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RNA interference protocols for gene silencing in the spittlebug *Philaenus spumarius*, vector of *Xylella fastidiosa*

Cecilia Parise[1,2], Luciana Galetto[2]✉, Simona Abbà[2], Nicola Bodino[1], Cristina Marzachì[2] & Domenico Bosco[1]

RNA interference (RNAi) is double stranded RNA (dsRNA)-based gene silencing mechanism. Exogenous dsRNAs application to crops has raised as a powerful tool to control agricultural pests. In particular, several sap-feeder are important plant pathogens vectors, such as *Philaenus spumarius*, known as main vector of *Xylella fastidiosa* (Xf), causal agent of olive quick decline syndrome (OQDS) in southern Italy. Here, dsATP synthase beta (dsATP), dsLaccase (dsLacc) and dsGreen Fluorescent Protein (dsGFP) as control, were provided to spittlebug adults by microinjection or to nymphs fed on dsRNA-treated plant shoots. Treated insects were collected at different time points to monitor silencing efficiency over time, describing significant reduction of transcript levels from 8 to 24 days post treatment. Downregulation of target genes ranged from 2- to 16-fold compared to the corresponding dsGFP controls, where highest silencing effects were generally noticed for ATP synthase beta. Sequencing of libraries obtained from total smallRNA (sRNA) showed the generation of dsRNA-derived sRNAs by RNAi pathway, with majority of reads mapping exclusively on the correspondent dsRNA. Also, we characterized components of a functional RNAi machinery in *P. spumarius*. Further research is needed to clarify such mechanism, screen effective target lethal genes to reduce vector population and improve delivery strategies.

Keywords Double stranded RNA, dsRNA delivery strategies, Microinjection, Plant-mediated feeding, smallRNA sequencing

Philaenus spumarius L. (Hemiptera: Aprophoridae) is a xylem-sap-feeder insect, with a very large distribution in the Palaearctic Region, from North Lapland to the Mediterranean Basin[1]. In Italy it is a common species, locally very abundant, probably due to its highly polyphagous behaviour[2]. After the discovery of *Xylella fastidiosa* subsp. *pauca*, Wells et al. (Xanthomonadales: Xanthomonadaceae) (*Xf*) in 2013[3], *P. spumarius* has been soon identified as the main vector of this plant pathogenic bacterium[4], causal agent of the Olive Quick Decline Syndrome (OQDS) observed in Apulia region[5,6]. Nowadays, control methods against *Xf* spreading are aimed to reduce spittlebug populations inside olive groves using insecticides[7] as well as soil tillage and cover crop management[8,9]. In late spring, newly emerged adults move from herbaceous plants to olive tree canopies. When they feed on infected olives, they acquire and readily transmit *X. fastidiosa* in a persistent manner, until the end of the summer, when males and females move back to the herbaceous vegetation for mating and laying eggs[1,10].

RNA interference (RNAi) is a gene silencing mechanism based on double stranded RNA (dsRNA) already documented in many insects[11–13]. Although RNAi was originally investigated mainly for functional genomic studies, more recently, it raised as a powerful and promising tool for agricultural pest management[14]. In this perspective, several issues have to be solved, related to dsRNA delivery[15] and the variable response to dsRNAs among insect orders[16]. Coleopterans, such as *Diabrotica virgifera virgifera*[17], *Tribolium castaneum*[18–20] and *Leptinotarsa decemlineata*, Colorado Potato Beetle, CPB[21], are particularly sensitive to RNAi. Indeed, Ledprona is the first double-stranded RNA-based biopesticide available in the market to control CPB[22]. On the other hand, insects belonging to Hemiptera, Orthoptera, Diptera, Hymenoptera and Lepidoptera orders show some variation in their responses to dsRNA administration[16,23]. Differences in silencing response have been ascribed

[1]Università degli Studi di Torino, DISAFA, Largo Paolo Braccini 2, 10095 Grugliasco, TO, Italy. [2]Istituto per la Protezione Sostenibile delle Piante, CNR, Strada delle Cacce 73, 10135 Torino, Italy. ✉email: luciana.galetto@ipsp.cnr.it

to biochemical and physiological characteristics of insects, like the presence of nucleases in their salivary glands, midgut and/or hemolymph degrading dsRNA molecules[23]. Also, molecular internalization, cell uptake and intracellular transport of dsRNAs vary among insect species together with the genes involved in the RNAi core machinery[16,23]. Nevertheless, to date RNAi technology has been explored against many hemipteran species, likewise sharpshooters[24], psyllids[25–27], aphids[28], planthoppers and leafhoppers[29–32], giving promising results. Despite the major importance of *P. spumarius* as a vector in Europe, to date, RNAi functioning and efficacy have never been investigated in this species.

The present work aims at exploring the occurrence and the effectiveness of RNAi mechanism in spittlebugs. As a first step, the core genes involved in RNAi response in insects were identified in silico. Then, ATP synthase beta and laccase (*LACC1*) were selected as possible target genes, because their silencing caused phenotypic alterations in other insect species[17,32]. ATP synthase beta is a major component of the multisubunit-enzyme ATP synthase and is involved in maintaining the proton gradient generated by the respiratory process, which is necessary for ATP production[33]. In the leafhopper *Euscelidius variegatus*, a well-known phytoplasma vector, it was demonstrated that silencing of ATP synthase beta gene increases insect mortality, reduces phytoplasma multiplication in vector bodies and induces female sterility[34,35]. On the other hand, laccase encodes a phenol oxidase, responsible for body pigmentation and sclerotization in coleopterans. Indeed, silencing of homologous gene induces a visible phenotype with reduced black pigmentation in larvae[17,18]. Finally, the work aims at optimizing dsRNA delivery methods for this species, exploring abdominal microinjection into adults and dsRNA treatment of nymphs through feeding in a plant-mediated assay. To prove efficacy of the mechanism the level of specific transcripts was measured over time and smallRNA libraries were sequenced.

Materials and methods

Insect collection and rearing

Philaenus spumarius nymphs and adults were collected in Piedmont Region in 2023 from the end of May until August. The collection sites were in Chieri (TO, 45.0154139 N, 7.79305 E, 355 m asl) and Bellino (CN, 44.574510 N, 6.959477 E, 2012 m asl). Gathered insects were reared on *Vicia faba* and *Cichorium intybus* plants (obtained from purchased seeds) as well as on *Pinus* spp. saplings (purchased from a nursery), inside net-plastic cages (Bugdorm, 45 × 45 × 90 cm, LxWxH). The growth chamber was maintained at temperature-lag from 18 to 25 °C and a photoperiod of 16:8 h (L: D).

Selection of target and reference genes

Preliminary bibliographic search was done to select target genes that, once silenced, might induce an overt phenotype. ATP synthase beta and laccase (*LACC1*) genes were selected as potential candidates, whereas elongation factor 1 and glutathione S transferase as reference genes to normalize the expression of target transcripts. Sequences of ATP synthase beta, elongation factor 1 and gluthatione S transferase were retrieved from *P. spumarius* transcriptome (BioProject: PRJNA272277) accessible at NCBI under the following accession numbers GCZA01079300.1, GCZA01082020.1 and GCZA01087749.1, respectively. Sequence of laccase (*LACC1*) gene was retrieved from *P. spumarius* genome (BioProject: PRJNA602656) accessible at NCBI under JAGFPH010007646.1 accession number (position 57603–60449). Derived dsRNAs sequences were in silico screened to spot identities with corresponding homologues of *Apis mellifera*, in order to avoid non-target effects on honeybee in a possible future field application.

