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Research Article

Novel bacterial plasmid produces small interfering RNAs (siRNAs) that induce effective gene silencing in the Asian citrus psyllid *Diaphorina citri*

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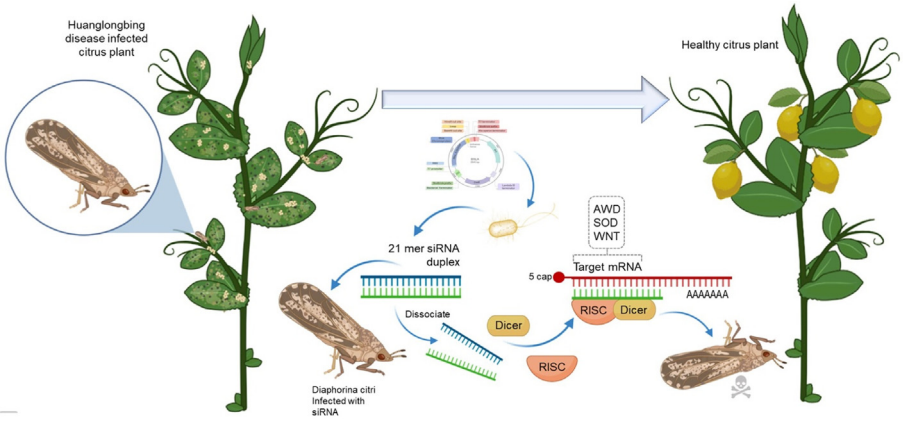
GRAPHICAL ABSTRACT



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ABSTRACT

Background: The Asian citrus psyllid, *Diaphorina citri*, is a vector of the plant pathogen *Candidatus* Liberibacter asiaticus (CLas), the causal agent of Huanglongbing disease. HLB represents the main threat to the citrus industry around the world due to its fast spreading, high infectivity, and incurability. An

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Small interfering RNAs

alternative for the control of this plague in *Rutaceae* is the implementation of RNA interference (RNAi). Here, we propose a novel method to produce small interfering RNA (siRNA) in *Escherichia coli*. The method includes a reporter system to visually demonstrate dsRNA expression. We produced and tested siRNAs against three *D. citri* genes: Abnormal Wing Disk (AWD), Superoxide dismutase 1 (SOD), and Wingless (WNT). These genes play key roles in psyllid development and maturity, and thus, represent promising targets for potential vector control.

Results: The blue coloration in bacterial cultures was easily observable, and it corroborated the generation of the dsRNA that is in the same transcript. We evaluated the dosage efficiency of siRNA using the AWD siRNA. The RNAi treatment was evaluated, and all the siRNAs tested were able to induce silencing (-3.05 for AWD, -2.60 for SOD and -2.57 for WNT).

Conclusions: The novel bacterial plasmid effectively produces siRNAs, and the blue color reporter is visually facilitated to check the expression of each dsRNA. Treating *D. citri* with the produced siRNAs resulted in a decrease in gene expression. In addition, psyllid mortality was observed, being the highest when treated with WNT-siRNA. Our results suggest the potential of siRNA treatment as a method for controlling Huanglongbing.

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1. Introduction

Huanglongbing (Chinese for "yellow dragon disease" or HLB) also known as "citrus greening" disease is the most severe citrus bacterial infection reported so far, causing global devastation in the citrus industry [1]. Unfortunately, HLB has become well established in many citrus-producing regions from over 40 countries in Africa, the Americas, and Asia, and continues to spread worldwide [2,3,4,5]. Since citrus plants produce some of the most important crops in the world, HLB represents billion-dollar losses to the citrus industry per year [6]. There is an urgent need for the development of effective strategies to prevent and mitigate the spread of HLB.

HLB is caused by three species of the Gram-negative, α-Proteobacteria *Candidatus* Liberibacter: *Candidatus* Liberibacter asiaticus (*C*Las), *Candidatus* Liberibacter americanus (*C*Lam), and *Candidatus* Liberibacter africanus (*C*Laf). Among them, *C*Las is the most predominant species [6]. *C*Las is a fastidious, phloem-limited, intracellular plant pathogen that has not yet been cultured *in vitro* [5]. These pathogens can be transmitted easily from infected to healthy citrus plants through either the Asian citrus psyllid (*Diaphorina citri*) or the African citrus psyllid (*Trioza erytreae*) [5]. The main HLB symptoms include phloem degradation, nutrient deficiencies, yellow shoots, leaves with blotchy mottle patterns, decreased fruit size, yield, and quality, and ultimately, death of the infected branches and the whole tree [7,8]. HLB has a long latency period that can last from several months up to two years [6,9], so infected trees may not show any visible symptoms at the initial stages of the infection, difficulting the early detection of the disease. Asymptomatic trees represent a potential reservoir for *C*Las and facilitate the spreading of the disease to other trees. The HLB infectious cycle begins when *D. citri* feeds from the phloem of an infected tree. *C*Las then resides in the salivary glands and the hemolyph of the psyllid, where it multiplies [10]. This mechanism allows the pathogen to be inoculated into another plant the next time the vector feeds, infecting it and thus, restarting the cycle [7]. Concerning, *C*Las-infected adults remain infective throughout their lives, becoming super-spreaders of the disease [11].

