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ORIGINAL PAPER

Transgenic plants expressing immunosuppressive dsRNA improve entomopathogen efficacy against *Spodoptera littoralis* larvae

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Abstract

Transgenic plants that express double-stranded RNA (dsRNA) targeting vital insect genes have recently emerged as a valuable new tool for pest control. In this study, tobacco plants were transformed to produce dsRNA targeting *Sl 102* gene that is involved in the immune response of *Spodoptera littoralis* larvae, a serious lepidopteran pest of several crops. Experimental larvae reared on transgenic tobacco lines showed (1) a strongly reduced level of *Sl 102* transcripts, which was positively associated with food consumption; (2) a substantial impairment of the encapsulation response mediated by hemocytes; and (3) a marked increase in the susceptibility to Xentari™, a *Bacillus thuringiensis*-based insecticide. Importantly, this approach may allow a reduction in the doses of *B. thuringiensis* used for field applications and enhance its killing activity on mature larvae. The results obtained thus support the use of immunosuppressive RNAi plants to enhance the performance of microbial insecticides on lepidopteran larvae.

Keywords RNAi-plants · dsRNA delivery · Entomopathogen · Insect control · Insect immunity

Key message

- RNAi-plants proved to be very effective vectors for silencing dsRNA molecules in insects.
- Tobacco plants expressing a dsRNA targeting *Sl 102* immune gene induce its silencing upon ingestion in *Spodoptera littoralis* larvae, a serious crop pest.
- The resulting immune suppression enhances the killing activity of a *Bt*-based biopesticide.
- Immunosuppressive RNAi-plants are environmental-friendly tools that can be used to enhance the effectiveness of *Bt* sprays or other entomopathogen-based insecticides in integrated pest management strategies.

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Introduction

Currently, recombinant DNA technology is routinely used for the production of genetically modified crops (also known as biotech crops) to enhance plant defenses against abiotic and biotic stress (Ricroch and Hénard-Damave 2016). Since their introduction in 1996, the area under biotech crop cultivation has increased more than 100-fold, reaching 191.7 million hectares in 2018 distributed across 26 countries worldwide, while another 44 countries imported these biotech crops (http://www.isaaa.org). Although the market for insect-resistant crops is dominated by plants expressing toxins from the entomopathogen *Bacillus thuringiensis* (i.e., Cry and Vip toxins alone or combined by gene pyramiding) (Anderson et al. 2019), it is worth mentioning that several efforts to introduce other effective transgenes into plants have been made.

Several "antinutritional" proteins (i.e., that impair digestion and/or absorption of nutrients), for instance, have been considered excellent options for expression in plant tissues to confer protection against phytophagous insects. Genes encoding for protease inhibitors (PIs) have been claimed to be good candidates for crop engineering (Dunaevsky et al. 2005; Schlüter et al. 2010). PIs are key defense metabolites produced by plants to fight against herbivorous insects,

and their mode of action involves the impairment of protein digestion, which in turn causes retarded growth or even death (Macedo et al. 2015). The expression of exogenous PIs in plants, in particular serine- and cysteine-PIs, has been proven to confer resistance to Lepidoptera and Coleoptera, respectively (Dunaevsky et al. 2005; Schlüter et al. 2010). Among plant defense metabolites with antinutritional properties, lectins have proven to be very effective especially against Hemiptera (Macedo et al. 2015). The expression of lectins in plants inhibits larval growth and induces moderate mortality in Lepidoptera while causing high mortality in hemipteran insects by different mechanisms (Vasconcelos and Oliveira 2004; Shahidi-Noghabi et al. 2010a, b; Vandenborre et al. 2011; Caccia et al. 2012; Macedo et al. 2015). Interest in lectins was also motivated by their ability to act as vectors for the delivery of insecticidal molecules with hemolymphatic targets, such as GNA-venom fusion proteins (Rao et al. 1998; Foissac et al. 2000; Sétamou et al. 2002; Wang et al. 2005; Nagadhara et al. 2004; Trung et al. 2006; Fitches et al. 2012; Mi et al. 2017), an approach that has also been exploited in plants for the expression of chimeric proteins (Liu et al. 2016; Nakasu et al. 2014; Rauf et al. 2019). It is worth mentioning the exploitation of the antinutritional properties of antivitamins (i.e., compounds that interfere with the biological function of vitamins) to develop insect-resistant plants. Avidin, a glycoprotein present in avian egg white, forms complexes with biotin (vitamin B_{12}), and this binding impedes molecular recognition and uptake of the vitamin, inducing strong biotin deficiency. The expression of avidin in transgenic maize, tobacco and wheat indeed conferred resistance against many species of stored-product coleopteran and phytophagous lepidopteran pests (Kramer et al. 2000; Burgess et al. 2002; Abouseadaa et al. 2015). In addition, some chitinolytic enzymes proved to be interesting candidates for *in planta* crop protection, as they do not affect non-target organisms that lack chitin (i.e., vertebrates and plants), but are active against many noxious insect pests (Wang et al. 1996; Ding et al. 1998; Corrado et al. 2008; Berini et al. 2018). By altering peritrophic matrix architecture, these enzymes affect the efficiency of macronutrient digestion in the insect midgut (Berini et al. 2018).

Undoubtedly, the so-called RNAi-plants represent the most promising new generation of insect-resistant plants. Discovered in 1998 in a nematode (Fire et al. 1998), RNA interference (RNAi) (i.e., the mechanism of gene silencing mediated by dsRNA molecules) has been proven to be almost ubiquitous in eukaryotes wherein it represents a mechanism of gene regulation and a key player in antiviral defense (Agrawal et al. 2003; Kim and Rossi 2008; Ghildiyal and Zamore 2009; Schuster et al. 2019). RNAi has been exploited as a valuable tool to study gene function in many organisms and for many other purposes (Agrawal et al. 2003; Zhu and Palli 2020), such as the production of insect-resistant plants expressing dsRNA molecules (Mamta and Rajam 2017; Zhang et al. 2017; Zhu and Palli 2020). When plant tissues expressing dsRNA targeting essential insect genes (e.g., genes encoding V-type ATPase subunits, acetylcholinesterase, actin and tubulin isoforms, and enzymes involved in detoxification processes or endocrine regulation) are ingested, the silencing induces growth retardation or even death of the insect (Baum et al. 2007; Mao et al. 2011; Zhang et al. 2015; Eakteiman et al. 2018; Guo et al. 2018; Fu et al. 2020). A proof-of-concept study was published in 2007 by Baum and colleagues that demonstrated the insecticidal effect of transgenic corn expressing dsRNA against the *V-ATPase* gene of *Diabrotica virgifera virgifera*, resulting in a decrease in feeding damage and an increase in crop protection (Baum et al. 2007). The great interest in RNAi-crop technology for insect control resides in the very targeted delivery (dsRNA molecules can be expressed in specific plant tissues that are damaged by the insect pest) and specificity of interfering dsRNA to phytophagous insects (that can reach the species and, in some cases, even the strain level or even a specific developmental stage); this specificity results in no off-target effects and increased environmental safety (Fletcher et al. 2020).