RNA extraction

P. spumarius total RNA was extracted from single insects using a Direct-zol RNA Mini Prep Kit (Zymo Research, Irvine, CA, USA). Samples were maintained in liquid nitrogen and separately crushed in a 1.5 mL Eppendorf tube using a sterile micropestle and homogenized in 500 µL QIAzol Lysis Reagent (QIAGEN Sciences, Germantown, MD, USA). Then, the instruction protocol furnished by the manufacturer was followed and finally RNA samples were eluted in 30 µL DNAse/RNAse-free sterile water. The optional DNAse treatment step was performed for all the RNA extractions, in order to avoid genomic DNA contamination. Evaluation of concentration, quality and purity of the extracted RNA was analysed with a Nanodrop ND-1000 spectrophotometer.

Synthesis of dsRNA molecules

Aliquots of RNAs extracted from spittlebugs were retrotranscribed (500 ng) using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The corresponding cDNA was the starting material for the dsRNA molecules production. Fragments of the two target sequences were amplified trough PCR using specific primers both flanked at their 5′-end by the T7 promoter sequence (Table 1).

The PCR products were purified with DNA clean and concentrator kit (Zymo Research, Irvine, CA, USA) and ligated into pGEM-T Easy vector (Promega, Madison, WI, USA). Resulting plasmids were then cloned and purified using the ZR Plasmid Miniprep kit (Zymo Research, Irvine, CA, USA) and Sanger sequenced (BMR Genomics, Padova, IT). The plasmids were then used as templates for the subsequent PCRs with the corresponding primer pairs and, averagely 1–2 µg of each purified PCR products were in vitro transcribed using the MEGAscript RNAi kit (Thermo Fisher Scientific, Waltham, MA, USA), resulting in double-stranded RNAs targeting ATP synthase beta (dsATP) and laccase (*LACC1*) (dsLACC). Also, dsRNA quality and quantity were checked with Nanodrop ND-1000 spectrophotometer.

Methods of dsRNA delivery

Molecules of dsRNA were delivered according to two protocols: abdominal microinjection to spittlebug adults and nymph feeding on dsRNA-treated plant shoots after petiole absorption (plant-mediated feeding assay).

Application	Primer name	5′–3′ sequences*	Target gene	Product size (nt)
dsRNA synthesis	ATP_F_T7	*TAATACGACTCACTATAG*CTGCCACTACATTCGCTCAC	ATP synthase beta	389
	ATP_R_T7	*TAATACGACTCACTATAG*GTGATCGTAGTCTCCTGCCA		
	Lacc_F_T7	*TAATACGACTCACTATAG*GAATTTGATCTGCCAGAACA	laccase (*LACC1*)	397
	Lacc_R_T7	*TAATACGACTCACTATAG*TCCAGTAGTTGTCAATATCT		
	GFP_F	*TAATACGACTCACTATAG*CTTTTCACTGGAGTTGTCCC	green fluorescent protein	402
	GFP_R	*TAATACGACTCACTATAG*GAAACATTCTCGGACACAAA		
Real-time PCR	ATP_F462	CCGTGATCAGGAAGGACAGG	ATP synthase beta	180
	ATP_R641	GAGCCCTTCTTTGTGGTGG		
	Lacc_F173	GAGGTTCCCCGTTTATGGAT	laccase (*LACC1*)	198
	Lacc_R370	GATCCGAATGAGGGTCTTGA		
	EF1_F701	CTATCCTCCCACCTAGCCGA	elongation factor 1	112
	EF1 _R812	ACACCAGTTTCGACTCGTCC		
	GST1_189	CCAGTGATGTTTGGTGGAGC	glutathione S transferase	116
	GST1_R304	ACATGATCTCCGGCTGCAAA		

Table 1. List of primers for this work. *T7 promoter is italicised.

Microinjection

Newly emerged adults of *P. spumarius* were anaesthetized with CO_2 and microinjected between two abdominal segments under a stereomicroscope using a fine glass needle connected to a Cell Tram Oil microinjector (Eppendorf, Hamburg, Germany). Insects were injected with 1 µL of filter sterilized Tris–EDTA buffer added with dsRNAs at the concentration of 80 ng μL^{-1}. In average, 30 insects were used in each treatment. Injected insects were then kept, in net-plastic cages (Bugdorm, 45 × 45 × 45 cm, L × W × H) on broad beans. During the assay, the spittlebugs were monitored daily and sampled at 3-, 8-, 15-, and 24-days post injection (dpi). Dead insects were removed periodically. For each treatment, head and thorax + abdomen of the collected insects were dissected and separately stored in 1.5-mL Eppendorf tube at − 80 °C.

Plant-mediated feeding assay

Healthy *Vicia faba* shoots (15–20 cm length) were collected and immersed in a falcon tube with 4 mL solution containing 20 µg of dsRNAs in DEPC water. The dsRNA solution was completely absorbed in 24 h by the plant shoots. Nine replicates were made, three for each treatment, ATP synthase beta, laccase (*LACC1*), and dsRNA targeting green fluorescent protein (dsGFP) synthetized as detailed in Abbà et al. (2019), as control. In each of the nine replicates 10 *P. spumarius* third/fourth instar nymphs, previously starved for one hour, were allowed to feed for 24 h. Then, the nymphs were moved onto fresh broad bean plants, whereas treated shoots were collected and stored at − 80 °C to check the presence of dsRNA molecules.

Gene expression analysis

Total RNAs were extracted from heads and thorax + abdomen of single injected adults as well as from fed nymphs, as detailed above. RNA samples were retro-transcribed and used to quantify the silencing efficiency of each dsRNA by quantitative PCR (qPCR). At least four biological replicates were analysed for each sampling date and treatment. cDNA was synthetized from 400 ng RNA for nymphs and 500 ng RNA for adults, using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The resulting cDNA was employed as template for real-time PCR in a total volume mix of 10 µL, containing 1× iTaq Universal Sybr Green Supermix (Bio-Rad, Hercules, CA, USA) and 1 µL of each primer diluted at 3 µM. Samples were run in duplicate in a CFX Connect Real-Time PCR Detection System (Bio-Rad). Cycling conditions were: 95 °C for 3 min, and 40 cycles at 95 °C for 15 s and 59 °C for 30 s of annealing/extension step. The specificity of the PCR products was verified by melting curve analysis for all samples. GFP controls were always included in each plate. Glutathione S transferase and elongation factor 1 of *P. spumarius* (Table 1) were used as reference genes to normalize transcript levels among samples. Normalized expression levels of each target gene for each sample were calculated by CFXMaestro™ Software (Bio-Rad), which integrates normalization of both reference genes.

Small RNA sequencing

Total RNAs extracted from abdomen + thorax samples after injection of either dsATP, dsLACC or dsGFP were sent to Macrogen Inc. (South Korea) for smallRNA (sRNA) library construction and sequencing. Three biological replicates were prepared for each condition. Briefly, libraries were prepared with SMARTer smRNA-Seq Kit for Illumina (Takara Bio, USA) and sequenced by NovaSeq X (2 × 150). Raw reads were trimmed from adapters with Cutadapt, version 4.6[36], and filtered out according to (a) quality and length (minimum 17 nt; maximum 34 nt) and (b) mapping onto the *P. spumarius* ATP synthase beta and laccase (*LACC1*) coding sequences and onto the GFP coding sequence (control). Bowtie version 1.1.2 software[37] with no mismatch allowed in the alignment was used to establish sRNA abundance profiles of the nine sequenced samples. Following alignment, the resulting SAM files were converted to BAM format, sorted by position and indexed using SAMtools version 1.9[38] and visualized with the Integrative Genomics Viewer (IGV)[39]. sRNA counts were normalized for differences in

sequencing depths to account for the technical differences across samples. The smallRNA datasets generated during the current study are available in the SRA NCBI repository, (BioSample accessions: SAMN41600531-39).

