Phytopathology® • 2024 • 114:1802-1809 • https://doi.org/10.1094/PHYTO-02-24-0058-KC

Key Challenges

Disease Control and Integrated Management

Antisense Oligonucleotide as a New Technology Application for *CsLOB1* Gene Silencing Aiming at Citrus Canker Resistance

Luiz Felipe Franco de Lima,[1,2] Isis Gabriela Barbosa Carvalho,[1] Reinaldo Rodrigues de Souza-Neto,[1,2] Larissa da Silva dos Santos,[1] César Augusto Nascimento,[1] Marco Aurélio Takita,[1] Fabiano Touzdjian Pinheiro Kohlrausch Távora,[3] Angela Mehta,[3] and Alessandra Alves de Souza[1,†]

[1] Citrus Research Center "Sylvio Moreira," Agronomic Institute–IAC, Cordeirópolis, Brazil
[2] Department of Genetics, Evolution and Bioagents, Institute of Biology, University of Campinas–UNICAMP, Campinas, Brazil
[3] Embrapa Recursos Genéticos e Biotecnologia, PBI, Av. W/5 Norte Final, CEP 70770917, Brasília, Brazil
Accepted for publication 11 May 2024.

Abstract

Citrus canker disease, caused by *Xanthomonas citri* subsp. *citri*, poses a significant threat to global citrus production. The control of the disease in the field relies mainly on the use of conventional tools such as copper compounds, which are harmful to the environment and could lead to bacterial resistance. This scenario stresses the need for new and sustainable technologies to control phytopathogens, representing a key challenge in developing studies that translate basic into applied knowledge. During infection, *X. citri* subsp. *citri* secretes a transcriptional activator-like effector that enters the nucleus of plant cells, activating the expression of the canker susceptibility gene LATERAL ORGAN BOUNDARIES 1 (*LOB1*). In this study, we explored the use of antisense oligonucleotides (ASOs) with phosphorothioate modifications to transiently inhibit the gene expression of *CsLOB1* in *Citrus sinensis*. We designed and validated three potential ASO sequences, which led to a significant reduction in disease symptoms compared with the control. The selected ASO3-*CsLOB1* significantly decreased the expression level of *CsLOB1* when delivered through two distinct delivery methods, and the reduction of the symptoms ranged from approximately 15 to 83%. Notably, plants treated with ASO3 did not exhibit an increase in symptom development over the evaluation period. This study highlights the efficacy of ASO technology, based on short oligonucleotide chemically modified sequences, as a promising tool for controlling phytopathogens without the need for genetic transformation or plant regeneration. Our results demonstrate the potential of ASOs as a biotechnological tool for the management of citrus canker disease.

Keywords: susceptibility gene, *Xanthomonas citri*

Citrus canker, a severe bacterial disease caused by *Xanthomonas citri* subsp. *citri*, poses a significant global threat to commercial citrus production, resulting in substantial economic losses (Behlau et al. 2020; Martins et al. 2020). Currently, copper-based chemicals are the main compounds for controlling citrus canker. However, their extensive use raises concerns about environmental impact, plant stress, and bacterial resistance (Behlau et al. 2020; Heydarpanah et al. 2020; Lamichhane et al. 2018). To overcome these challenges, biotechnology-based approaches have emerged as promising alternatives, offering sustainable methods to enhance plant resistance against citrus canker disease (Caserta et al. 2019; Huang et al. 2022a; Prado et al. 2024).