Given that *C*Las has not been successfully cultured *in vitro* yet, most of the current methods focus on protecting the citrus trees [12,13]. Some of these methods include the treatment with antibiotics, thermotherapy, plant stimulation, disease-resistant breeding, and bacterial inoculation; each one presents advantages and disadvantages. For example, the use of antibiotics may affect the fruit yields and residues may remain in the fruit and the surrounding ecosystem [13,14,15]. Likewise, temperature treatment may affect the microbial composition in the soil [13,16,17] and is not effective against *C*Las residing in the roots [18]. Moreover, the use of nutrients and compounds to stimulate plant growth and/or immune responses [13,15,19,20,21,22] seems to have no significant effect on late-stage HBL-positive trees [23,24,25]. With the increasing developments of genetic engineering, the breeding of disease-resistant varieties might represent the most promising and sustainable approach to controlling HLB. However, the cultivars obtained by gene-editing approaches are still many years away from producing desired results. In addition, there are strict national and international regulations regarding the use of GMOs for commercial production. Moreover, a lack of HLB-resistant germplasm hinders new varieties generated by traditional breeding [13,26]. Recently, it was demonstrated that inoculating a *C*Las-positive citrus tree with *Xylella fastidiosa* strain EB92-1 prevented the development of HLB symptoms and reduced the incidence of severe symptoms. Nevertheless, this method does not eliminate the *C*Las infection and reinoculation is needed every 2–3 years [27]. Despite these efforts to mitigate the spread of HLB, they are not effective enough to control the pathogen.

The availability of *Candidatus* Liberibacter and *Diaphorina citri* genomes has allowed to increase the understanding of the pathogenicity of *C*Las, both in citrus trees and the vector. Recombinant DNA technology represents a promising tool to develop novel, sustainable, cost-effective, and environmentally safe methods to fight the spread of HLB [13,15,22,27,28,29,30]. RNA interference (RNAi) technology is a highly versatile tool, as it allows to target virtually any gene of interest with high specificity since only a matching mRNA sequence will be targeted [31]. RNAi has been used to assess the effects of silencing multiple different *D. citri* genes. Reported targets include a homolog of *boule* associated with fertility [32] and members of the carboxylesterase family [33]; silencing of these targets resulted in increased mortality in adult psyllids [32] and nymphs [33]. Not only does RNA-induced silencing induce psyllid mortality, but double-stranded RNA (dsRNA) remains stable for at least 72 h after application [32]. For instance, the RNA interference-induced knockdown of the glutathione S-transferase (GST) gene increases the psyllid susceptibility to insecticides [34]. Following this approach, we selected three *D. citri* genes as targets for RNA-induced silencing: Superoxide Dismutase 1 (SOD), Abnormal Wing Disk (AWD), and Wingless (WNT). We selected these genes as they are all important for the development

and maturation of the psyllids, thus silencing them should result in increased psyllid mortality.

Previous reports suggest that small doses of double-stranded RNA (dsRNA) targeting SOD triggered the RNA interference (RNAi) pathway inducing psyllids mortality [35]. As SOD is involved in superoxide radical detoxification, its downregulation results in metabolic disruptions and psyllid death [35,36,37]. Likewise, the inactivation of AWD has been shown to interrupt the development of the nymphs and induce psyllid mortality [38]. This gene is related to wing development during the transition from nymph to adult, so its silencing results are lethal for *D. citri* [35,37,38,39,40]. Although these two genes have been previously targeted, we designed novel small interfering RNAs (siRNAs).

Besides SOD and AWD, we also chose WNT, a novel target that has not been studied yet. The WNT signal transduction pathway regulates critical events during development such as cell proliferation, tissue reparation, larvae development, organogenesis, regeneration processes, and fate specification in species like *Drosophila melanogaster,* and it has been involved in wing development [41,42]. The multiple roles played by WNT make it an attractive target for RNA-induced silencing, as it is expected to decrease the viability of the nymphs and increase mortality.

For siRNA technology to be used as a potential tool against *D. citri* and mitigate the spread of HLB, it is imperative to produce the dsRNA molecules in a cost-effective, environmentally friendly, and scalable process. Bacterial and yeast systems offer many of these advantages and have been used for the large-scale scale production of recombinant proteins for therapeutic use like human insulin. Following this approach, multiple *Escherichia coli* strains and plasmids for efficient dsRNA production have been described in the literature [43,44,45,46,47]. We designed a novel vector to produce siRNAs targeting the previously described *D. citri* genes. Our vector, BSLA, encodes for the blue chromoprotein (B) as a reporter gene, the sense (S) and antisense (A) sequences of the desired siRNA, and with a loop (L) in between them.

Hence, in this work, we designed novel siRNAs specific to *Diaphorina citri* SOD, AWD, and WNT genes. We cloned them and successfully produced them on our novel BSLA vector. After siRNA purification, we evaluated their effect on gene silencing and on psyllid mortality. Other parameters such as the new synthesis method and the effect of different AWD-siRNA concentrations were evaluated as well.