Translocation of dsRNAs in plant shoots

Vicia faba shoots treated with dsRNA molecules were collected 2 days post absorption (dpa) to analyse if dsRNA molecules were translocated through petiole adsorption to apical broad bean leaves. Total RNA from broad bean shoots were extracted using Spectrum Plant total RNA kit (SIGMA-ALDRICH, Germany), according to manufacturer's instructions. Plant cDNA was obtained through reverse transcription as detailed above, starting from 500 ng RNA and it was used for qualitative PCR analyses. The total PCR volume per reaction was 25 µL, 5 µL of Buffer 5X, 0.25 µL AMPIGENE Taq-DNA-polymerase (Enzo, Farmingdale, NY, USA) and 0.5 µL for each primer with T7 promoter at 10 µM concentration. Five µL of each PCR products were electrophoretically separated into 1% agarose gel stained with Ethidium bromide and visualized on a UV transilluminator.

Data analysis

Normalized gene expression data for each sample were transformed in natural logarithm. In the case of nymphs fed on plant, target expression levels higher than the mean of the corresponding GFP controls were excluded from the analysis as outliers. One-way ANOVA test or Kruskal–Wallis One Way Analysis of Variance on Ranks, when normality test failed, were used to compare grouped expression level for each sampling date and treatment. To isolate the group or groups differing from the others, Dunn's Method multiple comparison was used. Parametric t-test or nonparametric Mann–Witney test, in case of not normally distributed data, were applied to compare transcript level of each treatment and the respective control. SigmaPlot version 13 (Systat Software, Inc., San Jose, CA, USA) was used to run the statistical analyses.

Results

RNAi machinery in *Philaenus spumarius*

As a first step, to understand whether a functional RNAi machinery exists in *P. spumarius*, the transcriptome assembly (BioProject: PRJNA272277) was searched for genes involved in the RNAi pathway (Table 2). The presence of all major genes involved in the three main insect RNAi mechanisms (miRNA, siRNA and piRNA pathways) confirmed the presence of a potentially functional RNAi machinery in this species.

Down regulation of target genes in *Philaenus spumarius* adults through dsRNA microinjection

Abdominal microinjection of dsRNAs was performed on *P. spumarius* adults. The injected spittlebugs were monitored daily and sampled at 3-, 8-, 15- and 24-dpi. Gene expression analyses was carried out on RNA separately extracted from head and thorax + abdomen of each treated insect to determine the silencing level of each correspondent gene transcript. Since no significant differences on target gene expression were detected in dsGFP-treated bodies among sampling dates, all data from the control samplings were grouped (Figs. 1 and 2). Similarly, target transcripts measured in dsATP- and dsLACC-treated insects collected at different sampling dates were pooled together, as no relevant variation was observed over time. A significant downregulation of both target transcripts was measured after the injection of dsATP (Mann-Whitney, $U = 9.000$, $T = 297.00$, $P < 0.001$) and dsLACC (one tailed t test $P = 0.021$) in adult spittlebug bodies (Figs. 1a and 2a), showing on average a 7- and 5-fold reduction compared to the dsGFP controls, respectively. Moreover, ATP synthase beta transcripts were significantly reduced at 8 dpi and 15 dpi (Dunn's multiple comparison $P = 0.001$, $P = 0.021$) compared to the dsGFP controls (Fig. 1b). On the other hand, for laccase (*LACC1*), significant gene down regulation was not observed at different sampling dates compared with the controls (Fig. 2b).

In addition, to explore dsRNA translocation and silencing efficiency in body parts far from the injection site, heads were separated from the rest of the body and independently analyzed. Silencing effects were observed in the heads of the dsATP-injected insects (Fig. 1c), with an average 7-fold transcript reduction in comparison with dsGFP-treated insects over the entire time course of the experiment (Mann-Whitney, $U = 0.000$, $T = 306.000$,

Philaneus spumarius[a] hits	*Tribolium castaneum*	*Tribolium castaneum* gene description	Identity[b]	Query coverage[b]
GCZA01056520.1[c]	XM_015982502.1	argonaute-1	96%	53%
GCZA01058587.1[c, d]	XM_008194764.2	argonaute-2b	53%	51%
GCZA01057758.1	XM_008194203.2	argonaute-3	43%	95%
GCZA01074397.1	NP_001107840.1	dicer-2	37%	98%
GCZA01066818.1[c]	XM_008200208.2	RISC-loading complex subunit tarbp2	65%	90%
GCZA01056261.1[c]	XM_015980716.1	microprocessor complex subunit DGCR8	61%	66%
GCZA01080497.1	XM_015981934.1	aubergine	51%	87%
GCZA01057770.1	NP_001099012.1	Sid-1-related A precursor	35%	75%

Table 2. Names and NCBI accession numbers of the genes involved in the insect RNAi pathways and systemic RNA interference deficient-1 (SID1), responsible of dsRNA uptake. [a]*Philaenus spumarius* accession numbers are referred to NCBI TSA GCZA00000000.1 (BioProject: PRJNA272277). [b]Percentages of sequence identities and query coverage between *Tribolium castaneum* RNAi pathway genes and the best hits found in *Philaenus spumarius* transcriptome. [c]Incomplete transcript, at the 5′ or the 3′ or both ends. [d]Transcript with Ns.