In addition to the direct killing of the pest, the versatility of RNAi plants has been demonstrated by Mao and colleagues (Mao et al. 2007, 2011). Indeed, in the cotton bollworm *Helicoverpa armigera*, ingestion of dsRNA targeting a *cytochrome P450 monooxygenase* gene (i.e., *CYP6AE14*) increases the toxic effects of the cotton metabolite gossypol which is normally tolerated by this pest due to detoxification mediated by different enzymes (Mao et al. 2007). Cotton plants expressing dsRNA targeting *CYP6AE14* drastically impaired insect growth and significantly reduced plant damage (Mao et al. 2011). In the present work, we exploited plants as vectors for immunosuppressive dsRNA molecules to enhance insect pest susceptibility to entomopathogens. In particular, tobacco plants were transformed to express interfering dsRNA that specifically targeted *Sl 102 Spodoptera littoralis* gene (a gene involved in immune cellular responses, as microbial nodulation, in *S. littoralis* larvae) (Di Lelio et al. 2014; Caccia et al. 2016) and, were able to increase insect pest susceptibility against the entomopathogen *B. thuringiensis*. The results obtained in the present work have thus important implications for the effectiveness of microbial control agents (MCAs) in integrated pest management (IPM) strategies.

Materials and methods

Molecular cloning

Total RNA extracted from *S. littoralis* hemocytes was subjected to retrotranscription (Ambion® RETROscript® Kit,

Thermo Fisher Scientific, Waltham, Massachusetts, USA) and, then, used for PCR amplification of a 479 bp *Sl 102* fragment, with specific primers (i.e., *Sl102-att*B1 Fw and *Sl102-att*B2 Rv in Table 1 of the Supplementary material).

Recombinant plasmid molecules were obtained by Gateway® Recombination Cloning Technology (Thermo Fisher Scientific). An entry clone was generated by performing a BP recombination reaction between the *att*P-containing pDONR™/Zeo vector (Thermo Fisher Scientific) and the *att*B PCR product. The reaction mixture included 20–40 fmol of PCR products with *att*B sites, 150 ng of Gateway™ pDONR™/Zeo Vector (Thermo Fisher Scientific), 2 µl of BP Clonase® enzyme (Thermo Fisher Scientific) and TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) to a final volume of 8 µl. The mixture was incubated at room temperature overnight. Subsequently, 1 µl of Proteinase K (Thermo Fisher Scientific) was added, and samples were incubated at 37 °C for 10 min. For the creation of Gateway® Expression Clones, LR reactions were performed using 250 ng of *att*L-flanked entry clone, 150 ng of destination vector (containing *att*R sites), 2 µl of LR Clonase® enzyme and TE buffer to a final volume of 8 µl. The reaction mixture was incubated at room temperature overnight. The destination vector was pHellsgate12 (CSIRO Plant Industry, Canberra, Australia), a Gateway® adapted binary vector suitable for gene silencing in plants (Helliwell et al. 2002). Plasmid DNA was isolated using the alkaline lysis protocol of the Purelink™ Quick Plasmid Miniprep Kit (Thermo Fisher Scientific), quantified by densitometry with agarose gel electrophoresis (0.8% w/v) and screened by PCR for the presence and orientation, if necessary, of the inserts (Sambrook and Russell 2001). The sequence of the inserted DNA was verified by Sanger sequencing, performed at the Bio-Fab Research Company (Rome, Italy). The recombinant plasmids were introduced into *Escherichia coli* cells by electroporation at 2.5 kW for 5 milliseconds or heat shock (Sambrook and Russell 2001). Following transformation, the suspension of *E. coli* cells was placed on ice and 250 µl of Super Optimal broth with Catabolite repression (SOC) medium (Thermo Fisher Scientific) (Sambrook and Russell 2001) were added. Cells were then incubated at 37 °C for 30 min with agitation (150 rpm) in Luria–Bertani (LB) broth and plated on LB-agar with the appropriate antibiotic(s).

DNA samples were amplified in a final volume of 20 µl containing 0.5 U of Taq DNA polymerase (Promega, Milan, Italy), 1 × buffer (Promega), 1.5 mM $MgCl_2$, 10 mM dNTPs and 20 pmol of each primer (forward and reverse). The amount of DNA used as the template ranged from 10 ng (for the amplification of plasmid DNA templates) to 100 ng (for the amplification of plant genomic DNA). Colony PCR was also used in the screening for recombinant plasmids (Sambrook and Russell 2001). PCR products were verified by agarose gel (1% w/v) electrophoresis (Sambrook and Russell 2001). The marker used was a 1 kb Plus DNA ladder (Thermo Fisher Scientific). Primers were obtained from Thermo Fisher Scientific, and sequences are reported in Table 1 of the Supplementary material. Reactions were carried out in a Gene Amp 2700 PCR cycler (Thermo Fisher Scientific).

All primer pairs were designed using Primer Express 3.0 software (Thermo Fisher Scientific) following the standard procedure.

Plant transformation

The binary vector carrying *Sl 102* dsRNA was produced using Gateway® technology and mobilized in *Agrobacterium tumefaciens*. Tobacco (*Nicotiana tabacum* L., 'Samsun' NN) transformation and regeneration of kanamycin-resistant plants were carried out as previously described (Corrado et al. 2016). Putative transgenic plants were transferred in sterile soil and grown under controlled conditions (T_0 generation). Transgenic plants of the T_1 generation were identified by successive rounds of selection in kanamycin containing media and PCR analyses.

Molecular characterization of transgenic plants

DNA from putative transgenic plants was extracted according to Fulton's protocol (Fulton et al. 1995). Transgene presence was verified by PCR targeting the *Sl 102* gene (for primer sequences see Table 1 of the Supplementary material). Total RNA was isolated from the leaves of 4 week-old plants, first strand cDNA was synthesized, and qRT-PCR was performed as previously reported (Corrado et al. 2012) to detect the production of *Sl 102* dsRNA. Relative quantification was carried out using the ΔΔCt method (Livak and Schmittgen 2001; Pfaffl 2001; Pfaffl et al. 2002) using two technical replicates for each of the 3 biological replicates per sample. The housekeeping gene *elongation factor 1 α* (*EF-1α*) was used as an endogenous reference gene for the normalization of the expression levels of the target transcript. The expression analysis was carried out on the T_0 generation and later on the T_1 generation using a genotype with a low level of transgene expression as calibrator. Sequences of the primers used for qRT-PCR (i.e., *Sl102* Nt Rt Fw, *Sl102* Nt Rt Rv, *EF-1α* Rt Fw, and *EF-1α* Rt Rv) were designed using Primer Express 3.0 software (Thermo Fisher Scientific), following the standard procedure, and are reported in Table 1 of the Supplementary material.

Insect rearing and preparation of experimental larvae for feeding bioassays

S. littoralis larvae were reared on an 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, 70 ± 5% relative humidity, 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 reported above and using *S. littoralis* larvae that were maintained until the beginning of the experiment on subapical leaves of 4 week-old wild-type (WT) tobacco plants. These leaves were replaced daily in plastic boxes (30 × 40 × 15) bottom lined with 50 ml of 1.5% (w/v) agar in water to preserve leaf turgor. The experimental larvae were checked daily and those molting within 4 h formed experimental groups of synchronized larvae.

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

Different time course experiments were carried out on *S. littoralis* larvae fed transgenic plant leaves expressing *Sl 102* dsRNA to assess the level of silencing of the targeted gene. All the described experiments were carried out in plastic boxes, prepared as described above.