2. Methodology

2.1. siRNA design

The *D. citri* genes AWD, SOD, and WNT were selected as targets for RNA-induced silencing. The sequences were obtained from their GenBank accession numbers (Table 1). We evaluated the expression of the selected targets across different development stages, tissues, host plants, and in the presence and absence of *C*Las infection. We used publicly available RNA-seq datasets from previous studies reported through the Citrus Greening Expression Network (CGEN: https://cgen.citrusgreening.org/) [48,49,50,51]. SOD, AWD, and WNT are expressed consistently in adults (**Figure S1**), therefore validating our target selection for RNA-induced silencing. For siRNA design, we used Dharmacon siRNA design center (https://dharmacon.horizondiscovery.com/design-center/), InvivoGen siRNA wizard (https://www.invivogen.com/sirnawizard/), and OligoWalk (https://rna.urmc.rochester.edu/cgi-bin/server_exe/oligowalk/oligowalk_form.cgi) bioinformatic tools. We utilized empirical design parameters for siRNA previously reported by Birmingham et al. [52] and Yamaguchi et al. [53]. All obtained results with these methods were compared looking for matches, to create a pool of candidates.

We also evaluated siRNA specificity for *D. citri* to prevent undesired effects on other species. The candidates were tested for matches on other species using BLAST [54,55,56] and also aligned against homologous genes in *Citrus sinensis* (sweet orange), *Arabidopsis thaliana, Drosophila melanogaster, Anopheles gambiae* (mosquito), *Apis mellifera* (honeybee), and *Homo sapiens* (Table S1). We used Clustal Omega default parameters for alignments [57] (Fig. 1) and chose siRNA candidates with no significant alignments nor long patches of identical sequences in species other than *D. citri*.

Finally, the selected siRNAs were tested for both stability and spontaneous hybridization. The thermodynamic ensemble was tested using RNA Fold [58,59], and hybridization studies were performed using BiBiServ2 RNA Hybrid [60,61]. The selected siRNA sequences are included in Table 1.

2.2. siRNA expression plasmid construction synthesis

We designed and constructed a novel plasmid harboring a reporter gene to verify siRNA transcription in *E. coli.* We developed the BSLA plasmid (Blue chromoprotein, Sense, Loop, Antisense) based on previously reported sense/loop/antisense dsRNA expressing plasmids [44,45,46]. These plasmids were expressed in *Escherichia coli* HT115 (DE3), producing a stem-loop dsRNA linked with the Green Fluorescent Protein (GFP) sequence, which was then cleaved by digestion with ribonuclease A (RNase A).

Some of the components of our BSLA were taken from the iGEM Registry of Standard Biological Parts (http://parts.igem.org/) such as the high-copy plasmid backbone (pSB1C3), the T7 promoter (BBa_I712074) and terminator (BBa_K2246001), the strong RBS (BBa_B0034), and the blue chromoprotein coding region (BBa_K592009 T573A). The desired siRNA, a 7-base loop (5'-AAGTTCTCT-3') [62] and the siRNA antisense sequence were cloned between the BamHI and HincII restriction sites in the vector. BSLA also encodes for the chloramphenicol acetyltransferase CmR as a selection marker. We generated one vector encoding for each of the selected siRNAs (AWD, SOD, and WNT) and one encoding for GFP as a control. Successful siRNA transcription was denoted by the expression of blue chromoprotein. All constructs were verified by sequencing.

2.3. siRNA synthesis and extraction

To synthesize the siRNAs, we used *E. coli* HT115 (DE3), which was kindly donated by Dr. Rosa Estela Navarro González (Institute of Cellular Physiology, UNAM, Mexico). This strain has been widely used to produce dsRNA due to its genotype [47]. It contains the T7 polymerase-encoding gene, enabling it to generate dsRNA from T7 promoter-containing plasmids such as BSLA. More importantly, it has a mutated RNase III-encoding gene, allowing the expression of dsRNA without degradation, thus increasing the siRNA production efficiency [47].

E. coli HT115 (DE3) was propagated in agar plates with ampicillin (100 μg/mL), tetracycline (12.5 μg/mL). It was then transformed with each BSLA plasmid following the standard procedure and selected with ampicillin, tetracycline, and chloramphenicol (25 μg/ml). From the selection plate, a single colony was inoculated into 10 mL LB medium supplemented with the triple antibiotic selection and grown overnight at 37°C, 260 rpm. The overnight bacterial culture was then used for the total RNA extraction by TRIzol Reagent (Invitrogen, Cat# 15596026) method following the manufacturer's instructions, followed by treatment with an RNase A at high NaCl concentrations (0.3 M) for 5 min at 37°C to degrade all ssRNA [46,63].

Table 1
Selected *D. citri* genes and designed siRNA sequences.

Diaphorina citri gene	GenBank accession no.	Citrus Greening Solutions Gene ID	siRNA sequence (5' - 3')
AWD	XM_026830613.1	Dcitr07g08030.1.1	CCACATATTCTTTAGCGCCTAGC
WNT	XM_008486571.2	Dcitr04g11660.1.1	ATTACATTTGACCTCACAGC
SOD	XM_026824040.1	Dcitr05g05840.1.1	TGTCTTACTCAGTTCATGACCAC