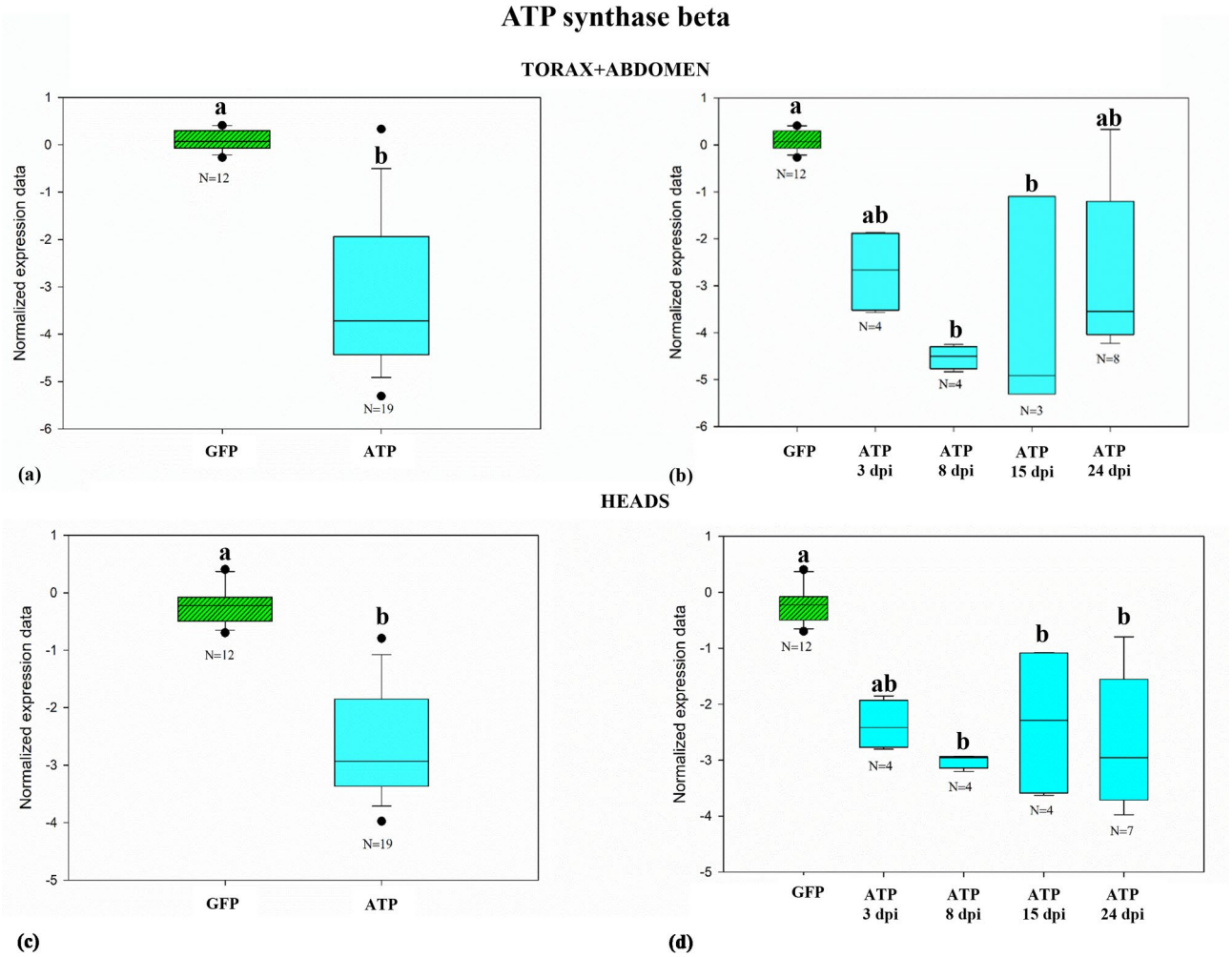

Fig. 1. Expression level of ATP synthase beta gene measured in *Philaenus spumarius* adults injected with dsATP or dsGFP. Data derived from torax + abdomen samples were evaluated considering all the sampling dates together (**a**) or separately (**b**). The same criteria were applied to results derived from spittlebug heads (**c**,**d**).

$P < 0.001$). In addition, ATP synthase beta transcripts measured in heads sampled at different dates post injection were significantly reduced at 8-, 15- and 24- dpi (Dunn's multiple comparison $P = 0.01$, $P = 0.043$, $P = 0.001$ respectively) compared to the dsGFP control (Fig. 1d). In the case of laccase (*LACC1*), if all time points were considered together, no reduction in the transcript levels was observed in heads (Fig. 2c). Despite a general reduction trend, there were no significant differences between the transcript level registered at different sampling dates and the dsGFP controls (Fig. 2d).

Possible off-target effects against honeybee of the produced dsRNA molecules were in silico tested. The percentage of shared identity between *A. mellifera* homologous genes and correspondent *P. spumarius* dsRNA designated sequences, plus the presence of at least three stretches of contiguous and identical 21 nt fragments[40] were the two criteria evaluated. *A. mellifera* ATP synthase beta and laccase sequences were 82% and 71% identical to dsATP and dsLACC, respectively. Stretches of 21 nucleotides identical to the honeybee sequences were completely absent in the designed dsRNAs targeting the selected *P. spumarius* correspondent genes.

Generation of dsRNA-derived smallRNA

Nine smallRNA libraries (three replicates for each treatment) were constructed from total RNA extracted at 8 dpi. On average, we obtained 19.1 M of clean PE reads per library (min. 15.8 M, max. 29,9 M). SmallRNA reads mapping onto the dsATP, dsLACC and dsGFP sequences were found in all the three replicates of each treatment (Table 3). These observations confirmed that the silencing mechanism was active at 8 dpi in all *P. spumarius* samples (dsATP, dsLACC and dsGFP). Moreover, smallRNA exclusively mapped onto the sequence fragment corresponding to the injected dsRNAs, although with an uneven distribution (Fig. 3a–c).

For each treatment the length of the target gene coding sequence, the length of the dsRNA molecules and the number of read counts separately obtained for the three replicates per treatment are reported.

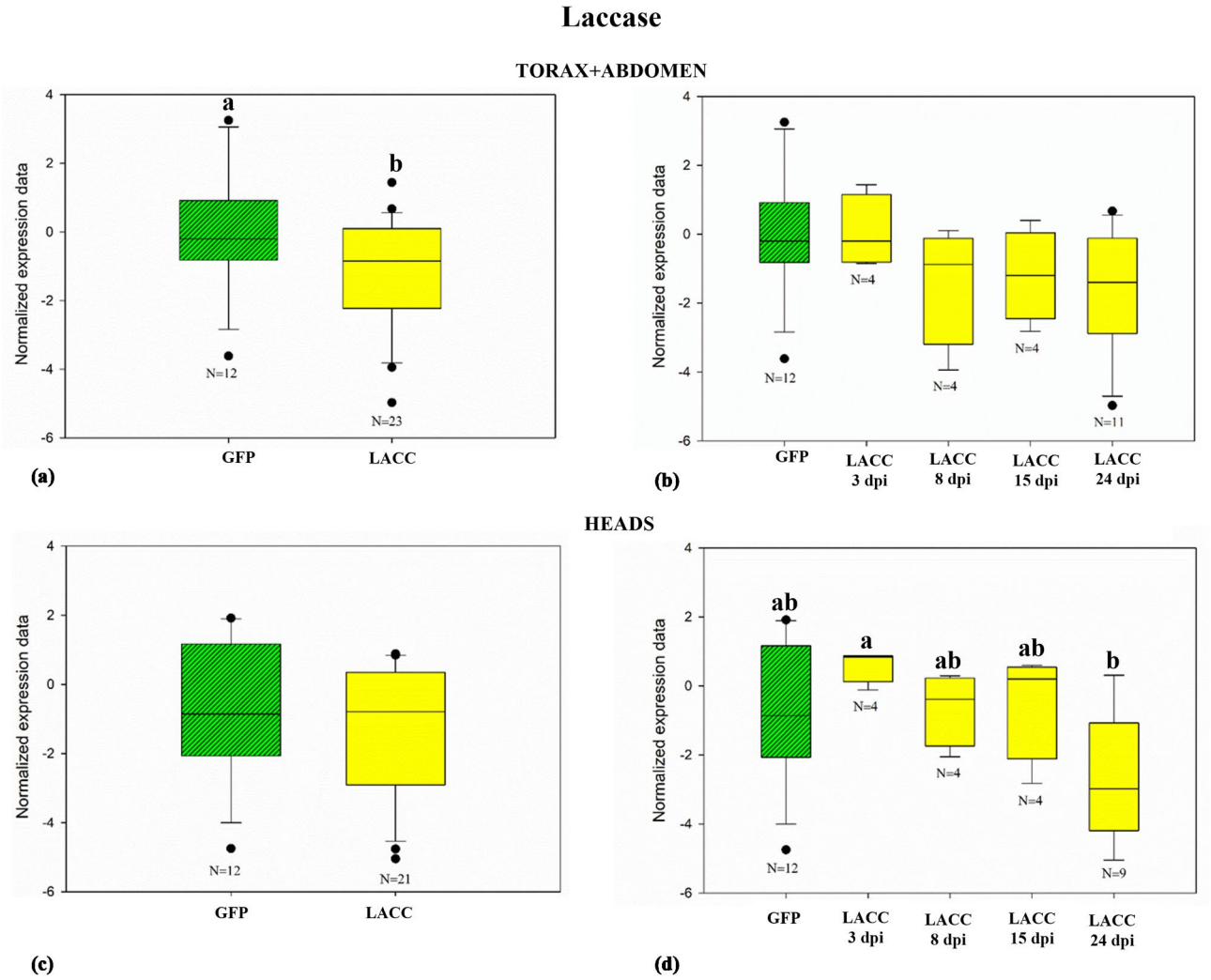

Fig. 2. Expression level of laccase gene measured in *Philaenus spumarius* adults injected with dsLACC or dsGFP. Data derived from torax + abdomen samples were evaluated considering all the sampling dates together (**a**) or separately (**b**). The same criteria were applied to results derived from spittlebug heads (**c**,**d**).