In the first time course experiment, *S. littoralis* eggs were left to hatch on WT tobacco leaves and larvae fed on those leaves for 12 h. First instar larvae were then grouped (100 larvae per box) and fed transgenic plant lines or WT tobacco (controls) leaves. Larvae were synchronized daily as described above, and at the end of the third instar (i.e., just before molting), experimental larvae were divided into additional boxes (25 larvae per box) to prevent cannibalism, and reared until pupation on experimental tobacco leaves. For total RNA extraction, whole larvae (fully grown second, third, fourth, and fifth instars, those on the first day of the sixth instar and those in the prepupal stage, i.e., 24 h after feeding cessation) were collected into TRIzol® reagent (Thermo Fisher Scientific). In the second time course experiment, *S. littoralis* eggs were left to hatch on WT tobacco leaves and larvae fed on WT tobacco leaves until the end of the third instar. Then, 150 newly molted fourth instar *S. littoralis* larvae (25 larvae per box) were reared on transgenic tobacco leaves (or on WT in control experiments) and allowed to develop until the pupal stage. The experimental larvae, i.e., those fully grown to the fourth and fifth instars, on the first day of the sixth instar and in the prepupal stage were processed to collect hemocytes into TRIzol® reagent and perform total RNA extraction (Di Lelio et al. 2019).

Silencing efficiency was evaluated by qRT-PCR, as described below, and the impact of gene silencing on cellular immune competence was assessed by measuring the encapsulation index of chromatography beads in fifth instar larvae fed, from the fourth instar, with the experimental tobacco leaves as described above. The encapsulation assay was carried out as previously described (Di Lelio et al. 2014; Becchimanzi et al. 2019).

RNA extraction and qRT-PCR

Total RNA was extracted from the whole larval body or from hemocytes in TRIzol® reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. The concentration and purity of total RNA were determined using a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific). Transcription of the *Sl 102* gene (Accession Number KJ544881.1) was measured by one-step qRT-PCR, using the SYBR Green PCR Kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

Relative qRT-PCR was carried out using *Sl 102* gene-specific primers (see Table 1 in Supplementary material for *Sl102* Rt Fw and *Sl102* Rt Rv sequences), which were designed to detect a segment of the *Sl 102* mRNA outside 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 (primer sequences are reported in Table 1 of the Supplementary material). All primer pairs were designed using Primer Express 3.0 software (Thermo Fisher Scientific), following the standard procedure. Negative controls (water) were included in each run of the qRT-PCR. The amount of target transcript relative to the endogenous control was determined using the ΔΔCt 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 [$\Delta Ct = Ct_{(Sl\ 102)} - Ct_{(\beta\text{-actin})}$] was plotted versus the log of tenfold 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 of less than 0.1 (Slope = 0.0154, $R^2 = 0.0776$), indicating that the efficiencies of the two amplicons were approximately equal.

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

A preliminary experiment was performed to determine the minimum feeding time on transgenic tobacco leaves to obtain gene silencing in the larvae used in bioassays, i.e., fourth and fifth instar larvae. Larvae were reared on WT tobacco leaves as described previously. Sixty-four newly molted fourth and fifth instar larvae were then transferred to transgenic tobacco leaves (4 and 6.25 cm^2 leaf squares were offered to fourth and fifth instar larvae, respectively) and silencing was monitored over time by qRT-PCR as previously described, using total RNA extracted from hemocytes collected in TRIzol® reagent (Di Lelio et al. 2019). Control larvae were fed on WT tobacco leaves. To search for a correlation between the silencing level of every single

larva, tobacco leaf pieces were collected after 14 h and the respective leaf consumption was arbitrarily defined as high, medium, or low.

For the bioassays with *Bt* bioinsecticide, synchronous fourth and fifth instar larvae, reared on WT tobacco plants or on the two transgenic lines, were obtained as described above, and singly transferred to multiwell plastic rearing trays (RT32W, Frontier Agricultural Sciences, USA) that were bottom-lined with 1 ml of 1.5% (w/v) agar in water, to keep the leaf squares turgid. The rearing wells, each containing a leaf square and a larva, were closed by perforated plastic lids (RTCV4, Frontier Agricultural Sciences, USA). The leaf squares were obtained by cutting leaves uniformly sprayed with Xentari™ (Valent BioSciences), a bioinsecticide based on *Bt* subsp. *aizawai*, that contains several Cry toxins and active on *Spodoptera* spp. Experimental tobacco leaves sprayed with distilled water were used as controls.

Preliminary bioassays were performed to determine the sublethal doses (i.e., causing growth retardation and/or weight decrease but no mortality) of Xentari™ in controls (larvae fed on WT tobacco). Sublethal doses were 1 and 3 µg/cm^2, for fourth and fifth instar larvae, respectively.

In the first bioassay (sequential treatment), for each experimental tobacco line, newly molted fourth instar larvae were fed ad libitum on leaves for 3 days. Then, 64 larvae were transferred to multiwell 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 as controls. Mortality was recorded daily from the beginning of the bioassay for 8 days, and the weight of the surviving larvae was recorded on Day 8.

Two other bioassays (concurrent treatment with *Bt*) were carried out on fourth and fifth instar larvae. Both concurrent bioassays were carried out by feeding fourth instar or fifth instar larvae leaf pieces from each experimental tobacco plant line for 24 h and then treating them with *Bt* for the following 3 days. Mortality was recorded daily from the beginning of the bioassay for 6 days, and the weight of the surviving larvae was recorded on Day 6.

Statistical analysis

Sl 102 dsRNA expression in tobacco transgenic lines was analyzed using unpaired Student's *t* test. *Sl 102* gene expression in larvae and data from the encapsulation assay were analyzed using one-way ANOVA, followed by Tukey's multiple-comparison post hoc test. Normality of the 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 and log-rank analysis. All data were analyzed using GraphPad Prism, version 6.0b (GraphPad software; San Diego, California, USA).

Results

Production of tobacco plants expressing *Sl 102* dsRNA

For the creation of an expression vector containing the construct to produce a dsRNA hairpin using the Gateway® Cloning System, a sequence of the *Sl 102* gene was amplified with *Sl102-att*B1 Fw and *Sl102-att*B2 Rv primers using RNA extracted from *S. littoralis* hemocytes as the template. Purified PCR products were then recombined with the pDONOR™/Zeo vector to obtain the pD*Sl102* entry clone. pD*Sl 102* was then recombined with the destination vector pHellsgate12, yielding the pH*Sl102* vector with the *Sl 102* dsRNA hairpin expression cassette (Fig. 1a). Recombinant pH*Sl 102* vectors were checked by PCR to ensure the presence of *Sl 102* fragments in both orientations (Fig. 1a and b) and used for stable tobacco transformation. Regenerated plants were transferred *in vivo* to an isolated growing chamber. The DNA of putatively transformed plants was isolated and analyzed by PCR using the AGRI64(2) Fw and *att*B2 Rv (Fig. 1a in Supplementary material), and the AGRI51(2) Fw and *att*B1 Rv (Fig. 1b in Supplementary material) primer combinations to check the presence and correct orientation of the *Sl 102* sequences. As expected, the expression analysis of the transgenic plants produced (T_0 generation) revealed genotypes showing different levels of transgene expression (Fig. 2 in Supplementary material). According to the transgene expression levels and to test a more stable genetic background, two transgenic genotypes were selected to obtain T_1 generations for subsequent investigations. Figure 2 shows the relative quantification of transgene expression on experimental transgenic tobacco plants (Line 1 and Line 2) and their phenotype compared to WT tobacco (Fig. 2a and b, respectively).

Silencing and immunosuppressive effects of *Sl 102* dsRNA transgenic plants

Both transgenic tobacco lines expressing *Sl 102* dsRNA induced a significant level of gene silencing when fed to *S. littoralis* larvae (Fig. 3a and b).