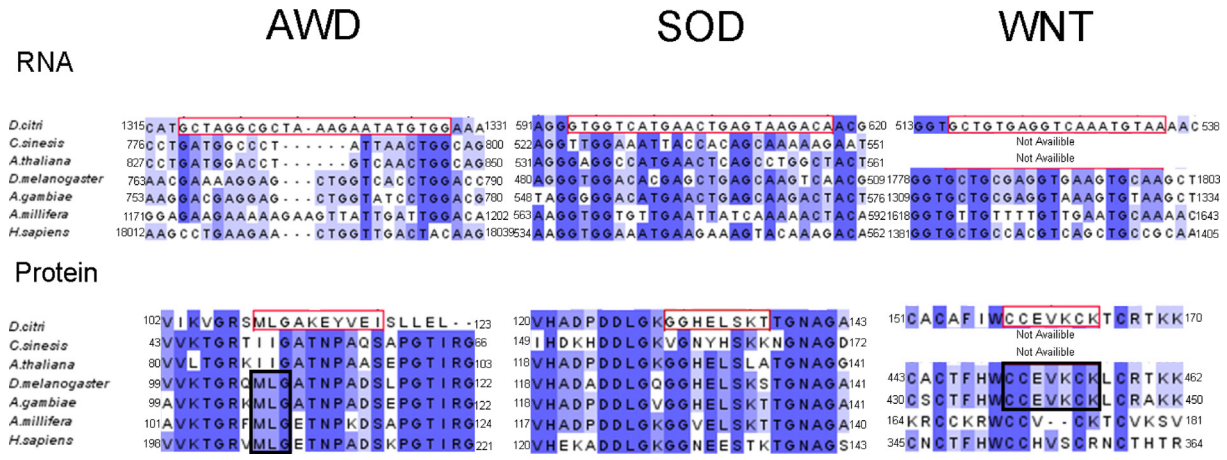

Figure 1. RNA and protein alignments. Alignments of the siRNA target regions at RNA and protein levels on *D. citri*, *C. sinensis*, *A. thaliana*, *D. melanogaster*, *A. gambiae*, *A. mellifera,* and *H. sapiens* genes. The siRNA target sequence and its translations are in red rectangles. Black boxes indicate conserved motifs. Darker shading indicates that a particular nucleotide or amino acid is conserved amongst more of the evaluated sequences. There is no homologous gene of WNT in *C. sinensis* and *A. thaliana.*

2.4. Diaphorina citri maintenance under controlled conditions

Diaphorina citri specimens were kindly donated by the Southeastern Regional Laboratory of *Tamarixia radiata* Massive Reproduction located in Mérida, Yucatán, Mexico. This institution also provided training for the safe and ethical handling of the psyllid, following the guidelines of the National Agro-Alimentary Health, Safety and Quality Service (Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria of Mexico in Spanish). The specimens were kept on custom-made cages double-lined with a fine white mesh, containing *Murraya paniculata* (orange jasmine) for feeding under a controlled photoperiod (16 h light/ 8 h dark), temperature (26-30°C) and humidity (50-80%). *Murraya paniculata* plants were cleaned using a containing 5% acetic acid and 70% ethanol solution, and the soil was covered with aluminum foil to prevent the presence of any other insect species. The population was monitored regularly to keep a record of the number of specimens, and to separate the adult specimens from the nymphs.

2.5. dsRNA delivery method by in vivo soaking tests

We performed the dsRNA delivery methodology based on a previous report by Yu et al. [64]. To optimize the siRNA concentration for further experiments, we tested different concentrations (20, 40, 60 and 100 ng/mL) of a siRNA solution containing AWD-siRNA. The dose range was adopted from a previously reported dsRNA titration assay [64]. For each of the three RNAi treatments, we used a 100 ng/mL siRNA solution. GFP-siRNA (5'-CCACATATT-CTTTAGCG CCTAGC-3') treatment was used as a negative control.

Ten young adult psyllids were starved for 2 h and cooled down to 4°C to reduce their movement. We applied an 8 µL droplet of siRNA solution on the middle abdomen of each psyllid. The droplet was left for 5 min to soak, and then, it was carefully dried using filter paper. The psyllids were placed on a fresh *Murraya paniculata* shoot in an isolated container for 48 h or until mortality was observed. The psyllids were retrieved and flash-frozen at -80°C for further RNA extraction and real-time PCR analysis.

2.6. Diaphorina citri RNA extraction

Twenty psyllids were frozen with liquid nitrogen and crushed with a mortar until a homogeneous mix was obtained. A total of 500 µL of TRIzol Reagent were added to the mix prior to being transferred to a microtube and incubated for 5 min at room temperature. A total of 160 µL of chloroform were added and incubated for 3 min. The mix was then centrifuged (12,000 × *g*, 4°C, 15 min); the supernatant was recovered and treated with 5 µL of RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, Cat# 10777019) and 400 µL of isopropanol. After a 10 min incubation at room temperature and centrifugation (10,000 × *g*, 4°C, 10 min), the pellet was resuspended in 800 µL of 75% ethanol and ultimately centrifuged (7,500 × *g*, 4°C, 5 min). The supernatant was discarded, and the pellet was resuspended in 40 µL of nuclease-free water.

2.7. Silencing analysis by RT-qPCR

After the total *D. citri* RNA extraction, we performed the cDNA synthesis with "RevertAid H Minus First Strand cDNA Synthesis Kit" from ThermoFisher (Cat#. K1631), according to manufacturer instructions using 3 µL of RNA normalized to a concentration of 250 ng/µL. A total of 2.5 µL of cDNA were then used for RT-qPCR, following the instructions of the kit Maxima SYBR GREEN/ROX qPCR (ThermoFisher Scientific Cat#. K0221). The cDNA was then amplified for 40 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 30 s, followed by 75°C for 5 min and 12°C as hold temperature. The primer sequences for each gene amplification are described in Table 2. We tested the specificity of the primers by evaluating their respective melting curves. For all of them, we observed only one peak, thus confirming primer specificity. All assays were done in triplicate.