To induce citrus canker symptoms, *X. citri* subsp. *citri* secretes PthA4, a transcriptional activator-like effector that enters the nucleus and activates the expression of the canker susceptibility gene LATERAL ORGAN BOUNDARIES 1 (*LOB1*), causing hypertrophy and hyperplasia on citrus tissues (An et al. 2020; Martins et al. 2020; Yan and Wang 2012). Activation of the LOB1 transcription factor induces the expression of plant genes associated with cell expansion, remodeling of the cell wall, and the development of citrus canker symptoms (Hu et al. 2014; Roeschlin et al. 2019; Zou et al. 2021). Recently, resistance to citrus canker was achieved in different citrus species by using CRISPR-Cas technology to target *LOB1* genes (Huang et al. 2022b; Jia and Wang 2020; Jia et al. 2017, 2022; Peng et al. 2017; Su et al. 2023). These different studies demonstrated the importance of *LOB1* as a target gene for citrus canker control. Although conventional transgenics and CRISPR genome editing are relevant strategies for building more durable disease resistance, both require plant transformation or regeneration techniques, in addition to propagation, to produce seedlings, making these approaches laborious and time consuming (Krasnodębski et al. 2023). Recently, the use of antisense oligonucleotides (ASOs) has been highlighted as a potential biotechnological tool to transiently silence genes of interest without modifying the plant genome (Dinç et al. 2011; Krasnodębski et al. 2023).

ASOs are short synthetic DNA or RNA polymers capable of affecting nucleic acid metabolism through physical and chemical alteration (Benizri et al. 2019; Dinç et al. 2011; Rinaldi and Wood 2018). ASOs reversely complement their target messenger RNA (mRNA), consequently inducing a gene silencing effect (Oberemok et al. 2022). Once within the cellular environment, they hybridize with their target transcript, forming a duplex structure (Chan et al. 2006). This process disrupts the mRNA's conformation and rearranges their own structure, ultimately leading to the establishment of a heteroduplex (Shao et al. 2006). The major silencing activity arises from recruiting the endogenous nuclease RNAse H, which identifies the newly formed hybridized structure. This recognition event triggers the mRNA's cleavage, thereby

†Corresponding author: A. A. de Souza; desouza@ccsm.br

Funding: Support was provided by the Fundação de Amparo à Pesquisa do Estado de São Paulo (2019/23217-1, 2020/04795-1 awarded to I. G. B. Carvalho, 2017/13885-1 awarded to R. R. de Souza-Neto, 2021/09689-8 awarded to L. S. dos Santos, 2022/04112-7 awarded to C. A. Nascimento, and 2021/096). L. F. F. de Lima is a master student from the Graduate Program in Genetics and Molecular Biology (State University of Campinas/Unicamp) supported by a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (grant 001). A. A. de Souza, A. Mehta, and M. A. Takita are recipients of research fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

***e*-Xtra:** Supplementary material is available online.

The author(s) declare no conflict of interest.

exerting a negative regulatory effect (Chan et al. 2006; Dinç et al. 2011; Oberemok et al. 2022). Recently, ASOs were employed to silence specific genes from '*Candidatus* Liberibacter asiaticus', a citrus-pathogen bacterium that causes citrus greening disease, also known as Huanglongbing (HLB). In this study, the authors demonstrated the potential of ASOs to be integrated into the management of agricultural pathogens aiming at HLB control (Sandoval-Mojica et al. 2021).

Despite the potential use of ASOs in plants, their application is limited when compared with mammalian cells (Horwich and Zamore 2008; Korobeynikov et al. 2022; McMahon et al. 2023). Thus, aiming to extend the application of ASOs in plants, we focused on *CsLOB1* as a target to reduce citrus canker disease. In this study, we developed a pipeline to design ASO sequences in the citrus genome and validate its interference in citrus canker development. The selected ASO3-*CsLOB1* was evaluated in detached leaves and whole plants using two methods of ASO delivery and bacteria inoculation. In both methods, ASO3-*CsLOB1* significantly reduced *CsLOB1* gene expression and citrus canker development. Together, this study confirms the potential use of ASOs as a potential technology for plant-pathogen control, which encompasses genetic manipulation without alterations in the plant genome (Dhuri et al. 2020; Lusser et al. 2011).