When the larvae were fed precociously with leaves of transgenic tobacco plants (from the first instar), a significant level of gene silencing was observed only when the experimental larvae attained the fourth instar (one-way ANOVA: second instar—$F_{(2,\ 25)} = 2.60$; $P = 0.00943$,

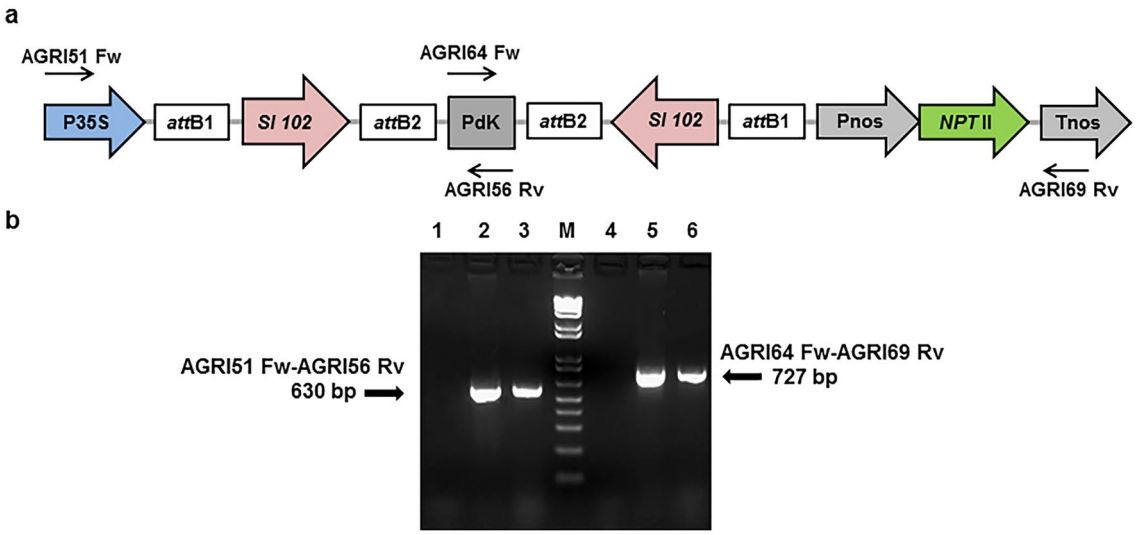

Fig. 1 Production of the *Sl 102* hairpin using the Gateway® cloning system. **a** Schematic representation of the expression cassette of the pH*Sl102* construct used for tobacco transformation (not to scale). P35S: *35S RNA CaMV* promoter sequence; *Sl 102* insert: *Sl 102* sequence flanked by two *att*R recombination sites in the sense orientation; PdK: *pyruvate orthophosphate dikinase* intron in the sense orientation fused to the *castor bean catalase-1* intron in the antisense orientation; *Sl 102* insert: *Sl 102* sequence flanked by two *att*R recombination sites in the antisense orientation; Pnos: *nopaline synthase* gene promoter; *nptII*: *neomycin phosphotransferase II* coding sequence; Tnos: *nopaline synthase* gene terminator. In **a** the AGRI primers to detect the sense (AGRI 51–56) and the antisense (AGRI 64–69) *Sl 102* fragments by PCR screening are shown. **b** Example of DNA fragment analysis using agarose gel electrophoresis of recombinant plasmids. Lanes 1 and 4: no template control; Lanes 2 and 3: PCR products of putative recombinant pH*Sl102* with the AGRI 51–56 primers; M: DNA ladder; Lanes 5 and 6: PCR products of putative recombinant pH*Sl102* with the AGRI 64–69 primers. Primers sequences are reported in Table 1 of the Supplementary material

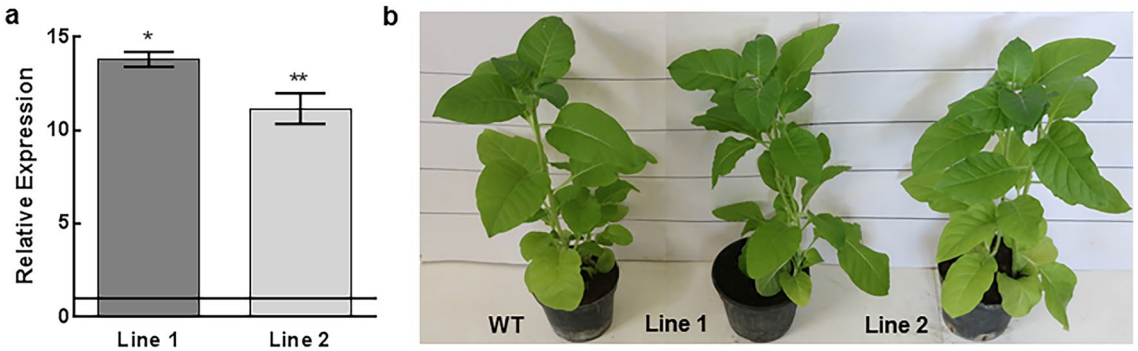

Fig. 2 Transcriptional analysis of *Sl 102* dsRNA by qRT-PCR in transgenic lines (**a**). The two transgenic plant lines showing the highest expression of *Sl 102* dsRNA (Line 1 and Line 2) were used in feeding bioassays. Transcript quantities were calibrated based on the transgenic genotype showing the lowest expression, which was arbitrarily set as 1 (*$P=0.000265$; **$P=0.0013$, Student's t test). The transgenic lines used in the experiments did not show phenotypic alterations with respect to wild type (WT) tobacco (**b**)

$dF=27$; third instar—$F_{(2, 26)}=0.483$; $P=0.6223$; $dF=28$; fourth instar—$F_{(2, 23)}=659.3$, $P<0.0001$, $dF=25$) (Fig. 3a). This reduction in the *Sl 102* gene transcription rate was consistently observed throughout the remainder of the bioassay, up to the prepupal stage (one-way ANOVA: fifth instar—$F_{(2, 26)}=488.5$, $P<0.0001$, $dF=28$; sixth instar—$F_{(2, 23)}=1114.0$, $P<0.0001$, $dF=25$; prepupal stage—$F_{(2, 24)}=8.0$, $P<0.0001$, $dF=26$) (Fig. 3a).

To check the hypothesis that the onset of gene silencing was influenced by the low amount of plant tissue (and thus of silencing *Sl 102* dsRNA) ingested by the earliest instar larvae, we performed the feeding bioassay starting with fourth instar larvae (Fig. 3b). The results supported our hypothesis, indeed, the occurrence of gene silencing was already observed at the end of the fourth instar and was statistically significant for all the following time points considered (one-way ANOVA: fourth instar—$F_{(2, 82)}=124.8$;