Table 2
Primers designed for the RT-qPCR.

Gene	Primer name	Sequence	Amplicon size
AWD	AWD F	5'-GGGGTTCAAGCTGATAGCGA-3'	180 bp
	AWD R	5'-AGCATGGATCGGCCAACTTT-3'	
SOD	SOD F	5'-TGCCGGACCTCACTTCAATC-3'	199 bp
	SOD R	5'-GTCATCAGGGTCAGCGTGTA-3'	
WNT	WNT F	5'-CTCAAAGATCGGTTTGACGGC-3'	200 bp
	WNT R	5'-ATTCCCAGTGCGGGATTCTT-3'	
α-tubulin 1	TUB F	5'-TTACTGTCTGGAGCACGGGA-3'	195 bp
	TUB R	5'-TGGAACAGCTGTCGGTAAGTA-3'	

The results from the RT-qPCR were analyzed by the comparative Ct or $2^{-\Delta\,\Delta Ct}$ method [65,66]. The commonly used housekeeping gene α-tubulin 1 (NCBI Gene ID: 103517567; Citrus Greening Solutions Id: Dcitr09g02650.1.1) was used as a reference gene to obtain the fold reduction of mRNA due to the application of each siRNA [32,35,38,40]. The data and results were visualized using the Python 3.9.4 library Matplotlib 3.4.1, and the statistical significance was obtained using a 2-sample t-student test with p-values 0.001, 0.01 and 0.05.

3. Results and Discussion

3.1. siRNA design

To confirm that the siRNAs were specific for *Diaphorina citri*, we performed sequence alignments against homologous genes in *C. sinensis*, *A. thaliana*, *D. melanogaster*, *A. gambiae*, *A. mellifera,* and *H. sapiens* (Fig. 1). The generated siRNAs were between 20 and 23 nt in size with maximum and minimum mismatches of 18 and 13 for AWD, 11 and 3 for SOD, and 8 and 3 for WNT, respectively. We aligned each siRNA's target mRNAs and their respective amino acid sequence to screen for possible binding with those of other organisms. None of the aligned sequences had high homology regions with the candidate targets. There are some conserved amino acid motifs in both AWD (MLG) and in WNT (CCEVKCK), which may play an important role for structure and/or function. Despite the presence of conserved motifs, the coding sequence is different between species (Fig. 1), therefore, the candidate siRNA sequences can be used safely to target *Diaphorina citri* genes.

The sequences shown in boxes share nucleotides or amino acids with other tested species but are not a perfect match. It has been proven that the siRNA-induced gene silencing mechanism has a low tolerance for mismatches, except for a few specific single nucleotide mismatches, especially if the mismatches are located near the 3' end of the guide RNA [62,67]. Although some 3-4 nucleotide mismatches may induce gene silencing by repressing translation, there must be adjacent matches with a maximum of two nucleotides separating groups of one or two adjacent mismatches [67]. All siRNA candidates used differed in more than three nucleotides with the target regions of the species tested. None of the 3-4 nucleotide mismatches observed for the WNT siRNA in *A. gambiae* and *D. melanogaster*, nor the 3-nucleotide mismatch observed for the SOD siRNA in *A. gambiae* fulfills the necessary characteristics to induce translation repression. Therefore, we conclude that the used siRNA sequences are specific for *D. citri*.

3.2. siRNA bacterial expression

We developed a new method for producing siRNA in RNase III-deficient *E. coli* HT115 (DE3) [47]. Our method resembles recombinant protein production and could potentially be scaled for production in bioreactors, reducing the cost of siRNA synthesis. Evidence of siRNA expression was observed by the expression of blue chromoprotein, resulting in blue bacterial colonies (Fig. 2). In the literature, we found several reports of long-dsRNA inducible expression and post-transcription assembly by RNA hybridization in bacterial systems [44,45,63,68,69,70]. The reported methods employ different types of purification processes, ranging High-Performance Liquid Chromatography to the standard bacterial lysis [44,45,63,68,69,70,71,72]. Even in the absence of a complex purification process, a strong silencing effect is observed, thus confirming the great potential of bacterial systems to reduce siRNA production costs. These methods produce long dsRNAs, which are then digested by Dicer, obtaining multiple siRNAs with varying sequences [44,45,63,64,65,66,69,71,72]. However, the risk of unintended targets increases since sequences are nonspecifically generated by Dicer. In contrast, we propose a system that enables us to track specific siRNA production by simply observing the color of the resulting colonies. Our BSLA system allows siRNA assembly with the independent formation of a hairpin from the reporter mRNA, enabling specific siRNA extraction by standard methods followed by enzymatic treatment.