Target	Lenght of gene coding sequence	dsRNA length	Read counts		
			Replicate 1	Replicate 2	Replicate 3
*P. spumarius*_ATP synthase beta	1116	389	30,240	11,162	84,461
*P. spumarius*_laccase (LACC1)	646	397	60,584	9291	28,930
GFP	720	402	416	193,080	168

Table 3. Derived smallRNAs profile from dsATP, dsLACC and dsGFP microinjected spittlebugs.

dsRNA uptake and translocation in broad bean shoots

Vicia faba shoots used in plant-mediated feeding assay (Fig. 4a), were screened to determine if dsRNA molecules were internalized in plants by petiole adsorption, and to assess if they could trigger RNAi in insects fed on treated plants, thus silencing the selected target genes of spittlebug. Total RNA extracted from apical leaves of treated broad bean shoots was analysed in RT-PCR to assess the presence of the dsRNAs. Samples from leaves collected 2 days after dsRNA treatment displayed bands of the expected size as the administered dsRNA molecules (Fig. 4b), indicating that these molecules translocated into broad bean vascular system and could be acquired by insects feeding in the xylem vessels.

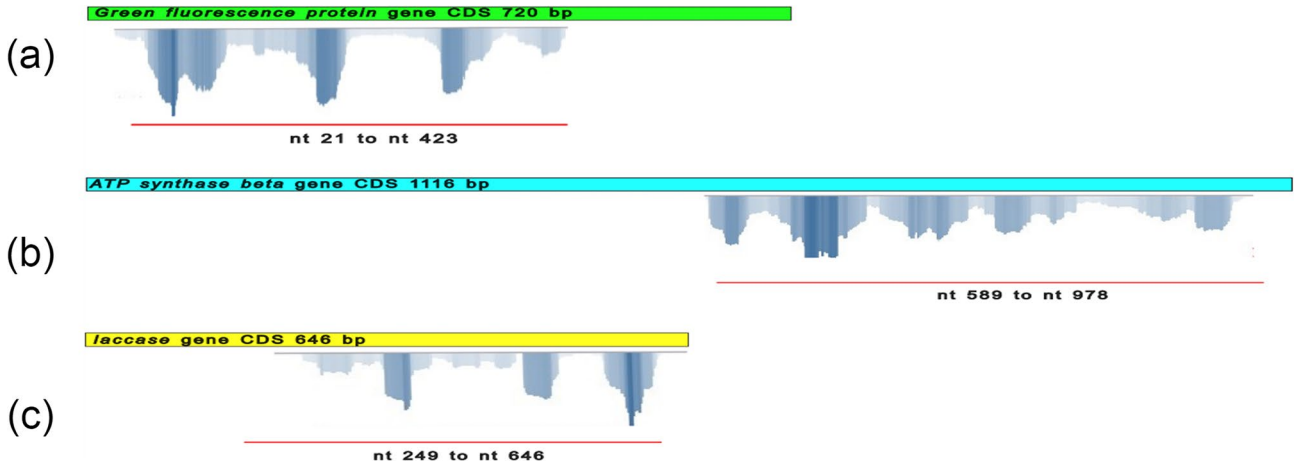

Fig. 3. Derived smallRNAs profile from dsATP, dsLACC and dsGFP microinjected spittlebugs. The smallRNA coverage along the produced dsRNA (red lines) and the full length coding sequences (green, blue and yellow boxes) were described for green fluorescent protein (**a**), ATP synthase beta (**b**) and laccase (**c**).

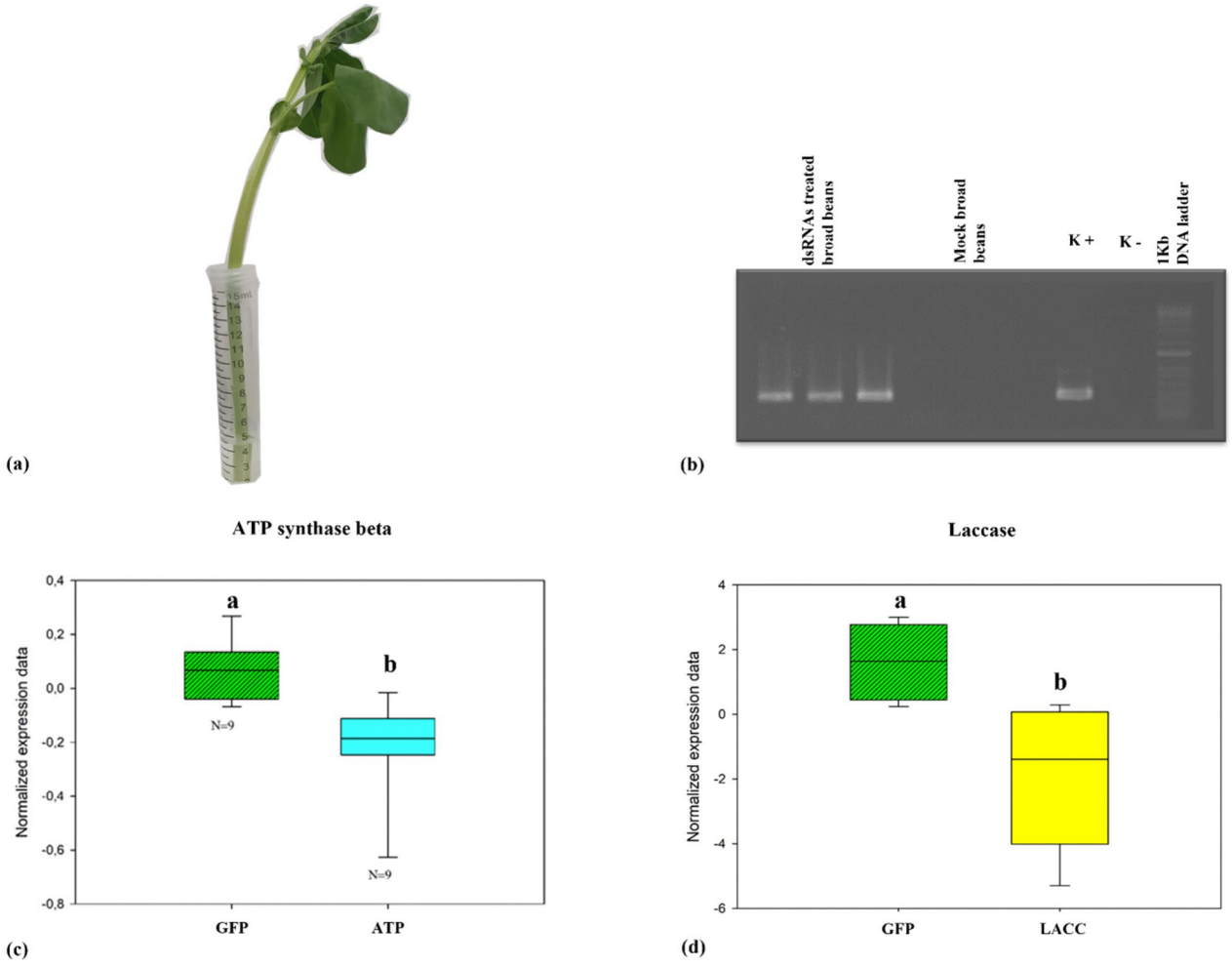

Fig. 4. Plant-mediated feeding assay. Experimental setting used in plant-mediated dsRNA feeding assay to *Philaenus spumarius* nymphs (**a**). Verification of the dsRNA acquisition and retention ability in broad bean apical leaves by RT-PCR (**b**, Supplementary Fig. S1). Target gene silencing in nymphs fed on dsATP- (**c**) and dsLACC- (**d**) treated plant shoots, in comparison with nymphs fed on dsGFP-treated shoots.