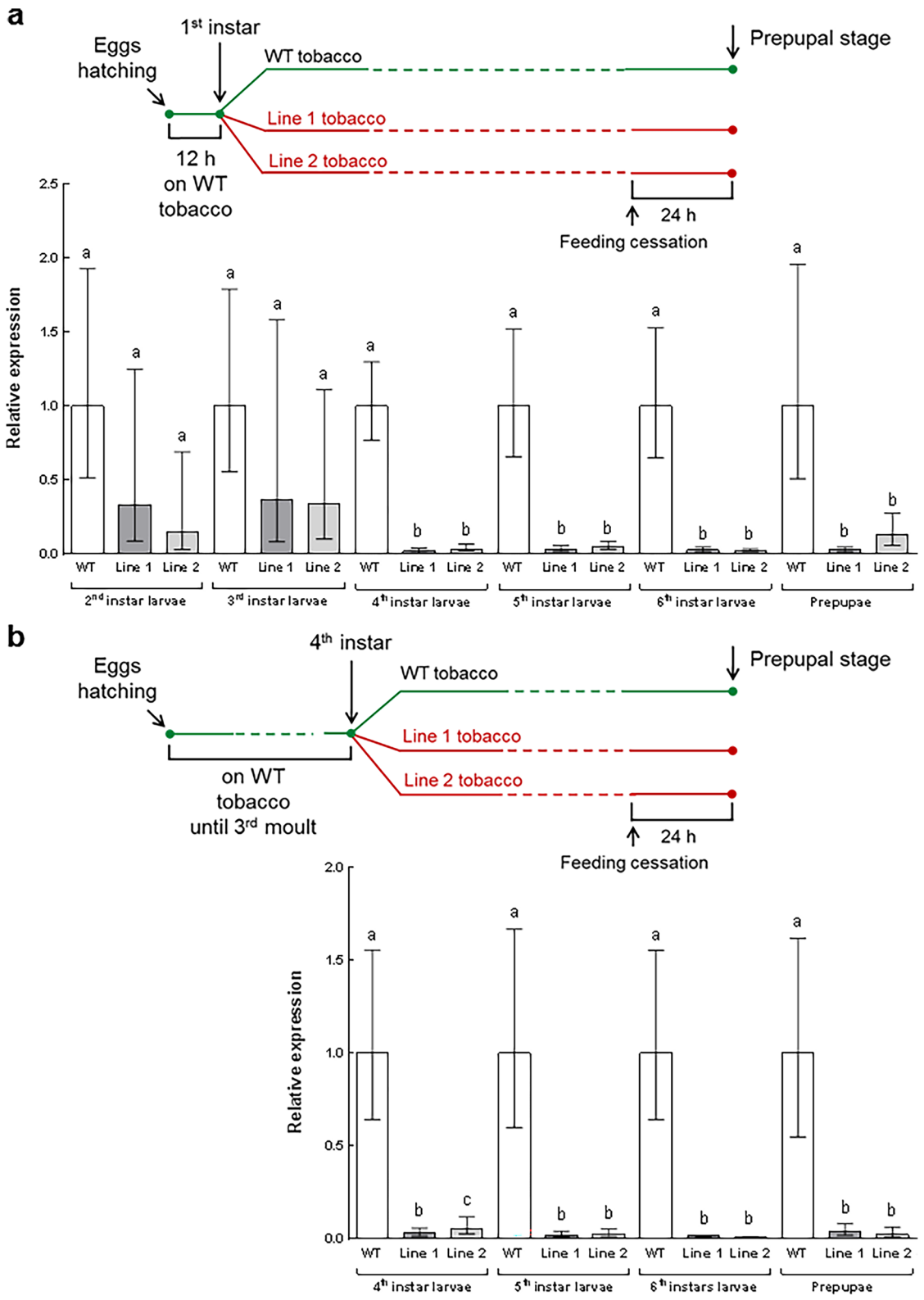

Fig. 3 Gene silencing in *Spodoptera littoralis* larvae reared on wild-type (WT) or *Sl 102* dsRNA-expressing (Line 1 and Line 2) tobacco plant leaves. Larvae were maintained on WT tobacco leaves and fed on transgenic plant leaves from the first (**a**) or fourth instar (**b**), as shown in the experimental plan above each histogram. Control larvae were reared on WT tobacco leaves. The comparison of the mean values was performed within each developmental instar, and those that were significantly different are denoted with different letters (*$P < 0.0001$, one-way ANOVA)

$P < 0.0001$; $dF = 84$; fifth instar—$F_{(2, 81)} = 240.6$; $P < 0.0001$; $dF = 83$; sixth instar—$F_{(2, 56)} = 665.6$; $P < 0.0001$; $dF = 58$; prepupal stage—$F_{(2, 44)} = 187.6$; $P < 0.0001$; $dF = 46$) (Fig. 3b). Moreover, gene silencing was measured in detail during the 3 days of fourth instar, demonstrating that although larvae do not feed continuously during the intermolt period, the significance of the silencing effect was maintained (14 h—Kruskal–Wallis: KW = 10.24; $P = 0.006$; 24 h—one-way ANOVA: $F_{(2, 52)} = 229.7$; $P < 0.0001$; $dF = 54$; 38 h—Kruskal–Wallis: KW = 42.94; $P < 0.0001$; 48 h—Kruskal–Wallis: KW = 42.34; $P < 0.0001$; one-way ANOVA: 62 h—$F_{(2, 56)} = 1733$; $P < 0.0001$; $dF = 58$; one-way ANOVA: 72 h—$F_{(2, 57)} = 645.5$; $P < 0.0001$; $dF = 58$) (Fig. 3 in the Supplementary material).

The level of *Sl 102* silencing observed was expected to induce an immunosuppressed phenotype, characterized by the impairment of both nodulation and encapsulation responses by hemocytes (Di Lelio et al. 2014; Caccia et al. 2016, 2020). Indeed, encapsulation and melanization reactions in silenced fifth instar larvae collected from the previous experiment (Fig. 3b) were significantly affected compared with those observed in control larvae fed WT tobacco (one-way ANOVA: $F_{(2, 112)} = 4568$; $P < 0.0001$; $dF = 114$) (Fig. 4). In particular, 24 h after hemocoelic injection, chromatographic beads recovered from the hemocoel of control larvae were completely encapsulated and melanized by hemocytes (Encapsulation Index, EI = 87.0%), while this cellular immune response completely failed in larvae fed the Line 1 (EI = 16.7%,) and Line 2 (EI = 18.0%) transgenic plant leaves.

Influence of leaf consumption on the silencing effect of *Sl 102* dsRNA transgenic plants

Prior to performing bioassays with *Bt* bioinsecticide, the minimum time interval of feeding required to induce a significant level of gene silencing in larval stages used for the bioassays (fourth and fifth instars) was assessed. Larvae were thus allowed to feed on plant tissue for 14 and 24 h. For both instars silencing was significant only after 24 h (one-way ANOVA: fourth instar—$F_{(2, 93)} = 279.6$, $P < 0.0001$; $dF = 95$; fifth instar—$F_{(2, 93)} = 525.7$; $P < 0.0001$; $dF = 95$) (Fig. 5a and b for fourth and fifth instars, respectively). Indeed after 14 h the silencing was not significant due to the very high variability in the rates of *Sl 102* transcription among larvae (Kruskal–Wallis test: fourth instar—KW = 5.769; $P = 0.056$; fifth instar—KW = 2.258; $P = 0.3234$) (Fig. 5a and b for fourth and fifth instar, respectively).

To check whether this variability was related to the amount of leaf consumption, the transcription data related to the 14 h time-point were grouped on the basis of leaf consumption. A clear dose–response effect on gene silencing was observed for both fourth instar larvae (one-way ANOVA: low leaf consumption level—$F_{(2, 60)} = 2.67$; $P = 0.0778$; $dF = 62$; medium leaf consumption level—$F_{(2, 47)} = 51.11$; $P < 0.0001$; $dF = 49$, high leaf consumption level—$F_{(2, 43)} = 170.0$; $P < 0.0001$; $dF = 45$) (Fig. 6a) and fifth instar larvae (one-way ANOVA: low leaf consumption level—$F_{(2, 55)} = 0.29$; $P = 0.7488$; $dF = 57$; medium leaf consumption level—Kruskal–Wallis test: KW = 35.32; $P < 0.0001$; high leaf consumption level—one-way ANOVA: $F_{(2, 41)} = 165.8$; $P < 0.0001$; $dF = 43$) (Fig. 6b).