Fig. 2 shows blue chromoprotein production linked to siRNA expression. Loop formation plays a key role in the assembly of the siRNA; it must form independently from the reporter mRNA and guides the vector design [73]. In addition, BSLA plasmid has a T7 constitutive promoter recognizable by T7 polymerase, inducible with Isopropyl β-d-1-thiogalactopyranoside (IPTG) in *E. coli* HT115 efficient for dsRNA production [63]. We used RNase A treatment under specific conditions to remove the reporter transcript and to obtain the dsRNA. This treatment effectively produces dsRNA, whilst degrading undesired RNA fragments [63].

3.3. siRNA effect on D. citri by soaking delivery

To evaluate the effect of the siRNAs on *D. citri* mortality, we treated the psyllids with each one of the three siRNAs (Fig. S2). As a negative control, we used GFP-siRNA, expecting no effects on any of the target genes as verified by multiple alignments (Fig. S3, Fig S4, Fig S5).

After siRNA treatment, we monitored the psyllids for 48 h and determined their mortality rate. When applied individually (800 ng of siRNA), the highest mortality rate was observed for WNT. WNT-siRNA treatment resulted in a 10% mortality rate after 24 h and increased to 20% and 40% after 36 and 48 h, respectively (Fig. 3). Both AWD-siRNA and SOD-siRNA resulted in an initial 10% mortality (24 h) and 30% mortality after 48 h.

3.4. D. citri RNA extraction and cDNA synthesis

Several ribosomal RNA subunits and RNA integrity were assessed from the different siRNA treatments by gel electrophoresis (Fig. S6A). We observed single bands for AWD, SOD, WNT, and α-tubulin that matched the expected size (180, 199, 200 and 196 bp, respectively) (Fig. S6B).

3.5. Soaking siRNA transfection

Previous reports of dsRNA application through a soaking method, where a range from 20 to 100 ng/μL was tested showed that the highest effect is obtained at dsRNA concentrations between 75 ng/μL and 100 ng/μL [64]. We performed optimizing assays using the AWD-siRNA to determine the optimal siRNA concentration for the soaking assays and to prevent RISC saturation. We tested concentrations ranging from 20 to 100 ng/μL. We found that the fold mRNA reduction increased as the concentration of siRNA increased (Fig. 4A). When a 20 ng/μL siRNA solution was applied, no significant mRNA reduction was observed. For concen-

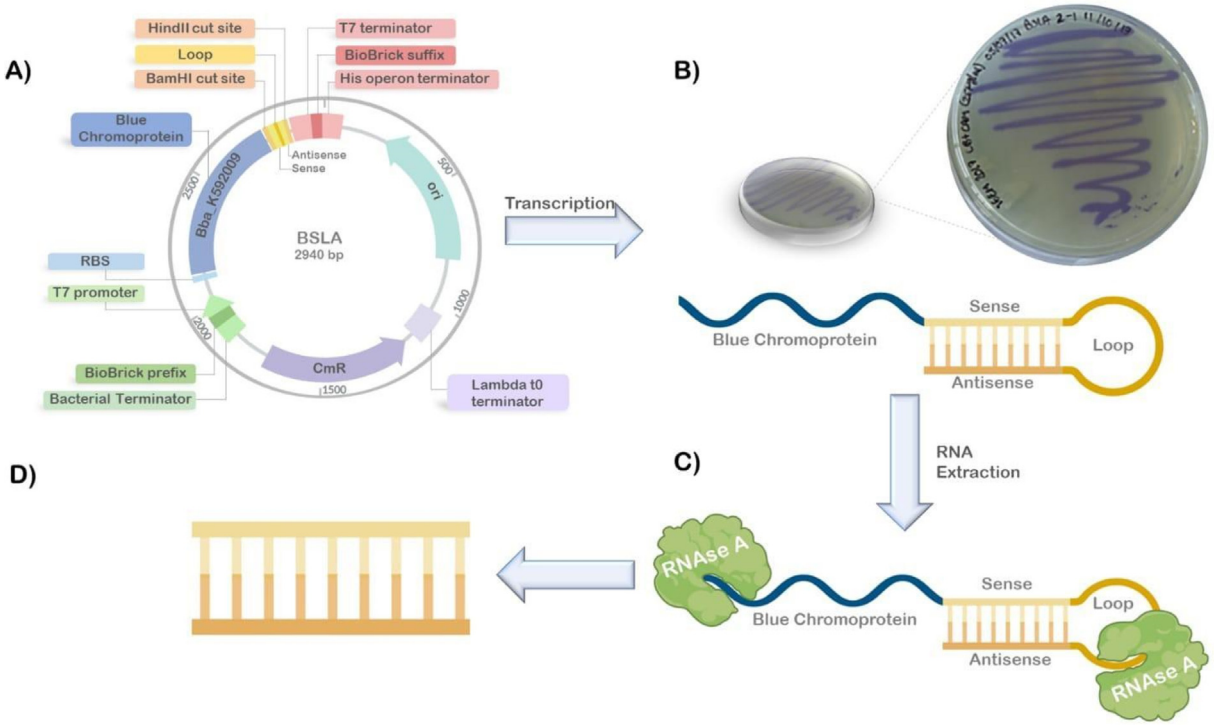

Figure 2. BSLA siRNA production method. (A) BSLA vector diagram. The plasmid is constituted by a T7 promoter, followed by a RBS (Ribosome binding site), the condign sequence of the chromoprotein blue fused to the sequence of the siRNA (sense, loop and antisense). (B) Secondary structure caused by the hybridization of siRNA sense and antisense sequences, whilst expressing a blue chromoprotein as a reporter, resulting in blue colonies. (C) Treatment with RNAse A after RNA extraction at NaCl concentrations higher than 0.3 M. (D) Assembled siRNA.

