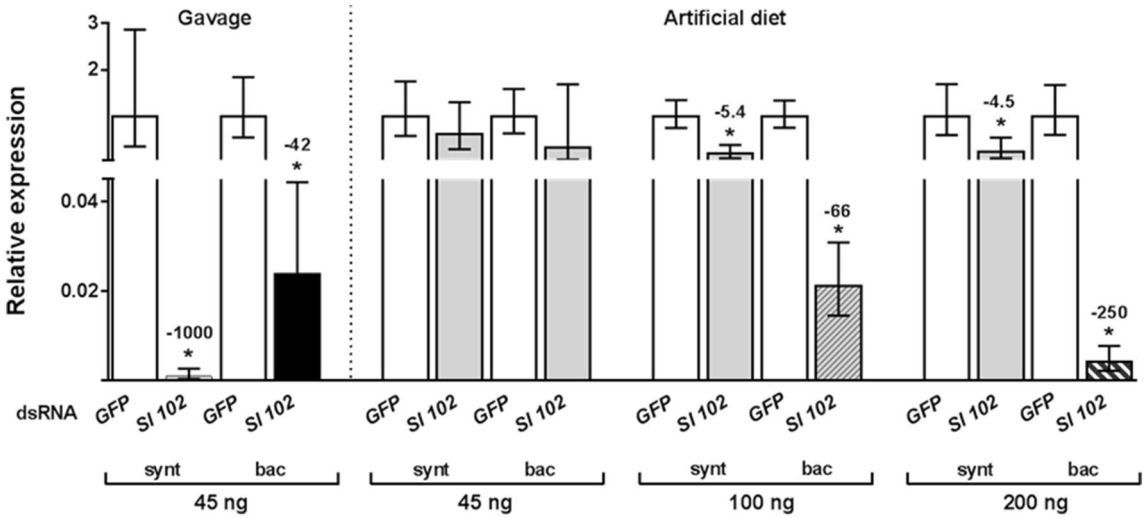

Fig. 2 Transcript levels of *Sl 102* gene in *S. littoralis* 4th instar larvae orally treated for 3 days with dsRNA. The *Sl 102* gene was down-regulated upon ingestion of *Sl 102* dsRNA administered by oral gavage, both in the case of dsRNA synthesized in vitro (*Sl 102* dsRNA-synt) and suspensions 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)

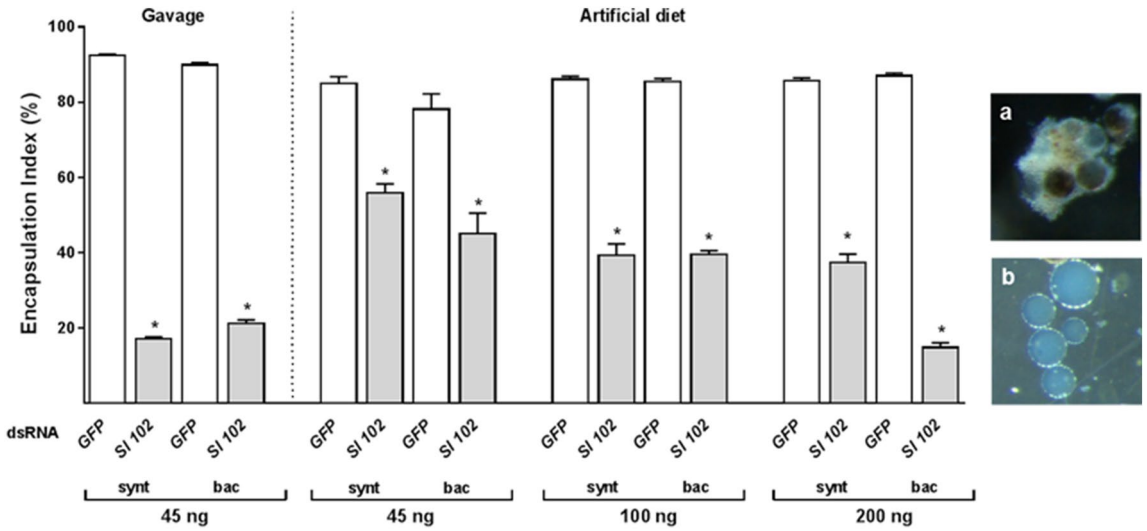

Fig. 3 Encapsulation assay in *S. littoralis* 4th larvae treated for 3 days with *Sl 102* dsRNA synthesized in vitro (*Sl 102* dsRNA-synt) or transformed HT115 *E. coli* expressing *Sl 102* dsRNA (*Sl 102* dsRNA-bac). Chromatography beads injected into the body cavity of control larvae were encapsulated 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).