Down regulation of target genes in *Philaenus spumarius* nymphs fed on dsRNA treated plant shoots

In the plant-mediated feeding assay, third/fourth instar nymphs were forced to feed on broad bean shoots previously immersed in 4 mL solution containing dsRNAs (Fig. 4a). At first, the t-test was performed for each treatment to highlight any difference in transcript levels among the two sampling dates (1 dpa and 4 dpa). No difference was observed between the two time points, so the corresponding data were grouped together. For both ATP synthase beta (Fig. 4c) and laccase (*LACC1*) (Fig. 4d) genes a significant lower level of corresponding transcripts were measured in comparison with those of dsGFP-treated control insects (Mann- Whitney, $U = 2.000$, $T = 47.00$, $P < 0.001$ for ATP synthase beta and $U = 1.00$, $T = 37.000$, $P < 0.001$ for laccase (*LACC1*)), confirming that RNAi occurred in both treatments. In average, a 16-fold reduction of target gene expression for nymphs fed on dsLACC-treated plant shoots, and a 1.3-fold downregulation for dsATP were measured. No reduction in black pigmentation was observed in treated nymphs.

Discussion

Gene silencing has been investigated in many insect species, firstly with the scope to elucidate gene function and regulation, then as a biotechnological promising tool for integrated pest management[11,24,26]. The present work represents the first attempt to cover the lack of knowledge about RNAi in spittlebugs, and namely in *P. spumarius*, the most important vector of *X. fastidiosa* in Europe. The main genes involved in RNAi core machinery were identified in this species, and the downregulation of target genes was evaluated after dsRNA delivery to nymphs and adults. In detail, a publicly available *P. spumarius* transcriptome was screened looking for the genes coding the major components of the RNAi machinery (i.e. argonaute-2, dicer-2, *RISC*), which are indeed expressed, as previously described for other hemipterans[34,41,42]. Beside the presence of the core RNAi machinery genes, target genes have been chosen and, corresponding dsRNA sequences designed. In our assay two target genes were selected, ATP synthase beta and laccase (*LACC1*). The microinjection of the corresponding dsRNAs into *P. spumarius* adults resulted in transcript downregulation for both genes, although with different efficiencies. ATP synthase is a mitochondrial protein, composed by five subunits, which, using proton gradient produced by the respiratory chain, synthesizes ATP[33]. It is essential for energy metabolism, lipids transport, juvenile hormone binding protein interaction, insect fitness and fertility[32,35,43–45]. In particular, silencing of ATP synthase beta subunit results in impaired larval growth, suppressed oogenesis and increased sterility[32,44,45]. On the other hand, laccase is responsible for body pigmentation and cuticular sclerotization in many species, and its downregulation determine visible phenotypes, lacking in black pigmentation[17,18,46]. In this work, we provided evidence of suppression of the cognate transcripts, whilst the effect of the downregulation of these genes on *P. spumarius* fitness (survival, fecundity, etc.) is currently under investigation. Diversity in RNAi efficiency of the two targets, could be explained by the laccase (*LACC1*) gene expression variability over time, especially before insect adulthood. Indeed, for the laccase (*LACC1*) gene, difference in temporal patterns of expression during insect development are described, with highest transcription levels just before moulting, when pigmentation is more intense, and lower levels right after moulting, when the insect is whitish and pale[18,46]. Interestingly, ATP synthase beta is expressed at different cellular districts, besides mitochondria[47,48], and its silencing was observed in many organs of treated insects[45]. Hence the derived protein is constantly expressed and active at a higher level compared to laccase, whose expression varies also between single insects and it may be linked to the well-known polymorphism of *P. spumarius* body pigmentation[49,50], resulting in various silencing efficiencies upon treatment with the appropriate dsRNA. In addition, silencing variability between dsATP and dsLACC could be addressed to the molecule design. Selecting species specific genes or not conserved regions[51] and avoiding stretches of 21 nt identical to homologous genes of not-target organisms[40] are possible strategies to minimize potential off-target effects of dsRNAs. In our case, dsLACC shared 71% homology with honeybee laccase and sequences of identical 21 nt have not been found. However, it could be hypothesized that the target gene fragment chosen for dsRNA construction was not the best in terms of RNAi efficacy. On the other hand, the homology between dsATP and ATP synthase beta gene of *A. mellifera* was 82%, sharing more similar sites than those shared by dsLACC and honeybee laccase, but still always below 21 identical nucleotides. The higher homology of dsATP could be due to the high conservation of ATP synthase beta among different organisms, even if the choice of a conserved gene as RNAi target could be risky in terms of undesirable off-target effect.

In microinjection assay, silencing effects were observed not only in spittlebug organs close to the administration site (thorax + abdomen), but also in head samples. Indeed, the downregulation of the ATP synthase beta gene was measured on both the body districts analysed, confirming the knockdown of target transcripts in tissues distal from the site of dsRNA application. The dsRNA transport in insect is indeed reported as a general phenomenon[52]. On the other hand, in the case of the laccase (*LACC1*) gene, specific silencing was less evident away from the dsRNA injection site, probably due to such gene expression variability along time[18,46] and between spittlebugs, instead of the microinjection location. In any case, dsATP molecules were able to translocate through spittlebug hemocoel to reach other organs, as also described in the kissing bug, *Rhodnius prolixus*[45], and in aphids[28], confirming systemic spread of the RNAi signal. Systemization of silencing message in insects remains unclear. In fact, systemic RNAi in plants is based on RNA dependent RNA polymerase (RdRp) and the resulting smallRNA displacement occurs through plasmodesmata[15]. RNA dependent RNA polymerases are present in nematodes[53], but not yet described in insects[54]. In nematodes, the systemic RNA interference deficient-1 (SID1) is an essential component involved in dsRNA uptake and translocation[55]. SID1 homologues have been reported in several insects[54] and also in silico found in spittlebug, but their function has not yet been clarified[15]. However, alternative translocation modes, including nanotube-like structure and exosome-like vesicle transporting secondary smallRNA derived from viral infection have been found in *Drosophila*[56,57], proving that RNAi systemization in insect body occurs but its mechanism remains largely unknown.

Transcript downregulation in *P. spumarius* was monitored over time, and the most efficient silencing effects were between 8 and 15 dpi, in line with what observed for other insects[35,45,58]. However, both local and systemic downregulation of ATP synthase beta transcripts occurred already at 3 dpi. Moreover, systemic gene silencing lasted up to 24 dpi for both transcripts, consistently with what observed for *Scaphoideus titanus*[32]. The presence of dsRNA-derived smallRNA in injected *P. spumarius* adults confirmed the efficient RNAi triggering signal. SmallRNAs mapping exclusively along the dsRNA molecule length were identified in insects treated with dsATP, dsLACC and dsGFP, sustaining the presence of an active core RNAi machinery in *P. spumarius*, even if a possible secondary mechanism for smallRNA production and spreading in insects still needs to be investigated in the absence of RdRp gene[41].

Efficient silencing was also achieved in spittlebug nymphs after feeding on dsRNA-treated broad bean shoots, confirming that plant-mediated and non-invasive administration of silencing triggers may be obtained for this xylem feeder. This approach is less laborious than microinjection and a step forward towards possible future applications in the field. Evidences of RNAi in insects achieved through feeding are many, especially for coleopterans[22,59,60]. On the other hand, some barriers jeopardise the efficient oral delivery of dsRNA to sap-feeders, mostly hemipterans, which are important plant pathogens vectors, and therefore a serious threat for agriculture[51,61]. Indeed, both the presence of dsRNA signals in new-grown sprouts of treated plants and the efficient downregulation of *D. citri*, and *E. variegatus* cognate transcripts confirm the suitability of this approach for sap feeders[27,62,63]. In fact, *in planta* delivery, either by trunk-injection, petiole absorption or root soaking, is based on exogenous dsRNAs uptake and systemization in the apoplast and xylem[64,65]. Moreover, in contrast to what was observed following adult microinjection, silencing of laccase (*LACC1*) gene was particularly intense upon feeding of nymphs on treated plants, probably due to the high expression level of this gene in the juvenile stages compared to the adult stage. Caging of adults on laccase (*LACC1*) dsRNA-treated plants could eventually validate this hypothesis.