Materials and Methods

Design and synthesis of ASOs

The ASOs were designed following the pipeline developed for *Citrus sinensis* in this study, based on Távora et al. (2021). In the first step, the fasta sequence (orange1.1g026556m) of the *CsLOB1* gene was obtained from the Citrus Genome Database (https://www.citrusgenomedb.org/). Subsequently, the Blast tool (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi-Global Align) was used to detect conserved homologous regions between *C. sinensis* varieties for ASO design. After selecting the fasta sequence, it was analyzed using both Sfold (https://sfold.wadsworth.org/cgi-bin/index.pl) and Oligowalk (http://rna.urmc.rochester.edu/cgi-bin/server_exe/oligowalk/oligowalk_form.cgi) software. Sfold and Oligowalk use algorithms to calculate the thermodynamic parameters of antisense hybridization by predicting changes in the free energy of oligonucleotide binding to the target RNA. The algorithms predict the best regions for accessing the target mRNA structure by evaluating the variation in the molecule's free energy. Thus, based on the input sequence, the software selects ASOs with a probability of successfully silencing the target mRNA. By default, Sfold generated a list of the top 20 ASOs, whereas Oligowalk produced a list of 140 oligos (Supplementary Tables S1 and S2). Subsequently, the ASOs generated by each software program were compared, and those common to both software programs were chosen (Supplementary Table S3). The oligonucleotides were then analyzed by BLASTn with the citrus genome to ensure there were no similarities with sequences of nonspecific genes, to avoid possible off-targets (cutoff = below 16 nucleotides of identity). The second check was performed by BLAST in the Citrus Genome Database to verify the location of the ASO sequences on the target mRNA and to ensure they were not located in a splicing region or untranslated region. Third, the ASO target site in the citrus genome was amplified and sequenced on the ABI PRISM 3730 sequencer (Applied Biosystems) to confirm homology between the ASO nucleotides and their respective genome target sequences. Finally, three ASOs for each gene were selected for synthesis (Supplementary Fig. S1). In addition to the selected ASOs, a random sequence ASO (random) was designed to serve as a negative control. This sequence was designed by randomizing the ASOs using the Shuffle DNA software (https://www.bioinformatics.org/sms2/shuffle_dna.html). Afterward, a BLASTn analysis was performed again for the random ASO to ensure it did not align with any other plant gene. The ASOs were ultimately synthesized by Integrated DNA Technologies, using phosphorothioate modification in all ASOs (https://www.idtdna.com/pages/education/decoded/article/antisense-oligonucleotides-(asos)). The modifications were introduced at both the 5′ and 3′ ends, with two modifications at each end.

Foliar petiole ASO absorption assay

Aiming to confirm the ASO absorption by plant vascular tissues, the random ASO was labeled with the fluorochrome 5′6-FAM (495 nm excitation, 520 nm green emission) (https://www.idtdna.com/site/Catalog/Modifications/Product/1108). For fluorescence microscopy analysis, a total of five sequential sectioned slices were performed using a LEICA SM2010R microtome. The slices were done 1 cm above the region of contact between the petiole and the ASO solution. The cross-sections were examined with a BX6 fluorescence microscope (Olympus) at magnifications of 40×. GFP fluorescence was captured using the U-MWB2/WB3 (Olympus) filter (band pass of 520 nm).