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

The high effectiveness of transgenic plants in the induction of *Sl 102* silencing and immune suppression prompted us to assess whether silenced *S. littoralis* larvae were more susceptible to a treatment with a *Bt*-based biopesticide (Xentari™), as previously reported in the case of *Sl 102* dsRNA delivered with different methods (Caccia et al. 2016, 2020) and of *Sl gasmin*, another immune gene (Di Lelio et al. 2019).

To take into account the potential discrepancy in Xentari™ efficacy when the bioinsecticide is used against larvae that have already fed and developed on a transgenic crop or larvae that come into contact with transgenic tobacco and the bioinsecticide more or less simultaneously, different bioassays were performed. Moreover, since the aim of the bioassays was measuring the effect of RNAi plants on Xentari™ efficacy, doses that were sublethal to controls, i.e., larvae fed WT plants, were used (see "*Sl 102* silencing by transgenic tobacco and effects on *Bt* killing activity" in Material and Methods for experimental details).

In the first set of experiments (sequential treatments), fourth instar *S. littoralis* larvae were alimented for 3 days on leaf squares of transgenic plants expressing *Sl 102* dsRNA and on Day 4 they were exposed with Xentari™ for 3 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 compared to controls (log-rank test: $\chi^2 = 271.5$, $P < 0.0001$, $dF = 5$) (Fig. 7a). Moreover, surviving larvae showed a significant reduction in the weight increase (one-way ANOVA: $F_{(5, 199)} = 448.7$, $P < 0.0001$; $dF = 204$) (Fig. 7d).

Based on the results obtained above (Fig. 5 and Fig. 3 in Supplementary material), after 24 h of feeding on transgenic plants, a significant level of gene silencing in fourth and fifth instar larvae was observed; thus, *Bt* treatment was performed on leaf squares for 3 consecutive days, starting 24 h after the onset of the bioassay. For both fourth and fifth instar larvae, the administration of *Sl 102* dsRNA-transgenic plants and Xentari™ caused significantly higher mortality in *Sl 102*-silenced larvae compared to controls (log-rank test—fourth instar larvae—$\chi^2 = 235$; $P < 0.0001$; $dF = 5$;

fifth instar larvae—$\chi^2 = 230.2$; $P < 0.0001$; $dF = 5$) (Fig. 7b and c). This was accompanied by a significant developmental impairment for both fourth instar (Kruskal–Wallis: KW = 119.0; $P < 0.0001$) (Fig. 7e) and fifth instar larvae (Kruskal–Wallis: KW = 135.1; $P < 0.0001$) (Fig. 7f).

Upon silencing, older instar larvae are thus more susceptible to *Bt* treatment, with minor changes depending on the duration of the feeding period on dsRNA-expressing plants. Indeed, although 24 h of feeding on *Sl 102* dsRNA-transgenic lines was sufficient to obtain an increase in Xentari™ effectiveness in fifth instar larvae (Fig. 7c), larvae fed on transgenic plants for all fourth instar resulted in significantly higher mortality on the last day (one-way ANOVA: $F_{(2, 6)} = 8.85$, $P = 0.0162$, $dF = 8$ for Line 1; $F_{(2, 6)} = 15.28$, $P = 0.0044$, $dF = 8$ for Line 2) (Fig. 7a, Table 2 in the Supplementary material).

Regardless of the administration protocol, bioassays clearly show that the application of the *Bt* formulate causes in a few days a dramatic drop of survival (around −50%) only in larvae fed transgenic tobacco lines expressing *Sl 102* dsRNA (Fig. 7a, b and c).

Discussion

Although a number of potential traits have been tested for plant engineering against insect pests, the expression of *Bt* toxins remains the most widely used (Anderson et al. 2019) and RNAi-plants are the focus of most research efforts and market considerations (Zhang et al. 2017; Anderson et al. 2019; Fletcher et al. 2020; Liu et al. 2020). The reasons for the lack of success of the "antinutritional" traits appear evident in some cases. For instance, although early achievements in PI-expressing plants were encouraging, coevolution between insect herbivores and host plants has led to the development of reciprocal adaptations (Zhu-Salzman and Zeng 2015; Singh et al. 2020). The ability of insects to cope with plant defense metabolites by a variety of mechanisms (e.g., remodeling of the digestive proteasome, expression of inhibitor-insensitive proteases, inactivation of PI) has thus hampered interest and discouraged further research on this strategy (Zhu-Salzman and Zeng 2015; Singh et al. 2020). On the other hand, the use of feed or food based on transgenic crops with antivitamins, such as avidin, was not favored. These compounds combine with vitamins reducing their availability; thus, supplementation of the vitamins in the diet or processing of the crop for the release of the vitamins from complexes is required. In addition, since a thorough risk assessment is still lacking, the presence of antivitamins in crops may suffer from a lack of public acceptability. The low success of lectin-expressing plants is likely because these proteins are potential food allergens (Barre et al. 2020). Although they proved to be very effective against pests, especially when fused with proteins from insect specific and even insect pest-specific arthropod venoms, no impact on beneficial insects has been observed (Gatehouse et al. 2011; Nakasu et al. 2014).

It is worth mentioning that the *in planta* coexpression of genes encoding insecticidal molecules with different modes of action can strongly enhance control effectiveness and delay the development of pest resistance (Sainsbury et al. 2012; Dormatey et al. 2020). This strategy (i.e., stacking or pyramiding), which is now routine for commercialized *Bt*-crops expressing multiple toxins, has also been attempted for antinutritional molecules (Boulter et al. 1990; Abdeen et al. 2005; Senthilkumar et al. 2010). Furthermore, plants have been transformed to express protease inhibitors or lectins with *Bt* toxins, leading to an enhancement of protection against both chewing and sucking insects (Fan et al. 1999; Maqbool et al. 2001; Su et al. 2011; Zhang et al. 2011; Boddupally et al. 2018).

Herein we have attempted a novel strategy of using transgenic plants as shuttles for molecules that can prepare insects for treatment with microbial control agents (MCAs) such as *B. thuringiensis*. The rationale behind the present work relies on the evidence that immunosuppressed insects are more susceptible to entomopathogens. Chen and coworkers have demonstrated that recombinant *Isaria fumosorosea* fungi expressing dsRNA targeting an immune gene (i.e., *Toll-like receptor 7*) in *Bemisia tabaci* are more virulent against this pest (Chen et al. 2015). Similarly, RNAi silencing of *AgraRelish*, a transcription factor of the IMD pathway in the Coleoptera *Anthonomus grandis*, improves the efficacy of the fungus *Metarhizium robertsii* (Moreira-Pinto et al. 2021). Interestingly, molecules of parasitic origin that impair immune responses in lepidopteran larvae have been proven to enhance entomopathogen performance against these insects. rVPr1, a protein from the venom of the endoparasitic wasp *Pimpla hypochondriaca* that affects hemocyte responses in several lepidopteran species, was able to enhance the susceptibility of *Lacanobia oleracea* and *Mamestra brassicae* larvae to *B. thuringiensis* and *Beauveria bassiana* (Dani et al. 2004; Richards et al. 2011, 2013; Richards and Dani 2010). The use of polydnaviruses or polydnavirus-derived factors to disrupt immune responses resulted in an increase of baculovirus infection in *S. littoralis* and *Manduca sexta* larvae (Rivkin et al. 2006; Washburn et al. 2000). Moreover, previous studies from our group have demonstrated that immunosuppressed *S. littoralis* larvae are more susceptible to *B. thuringiensis*. In particular, silencing of the *Sl 102* and *Sl gasmin* immune genes, both individuated in the framework of studies on host-parasitoid interactions (Di Lelio et al. 2014, 2019; Gasmi et al. 2015), led to an increase in the mortality of larvae treated with *B. thuringiensis* or *Bt* toxins (Caccia et al. 2016, 2020; Di Lelio et al. 2019).