Conclusion

Several lines of evidence confirmed the existence of an active RNAi mechanism in *P. spumarius*: the in silico identification of the main genes involved in RNAi core machinery, the downregulation of two target genes and the presence of specific smallRNAs. Silencing efficiency upon microinjection of dsRNAs depended on the target gene, but, when present, was (i) durable, thus suggesting the existence of an enduring RNAi machinery activity, and (ii) measurable both at the site of injection and at other body districts, hence suggesting translocation of dsRNA molecules into the haemocoel and the presence of an active smallRNA production mechanism. Systemic silencing was also achieved when *P. spumarius* nymphs fed on dsRNA-treated plants. These findings pave the way for the development of an RNAi-based strategy to control *P. spumarius*, the main vector of *X. fastidiosa* to olive. To this purpose, two main lines of research should be followed: (i) target lethal genes should be identified to elaborate a biopesticide formulation, (ii) dsRNA delivery to the plant vascular system needs to be further explored in order to develop an efficient application field protocol.

Data availability

The smallRNA datasets generated during the current study are available in the SRA NCBI repository, (BioSample accessions: SAMN41600531-39)

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References

1. Cornara, D., Bosco, D. & Fereres, A. *Philaenus spumarius*: when an old acquaintance becomes a new threat to European agriculture. *J. Pest Sci.* **91**, 957–972 (2018).
2. Thompson, V., Harkin, C. & Stewart, A. J. A. The most polyphagous insect herbivore? Host plant associations of the meadow spittlebug, *Philaenus spumarius* (L). *PLoS One.* **18**, e0291734 (2023).
3. Saponari, M., Boscia, D., Nigro, F. & Martelli, G. P. Identification of DNA sequences related to *Xylella fastidiosa* in oleander, almond and olive trees exhibiting Leaf Scorch symptoms in Apulia (southern Italy). *J. Plant. Pathol.* **95**, 668 (2013).
4. Saponari, M. et al. Infectivity and transmission of *Xylella fastidiosa* by *Philaenus spumarius* (Hemiptera: Aphrophoridae) in Apulia, Italy. *J. Econ. Entomol.* **107**, 1316–1319 (2014).
5. Martelli, G. P., Boscia, D., Porcelli, F. & Saponari, M. The olive quick decline syndrome in south-east Italy: a threatening phytosanitary emergency. *Eur. J. Plant. Pathol.* **144**, 235–243 (2016).
6. Cornara, D. et al. Spittlebugs as vectors of *Xylella fastidiosa* in olive orchards in Italy. *J. Pest Sci.* **90**, 521–530 (2017).
7. Almeida, R. P. P., Blua, M. J., Lopes, J. R. S. & Purcell, A. H. Vector Transmission of *Xylella fastidiosa*: applying fundamental knowledge to generate disease management strategies. **98**, 775–786 (2005).
8. Sanna, F. et al. Ground cover management in olive groves reduces populations of *Philaenus spumarius* (Hemiptera: Aphrophoridae), vector of *Xylella fastidiosa*. *J. Econ. Entomol.* **114**, 1716–1721 (2021).
9. Morente, M. et al. Habitat manipulation for sustainable management of *Philaenus spumarius*, the main vector of *Xylella fastidiosa* in Europe. *Pest Manag. Sci.* **78**, 4183–4194 (2022).
10. Bodino, N. et al. Temporal dynamics of the transmission of *Xylella fastidiosa* subsp. pauca by *Philaenus spumarius* to olive plants. *Entomologia.* **41**, 463–480 (2021).
11. Roignant, J. Y. et al. Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in Drosophila. *RNA.* **9**, 299–308 (2003).
12. Fujita, K., Shimomura, K., Yamamoto, K., Yamashita, T. & Suzuki, K. A chitinase structurally related to the glycoside hydrolase family 48 is indispensable for the hormonally induced diapause termination in a beetle. *Biochem. Biophys. Res. Commun.* **345**, 502–507 (2006).
13. Saleh, M. C. et al. Antiviral immunity in *Drosophila* requires systemic RNA interference spread. *Nature.* **458**, 346–350 (2009).
14. Mezzetti, B. et al. RNAi: what is its position in agriculture? *J. Pest Sci.* **93**, 1125–1130 (2020).