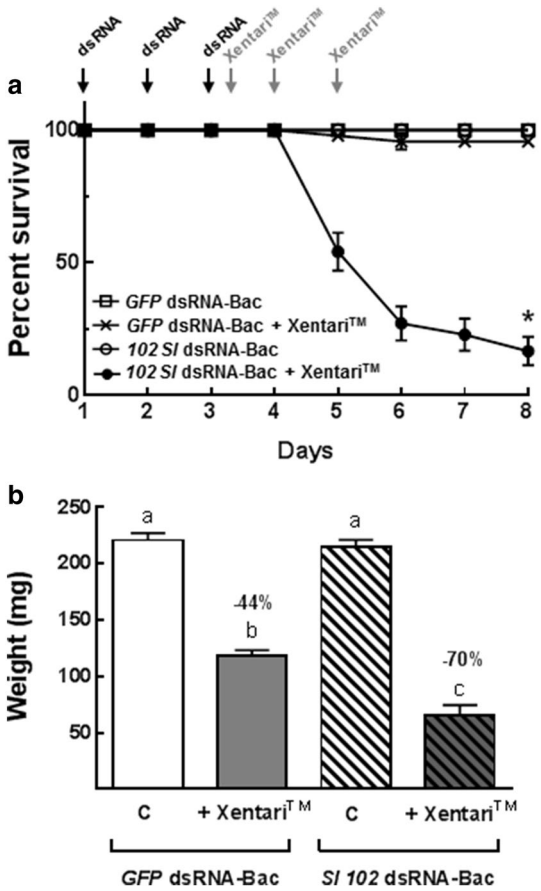

Fig. 4 Bioassay with *S. littoralis* 4th instar larvae exposed to dsRNA before *Bt* treatment. Newly molted larvae were treated for 3 days with artificial diet layered with transformed HT115 *E. coli* expressing *Sl 102* dsRNA (*Sl 102* dsRNA-Bac, corresponding to 200 ng of dsRNA) and then with 12 µg/cm^2 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)

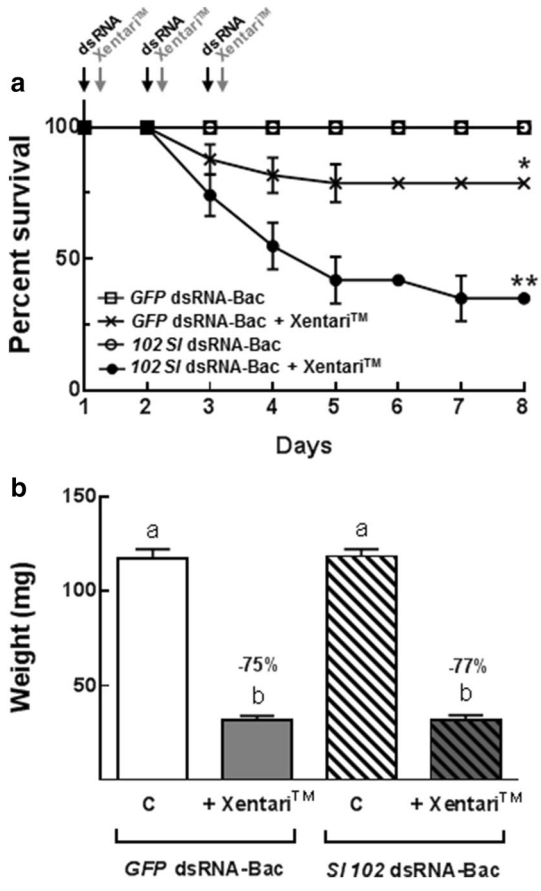

Fig. 5 Bioassay with *S. littoralis* 4th instar larvae simultaneously exposed to dsRNA and *Bt*. Newly molted larvae were treated for 3 days with artificial diet layered with transformed HT115 *E. coli* expressing *Sl 102* dsRNA (*Sl 102* dsRNA-Bac, corresponding to 200 ng of dsRNA) and with 9 µg/cm^2 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

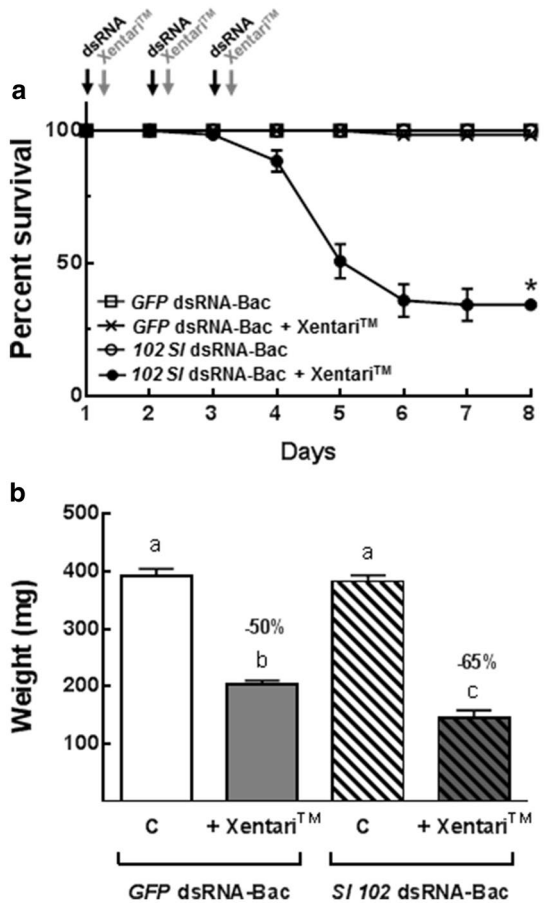

Fig. 6 Bioassays with *S. littoralis* 5th instar larvae simultaneously exposed to dsRNA and *Bt*. Newly molted larvae were treated for 3 days with artificial diet layered with transformed HT115 *E. coli* expressing *Sl 102* dsRNA (*Sl 102* dsRNA-Bac, corresponding to 200 ng of dsRNA) and with 12 µg/cm^2 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.

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