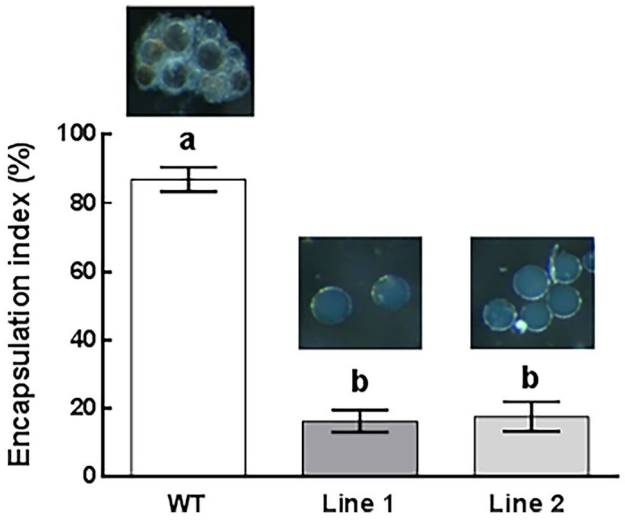

Fig. 4 Encapsulation index of *Spodoptera littoralis* larvae reared on wild type (WT) (controls) or *Sl 102* dsRNA-expressing (Line 1 and Line 2) tobacco plant leaves. The encapsulation response was significantly inhibited in *S. littoralis* larvae showing *Sl 102* silencing. Chromatography beads collected from the hemolymph of control larvae were completely encapsulated and melanized while hemocyte capsule formation was not observed in larvae fed transgenic plant lines. Mean values with different letters are significantly different ($P < 0.0001$, one-way ANOVA)

This evidence prompted us to use transgenic plants as vectors for immune suppressive molecules to impair the response of a phytophagous pest against entomopathogens. In the present study, transgenic tobacco lines that express dsRNA molecules targeting a *S. littoralis* gene (i.e., *Sl 102*) were produced. The target gene is involved in major cellular immune responses (i.e., nodulation and encapsulation) in *S. littoralis* larvae (Di Lelio et al. 2014; Caccia et al. 2016). When larvae were fed transgenic tobacco lines, *Sl 102* gene expression was reduced, and this reduction was reflected in the impairment of cellular immune responses in experimental larvae, as observed in our previous studies with naked and bacteria-delivered *Sl 102* dsRNA (Di Lelio et al. 2014; Caccia et al. 2016, 2020). Additionally, silencing was influenced by the amount of plant tissue consumed by the larvae, confirming the results of previous studies. Indeed, feeding bioassays with plants expressing dsRNA demonstrated the importance of the amount or duration of food consumption to obtain significant gene silencing and relative phenotypic alterations in several insects (Baum et al. 2007; Zhu et al. 2012; Mao and Zheng 2014; Han et al. 2017; Hou et al. 2019).

Importantly, the present work showed that once silencing was effective, *S. littoralis* larvae were more susceptible to the *Bt*-based insecticide Xentari™ and this effect was

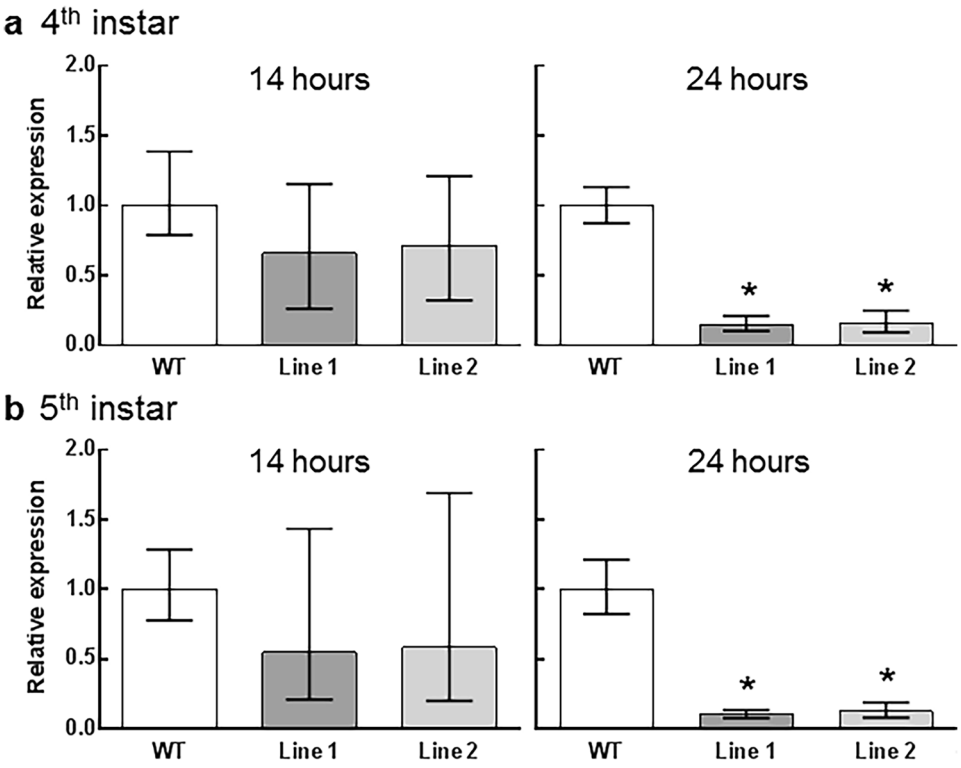

Fig. 5 Relative expression of the *Sl 102* gene in fourth and fifth instar larvae. The *Sl 102* transcript level was not affected after 14 h in larvae fed transgenic tobacco lines for either fourth instar larvae ($P = 0.056$, Kruskal–Wallis test) (**a**) or fifth instar larvae ($P = 0.3234$, Kruskal–Wallis test) (**b**). A significant reduction in *Sl 102* gene expression resulted after 24 h of feeding on transgenic plants for fourth instar larvae (**a**) and fifth instar larvae (**b**) ($P < 0.0001$, one-way ANOVA)