15. Huvenne, H. & Smagghe, G. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *J. Insect. Physiol.* **56**, 227–235 (2010).
16. Zhu, K. Y. & Palli, S. R. Mechanisms, applications, and challenges of insect RNA interference. *Annu. Rev. Entomol.* **65**, 293–311 (2020).
17. Miyata, K. et al. Establishing an *in vivo* assay system to identify components involved in environmental RNA interference in the western corn rootworm. *PLoS One*. **9**, e101661 (2014).
18. Arakane, Y., Muthukrishnan, S., Beeman, R. W., Kanost, M. R. & Kramer, K. J. Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 11337–11342 (2005).
19. Miller, S. C., Miyata, K., Brown, S. J. & Tomoyasu, Y. Dissecting systemic RNA interference in the Red flour beetle *Tribolium castaneum*: parameters affecting the efficiency of RNAi. *PLoS One*. **7**, e47431 (2012).
20. Knorr, E. et al. Gene silencing in *Tribolium castaneum* as a tool for the targeted identification of candidate RNAi targets in crop pests. *Sci. Rep.* **8**, 2061 (2018).
21. Zhu, F., Xu, J., Palli, R., Ferguson, J. & Palli, S. R. Ingested RNA interference for managing the populations of the Colorado potato beetle, *Leptinotarsa decemlineata*. *Pest Manag. Sci.* **67**, 175–182 (2011).
22. Rodrigues, T. B. et al. First sprayable double-stranded RNA-ased biopesticide roduct targets proteasome subunit beta Type-5 in Colorado potato beetle (*Leptinotarsa decemlineata*). *Front. Plant. Sci.* **12**, 728652 (2021).
23. Christiaens, O., Whyard, S., Vélez, A. M. & Smagghe, G. Double-stranded RNA technology to control insect pests: current status and challenges. *Front. Plant. Sci.* **11**, 451 (2020).
24. Rosa, C., Kamita, S. G. & Falk, B. W. RNA interference is induced in the glassy winged sharpshooter *Homalodisca vitripennis* by actin dsRNA. *Pest Manag. Sci.* **68**, 995–1002 (2012).
25. Yu, X. & Killiny, N. RNA interference of two glutathione S-transferase genes, *Diaphorina citri DcGSTe2* and *DcGSTd1*, increases the susceptibility of Asian citrus psyllid (Hemiptera: Liviidae) to the pesticides fenpropathrin and thiamethoxam. *Pest Manag. Sci.* **74**, 638–647 (2018).
26. Yang, S. et al. Knockdown of hexokinase in *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) by RNAi inhibits chitin synthesis and leads to abnormal phenotypes. *Pest Manag. Sci.* **78**, 4303–4313 (2022).
27. Li, H. et al. IPS (In-Plant System) delivery of double-stranded Vitellogenin and Vitellogenin receptor via hydroponics for pest control in *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae). *IJMS* **24**, 9497 (2023).
28. Yu, X. et al. RNAi-mediated plant protection against aphids. *Pest Manag. Sci.* **72**, 1090–1098 (2016).
29. Matsumoto, Y., Suetsugu, Y., Nakamura, M. & Hattori, M. Transcriptome analysis of the salivary glands of *Nephotettix cincticeps* (Uhler). *J. Insect. Physiol.* **71**, 170–176 (2014).
30. Yang, M. et al. Knockdown of two trehalose-6-phosphate synthases severely affects chitin metabolism gene expression in the brown planthopper *Nilaparvata lugens*. *Pest Manag Sci.* **73**, 206–216 (2017).
31. Peng, L. Y. et al. NADPH oxidase 5 is essential for molting and oviposition in a Rice planthopper *Nilaparvata lugens*. *Insects*. **11**, 642 (2020).
32. Ripamonti, M. et al. Silencing of ATP synthase β impairs egg development in the leafhopper *Scaphoideus titanus*, vector of the phytoplasma associated with grapevine Flavescence dorée. *IJMS*. **23**, 765 (2022).
33. Leyva, J. A., Bianchet, M. A., Amzel, L. M. & Understanding ATP synthesis: structure and mechanism of the F1-ATPase (review). *Mol. Membr. Biol.* **20**, 27–33 (2003).
34. Abbà, S., Galetto, L., Ripamonti, M., Rossi, M. & Marzachì, C. RNA interference of muscle actin and ATP synthase beta increases mortality of the phytoplasma vector *Euscelidius Variegatus*. *Pest Manag. Sci*. **75**, 1425–1434 (2019).
35. Galetto, L. et al. Silencing of ATP synthase β reduces phytoplasma multiplication in a leafhopper vector. *J. Insect. Physiol.* **128**, 104176 (2021).
36. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **17**, 10 (2011).
37. Langmead, B. Aligning short sequencing reads with Bowtie. *CP Bioinform.* **32**, (2010).
38. Li, H. et al. The sequence Alignment/Map format and SAMtools. *Bioinformatics*. **25**, 2078–2079 (2009).
39. Robinson, J. T., Thorvaldsdóttir, H., Wenger, A. M., Zehir, A. & Mesirov, J. P. Variant review with the Integrative Genomics Viewer. *Cancer Res.* **77**, e31–e34 (2017).
40. Bachman, P. M. et al. Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). *Transgenic Res.* **22**, 1207–1222 (2013).
41. Cagliari, D. et al. First transcriptome of the neotropical pest *Euschistus heros* (Hemiptera: Pentatomidae) with dissection of its siRNA machinery. *Sci. Rep.* **10**, 4856 (2020).
42. Yu, X. & Killiny, N. RNA interference-mediated control of Asian citrus psyllid, the vector of the huanglongbing bacterial pathogen. *Trop. Plant. Pathol.* **45**, 298–305 (2020).
43. Zalewska, M. et al. Juvenile hormone binding protein traffic — Interaction with ATP synthase and lipid transfer proteins. *Biochim. Biophys. Acta BBA Biomembranes*. **1788**, 1695–1705 (2009).
44. Chen, Y. et al. Knockdown of *ATPsyn-b* caused larval growth defect and male infertility in *Drosophila*. *Arch. Insect Biochem. Physiol.* **88**, 144–154 (2015).
45. Almeida-Oliveira, F. et al. ATP synthase affects lipid metabolism in the kissing bug *Rhodnius prolixus* beyond its role in energy metabolism. *Insect Biochem. Mol. Biol.* **158**, 103956 (2023).
46. Futahashi, R. et al. Laccase2 is required for cuticular pigmentation in stinkbugs. *Insect Biochem. Mol. Biol.* **41**, 191–196 (2011).
47. Collins, C. M., Malacrida, B., Burke, C., Kiely, P. A. & Dunleavy, E. M. ATP synthase F1 subunits recruited to centromeres by CENP-A are required for male meiosis. *Nat. Commun.* **9**, 2702 (2018).
48. Fruttero, L. L. et al. The fat body of the hematophagous insect, *Panstrongylus megistus* (Hemiptera: Reduviidae): histological features and participation of the β-chain of ATP synthase in the lipophorin-mediated lipid transfer. *J. Insect Sci.* **19**, 16 (2019).
49. Drosopoulos, S. New data on the nature and origin of colour polymorphism in the spittlebug genus *Philaenus* (Hemiptera: Aphorophoridae). *Ann. de la. Société entomologique de France (N S)*. **39**, 31–42 (2003).
50. Rodrigues, A. S. B. et al. Assessing genotype-phenotype associations in three dorsal colour morphs in the meadow spittlebug *Philaenus spumarius* (L.) (Hemiptera: Aphrophoridae) using genomic and transcriptomic resources. *BMC Genet.* **17**, 144 (2016).
51. Jain, R. G., Robinson, K. E., Asgari, S. & Mitter, N. Current scenario of RNAi -based hemipteran control. *Pest Manag. Sci.* **77**, 2188–2196 (2021).
52. Vélez, A. M. & Fishilevich, E. The mysteries of insect RNAi: a focus on dsRNA uptake and transport. *Pestic. Biochem. Physiol.* **151**, 25–31 (2018).
53. Guo, X., Li, W. X. & Lu, R. Silencing of host genes directed by virus-derived short interfering RNAs in *Caenorhabditis elegans*. *J. Virol.* **86**, 11645–11653 (2012).
54. Gordon, K. H. J. & Waterhouse, P. M. RNAi for insect-proof plants. *Nat. Biotechnol.* **25**, 1231–1232 (2007).
55. Feinberg, E. H. & Hunter, C. P. Transport of dsRNA into cells by the transmembrane protein SID-1. *Science*. **301**, 1545–1547 (2003).
56. Karlikow, M. et al. *Drosophila* cells use nanotube-like structures to transfer dsRNA and RNAi machinery between cells. *Sci. Rep.* **6**, 27085 (2016).
57. Tassetto, M., Kunitomi, M. & Andino, R. Circulating immune cells mediate a systemic RNAi-based adaptive antiviral response in *Drosophila*. *Cell*. **169**, 314–325e13 (2017).
58. Nishide, Y. et al. Diversity and function of multicopper oxidase genes in the stinkbug *Plautia stali*. *Sci. Rep.* **10**, 3464 (2020).

59. Baum, J. A. et al. Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* **25**, 1322–1326 (2007).
60. Haller, S., Widmer, F., Siegfried, B. D., Zhuo, X. & Romeis, J. Responses of two ladybird beetle species (Coleoptera: Coccinellidae) to dietary RNAi. *Pest Manag. Sci.* **75**, 2652–2662 (2019).
61. Chougule, N. P. & Bonning, B. C. Toxins for transgenic resistance to hemipteran pests. *Toxins*. **4**, 405–429 (2012).
62. Andrade, E. C. & Hunter, W. B. RNAi feeding bioassay: development of a non-transgenic approach to control Asian citrus psyllid and other hemipterans. *Entomol. Exp. Appl.* **162**, 389–396 (2017).
63. Rossi, M. et al. Lab-scale method for plant-mediated delivery of dsRNAs to phloem-feeding leafhoppers. *J. Pest Sci.* **97**, 455–467 (2024).
64. Dalakouras, A. et al. Delivery of Hairpin RNAs and small RNAs into woody and herbaceous plants by trunk injection and petiole absorption. *Front. Plant. Sci.* **9**, 1253 (2018).
65. Bragg, Z. & Rieske, L. K. Feasibility of systemically applied dsRNAs for pest-specific RNAi-Induced Gene silencing in White Oak. *Front. Plant. Sci.* **13**, 830226 (2022).

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Author contributions

C.P., L.G. and D.B. conceptualized and designed the experiment. S.A. retrieved the gene sequences used and made all the bioinformatics analysis. N.B. collected and reared the spittlebugs. C.P. was responsible of the laboratory work, data collection and analysis. C.P., L.G. and S.A. wrote the original draft. L.G., S.A., C.M. and D.B. reviewed and edited the manuscript. All the authors contributed to the article and approved the submitted version.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to L.G.