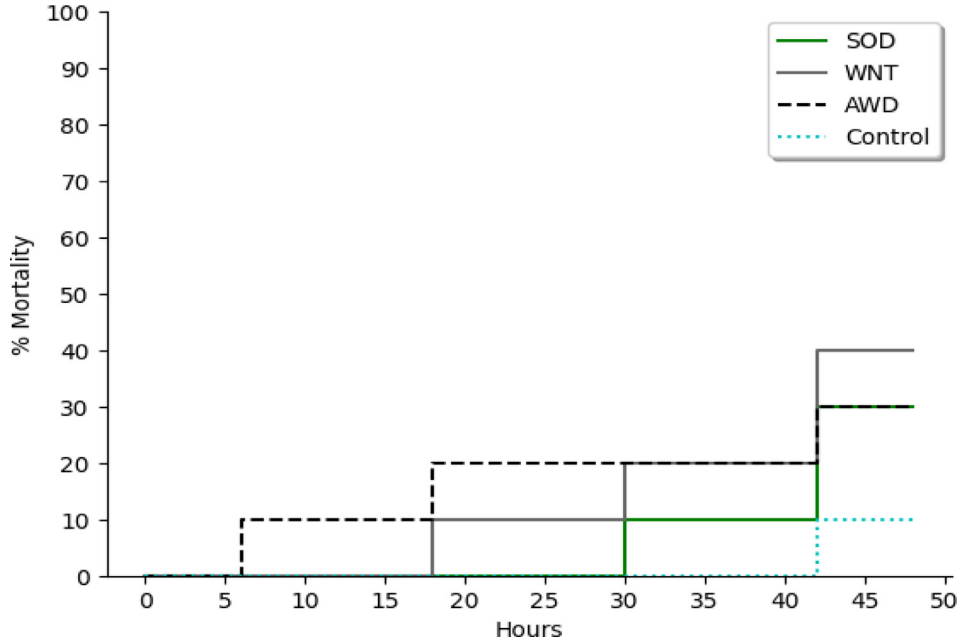

Figure 3. Biological effect of siRNA by soaking method on *Diaphorina citri* survival rate. After siRNAs treatment, the psyllids were monitored 48 hours posttreatment to determine the mortality rate.

trations of 40, 60, and 100 ng/μL, we observed a -2.37, -2.53, and -3 fold reduction in AWD mRNA, respectively.

dsRNA administration via soaking on *Diaphorina citri* allows siRNA uptake through spiracles and cuticular permeation, which in turns, spreads throughout the whole body and initiates a systemic and persistent RNAi response [64]. Soaking silencing efficiency depends on the dosage of siRNA applied. Previous reports demonstrated that higher doses of dsRNA-AWD (10-10,000 ng/nymph) increased mortality [38]. Likewise, other studies using the soaking method on *D. citri* found that increasing dosages of dsRNA yielded higher silencing effects between 10 and 300 ng/μL [64,74,75,76]. In our study, the highest silencing effect for the

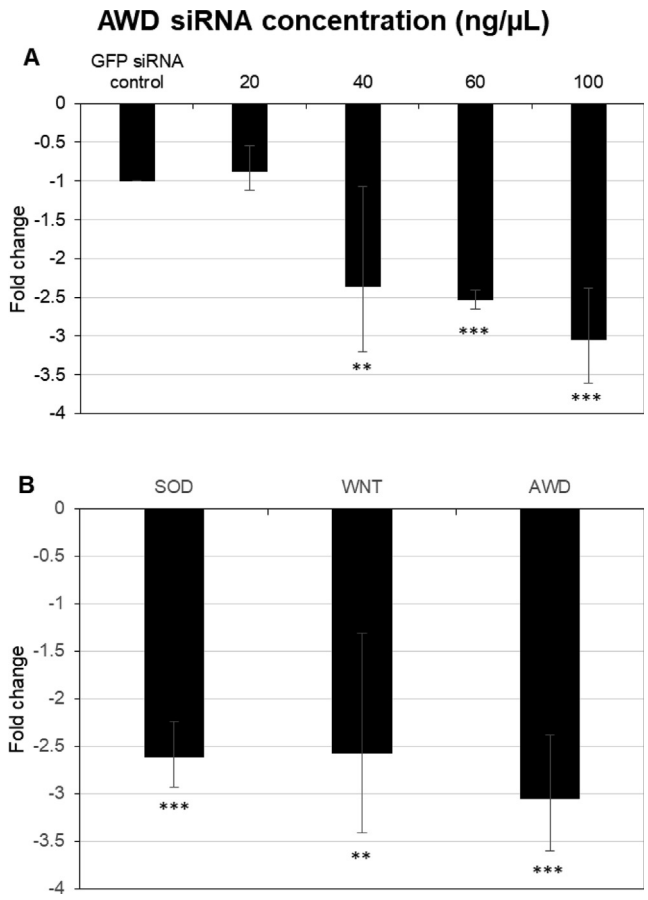

Figure 4. Fold change (log2) by siRNA application through soaking. Error bars represent a 95% confidence interval. ** statistically significant reduction to 1%, *** statistically significant reduction to 0.1%. In all the cases GFP siRNA treatment was used as a negative control. (A) Change in AWD expression after siRNA treatment at varying concentrations (20, 40, 60 and 100 ng/µL). (B) Fold mRNA reduction for each of the target genes after treatment with its corresponding siRNA (100 ng/µL of each siRNA).