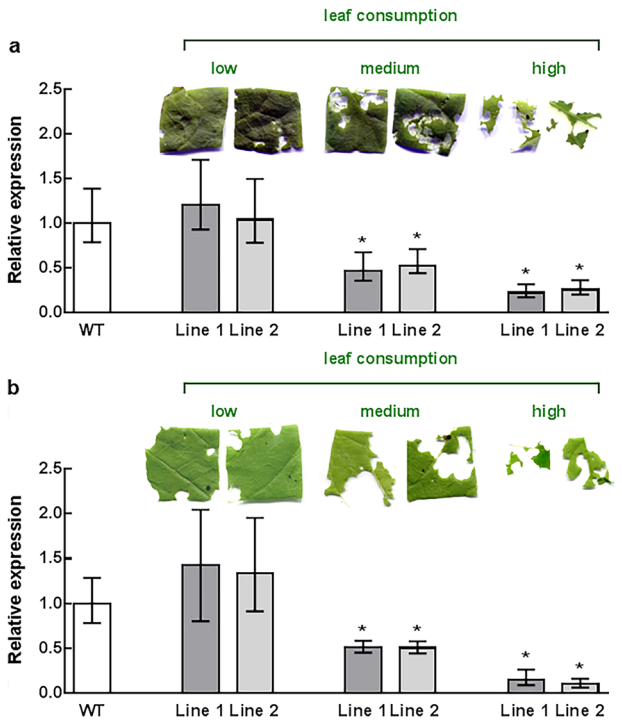

Fig. 6 Relative expression of the *Sl 102* gene in fourth and fifth instars larvae after 14 h, as affected by leaf consumption. The expression of the *Sl 102* gene was significantly lower, both for fourth (**a**) and fifth (**b**) instar larvae, only for medium and high leaf tissue consumption, compared to controls. Mean values with an asterisk are significantly different (*$P < 0.0001$, one-way ANOVA or Kruskal–Wallis test)

observed in advanced instars. This issue is very relevant from an application point of view since *Bt* sprays are less active on older instar larvae that cause major damage to the crop due to increased feeding (Bryant 1994; Navon 2000). This evidence also corroborates our recently proposed model on the role of septicemia in *Bt* mode of action (Caccia et al. 2016), and in particular the importance of insect immune responses to counteract the microbial invasion of the hemocoel through midgut *Bt*-induced midgut lesions.

The strategy proposed herein would be extremely valuable to control pest populations with overlapping generations that require very high doses of *Bt* product, as high doses are necessary to kill older larvae but are completely unneeded for younger larvae (Bryant 1994; Navon 2000). In this context, immunosuppressive plants would minimize the dose of *Bt* products (i) to obtain high mortality in all larval stages and (ii) to reduce selective pressure and retard the development of resistance in susceptible insects. In fact, although less frequent than in *Bt*-crops, resistance outbreaks to *Bt* formulations should be considered a major concern for the long-term use of such an effective bioinsecticide (Tabashnik et al. 1990; Janmaat and Myers 2003; Hernández-Martínez et al. 2010; Zago et al. 2013; Jurat-Fuentes et al. 2021).

Immune suppressive RNAi-plants may therefore represent a significant component of future IPM strategies against lepidopteran larvae that involve MCA application. In conclusion, the present work provides a proof of concept for the

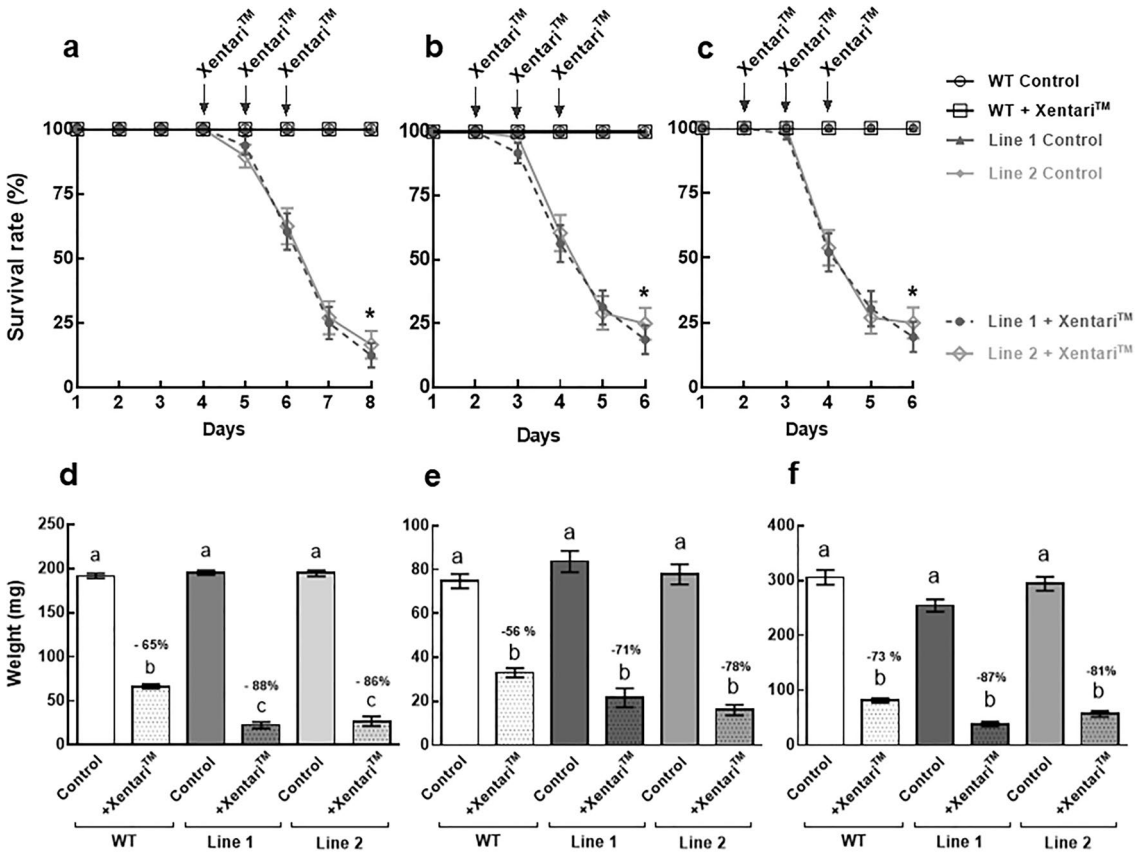

Fig. 7 Bioassay with *Spodoptera littoralis* larvae. In **a** fourth instar larvae were fed *Sl 102* dsRNA-transgenic plants before *Bt* treatment. Newly molted fourth instar larvae were alimented for 3 days with leaf squares from the experimental tobacco plants and then, soon after molting to the fifth instar, with 3 µg/cm^2 of Xentari™ for 3 additional days (see "Materials and methods" section for experimental details). In **b, c** larvae were concurrently exposed to dsRNA and *Bt*. Newly molted fourth instar (**b**) or fifth instar (**c**) larvae were alimented for 24 h with untreated experimental plant leaves and, for the following 3 days, with leaf squares sprayed with 1 µg/cm^2 (**b**) or 3 µg/cm^2 (**c**) of Xentari™, which were sublethal doses for larvae fed WT tobacco (see the "Materials and methods" section for experimental details). Survival was monitored until Day 8 (**a**) or Day 6 (**b, c**) when the weight of the surviving experimental larvae was assessed (**d–f**). The timing of *Bt* treatments is indicated by arrows. The values reported are the mean ± standard errors: curves that significantly differ from controls are indicated with an asterisk in (**a–c**) ($P < 0.0001$, log-rank Mantel–Cox test) and with different letters in (**d–f**) ($P < 0.0001$, Kruskal–Wallis test)

exploitation of RNAi-plants that create conditions necessary to boost MCA effectiveness against poorly susceptible larval stages of lepidopteran pests rather than directly killing agricultural and horticultural lepidopteran pests.

Authors' contributions

SC and RR conceived and designed research. EB, MC, SC, GC and IDL performed experiments. EB, MC, IDL and SC analyzed data. SC wrote the manuscript. All authors read and approved the manuscript.

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Declarations

Conflict of interest The authors declare no conflicts of interest/competing interests.

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