AWD gene was observed using a concentration of 100 ng/µL (Fig. 4A). We confirmed that the application of siRNA through the soaking method allowed effective silencing in a concentration-dependent trend. Therefore, we decided to use 100 ng/µL to test all target genes.

No silencing effects were observed for the GFP-siRNA treatment, as expected for negative controls. All three siRNA applications showed significant fold mRNA reduction (−3.05 for AWD, −2.60 for SOD and −2.57 for WNT, Fig. 4B). Remarkably, SOD mRNA was reduced by 61.6% after siRNA treatment when compared to normal expression levels (Fig. 4B). This reduction is consistent with the reported 69.6% mRNA reduction observed by Taning et al. [35], which also included randomly generated targets. In our case, we administered small dsRNA molecules instead of longer ones to be pre-processed by Dicer thus avoiding the generation of unspecific products and keeping only one possible target. SOD-siRNA treatment resulted in effective silencing with 30% psyllid mortality after 48 h (Fig. 3). Since SOD is overexpressed in the hemolymph during CLas infection, it represents a promising silencing candidate [36].

Similarly, treatment with AWD-siRNA resulted in an mRNA reduction of 67.2% (Fig. 4B) comparable to another report of 70% using long dsRNA [38]. Even though AWD is suggested to be involved in wing development [38], we show that silencing this gene on adult psyllids leads to mortality (around 30% after 48 h).

We propose WNT as a novel target for gene silencing in *D. citri*. In the literature, it is reported that this gene is also overexpressed in the hemolymph of CLas-infected *D. citri* [36,37]. We observed an mRNA reduction of 61% after WNT-siRNA treatment (Fig. 4B), which resulted in a 40% mortality rate after 48 h (Fig. 3). These results show that WNT is a promising target for silencing as a siRNA-based *D. citri* control method.

In this work, we propose our novel BSLA plasmid as a versatile tool that can be used to produce siRNA molecules. We also demonstrate that the bacterial-produced siRNAs effectively induce gene silencing in *Diaphorina citri*. Our results not only confirm the potential applications of siRNA technology targeting AWD and SOD but also show that WNT is a promising target. All the tested siRNAs induced gene silencing effectively, achieving decreases in mRNA levels comparable to other reports in literature. Moreover, siRNA treatment and gene silencing could lead to an increase in psyllid mortality, but further population-wide studies over a longer period are still required. This method could have advantages over other methods to prevent HLB spread and infection by controlling the population of the vector in a species-specific way and without antibiotic or pesticide use.

4. Conclusions

We designed a novel plasmid, BSLA, expressing a blue chromoprotein as a reporter of successful siRNA transcription. BSLA was used to produce dsRNA molecules against three *Diaphorina citri* genes: AWD, SOD, and WNT. The BSLA siRNA expression system demonstrated the viability of producing siRNA in bacterial systems, which opens the possibility for large-scale production. The designed siRNAs showed specificity for *Diaphorina citri* genes *in silico* and exhibited a high silencing efficiency when tested in vivo. A positive correlation was observed between AWD-siRNA concentration and the silencing effect. The highest silencing effect (−3 fold reduction) was observed using a siRNA concentration of 100 ng/µL.

The siRNAs applied to *Diaphorina citri* through the soaking method resulted in more than 50% mRNA reduction in all target genes and caused between 10-40% mortality in adult psyllids. The highest mortality was obtained with WNT-siRNA, a gene that was not targeted previously in literature. We report the effects of targeting WNT for the first time, showing promising results in both gene silencing and increased mortality. All the tested target genes are potential silencing candidates for a siRNA-based method for controlling *D. citri* populations and subsequently, the spread of HLB disease.

Author contributions

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  - o Certification for safe and ethical *D. citri* handling and maintenance: LF Durán-Armenta.
  - o *M. paniculata* and *D. citri* maintenance: LF Durán-Armenta, LI González-Campos, V Rico-Torres, NE Saucedo-Tavitas.
  - o *D. citri* treatment with siRNA: LI González-Campos, V Rico-Torres, AL Torres-Huerta.

- o Analysis and interpretation of results: JC Rueda-Silva, LI González-Campos, LF Durán-Armenta, AL Torres-Huerta.
- Draft manuscript preparation: JC Rueda-Silva, LF Durán-Armenta, A Karam-Coppola, A Antonio-Pérez, YG García-Huante, AL Torres-Huerta.
- Diagram design: A Karam-Coppola, A Antonio-Pérez.
- Research supervision: A Antonio-Pérez, YG García-Huante, C Cruz-Cruz, JC Amador-Molina, JI Tapia-Ramírez, AL Torres-Huerta.
- Revision of the results and approval of the final version of the manuscript: JC Rueda-Silva, LI González-Campos, LF Durán-Armenta, A Antonio-Pérez, AL Torres-Huerta.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary material

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Data availability

Several files of the experimental assays carried out are provided in the supplementary material.

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