Effect of ASOs by absorption in detached leaves

Eighteen young leaves from the *C. sinensis* 'Hamlin' variety were used in the experiment. Each leaf was incubated in a 50-ml Falcon tube with 250 μl of 10 μM ASO solution (10 μM ASO diluted in autoclaved water). This experiment was conducted with three biological replicates for each ASO (ASO1, ASO2, and ASO3) and controls (water and random). The leaves were incubated for 24 h with the ASO solution before inoculation with *X. citri* subsp. *citri* strain 306. In parallel, the *X. citri* subsp. *citri*_GFP strain 306 (Rigano et al. 2007) was grown on nutrient broth yeast extract culture medium containing ampicillin (100 mg/liter) and gentamicin (100 mg/liter). The plates were incubated at 28°C. A colony was used to prepare the pre-inoculum, which was inoculated in 10 ml of liquid nutrient broth yeast extract medium plus 100 mg/liter of gentamicin in a 50-ml Falcon tube and placed under agitation at 180 rpm at 28°C overnight. This inoculum was used for leaf infiltration, where 100 μl of the bacterial suspension (10^4 CFU/ml) was applied on each side of the leaf using a needleless syringe. A total of three leaves were used for each ASO. The leaves were collected for RNA extraction at specific time points: T0 (before ASO infiltration) and T1, T2, and T3 (1, 2, and 3 days after ASO infiltration). The symptoms were evaluated through photographic records and symptomatic area using the Quant software (Vale et al. 2003). The experiment was repeated twice with three internal replicates, and the statistical significance of the means was analyzed using Student's *t* test ($P < 0.05$).

Effect of ASOs by leaves infiltration in planta

The bacterium was harvested from solid nutrient broth yeast extract medium and incubated for 2 days at 28°C. The inoculum was prepared as described above. The inoculum was infiltrated into young leaves in whole plants of *C. sinensis* and kept in a plastic chamber to improve the humidity condition. After 48 h, solutions containing 10 μM of ASO1, 2, or 3 were infiltrated. This time point was chosen based on studies that demonstrated that *CsLOB1* is highly induced 48 h after *X. citri* subsp. *citri* inoculation (Abe and Benedetti 2016; An et al. 2020). As controls, water and random ASO infiltrations were used. The treatments and their respective controls were inoculated in the same leaf, totaling three infiltrations per leaf (see Supplementary Fig. S2). At least three independent experiments were performed with five replicates, and the statistical significance of the means was analyzed using Student's *t* test ($P < 0.05$).

Bacterial inoculation by pin-roller device

For experiments with ASO3, infiltration in whole plants was performed as described above. The bacteria were inoculated by a pin-roller, as described by Nascimento et al. (2022). This methodology induces microinjuries that facilitate bacterial entry while minimizing mechanical damage, thus reducing experimental variation. Moreover, as *X. citri* subsp. *citri* naturally enters plant cells

through natural openings or microinjuries, this methodology closely mimics what occurs under natural conditions (Nascimento et al. 2022). Briefly, the inoculations were performed using a pin-roller device (Dermaroller 540 System, ELON-YC) containing 540 microneedles 0.5 mm in length rolled over the leaves. Subsequently, sterile cotton soaked in a bacterial solution (10^8 CFU/ml) was applied to the top of the microinjuries on all the leaves. The leaves were collected for RNA extraction at specific time points: T0 (before ASO infiltration) and T1, T2, and T3 (1, 2, and 3 days after ASO infiltration). The symptomatology of canker lesions was photographed and analyzed using an Olympus MVX10 (U-MGFPHQ filter) stereomicroscope, and pustule analysis was performed using ImageJ software (Schneider et al. 2012). Samples containing canker pustules were collected from both treatments (ASO3 and control) at 5, 7, 9, 11, and 14 days after inoculation. For CFU counting, the samples were collected at 7 and 14 days after bacteria inoculation. The experiment was repeated three times for symptom analysis and twice for CFU counting and RNA extraction. Three internal replicates were used for each experiment, and the statistical significance of the means was analyzed using Student's *t* test ($P < 0.05$).

Quantitative real-time PCR

To access the gene expression of *CsLOB1*, the mRNA was reverse transcribed following quantitative real-time PCR (RT-qPCR) analysis. The total RNA of the leaf samples was extracted using the PureLink RNA Mini Kit (Invitrogen). The RNA quality and concentration were determined by spectrophotometry using NanoDrop ND-8000 (Thermo Fisher Scientific) and electrophoresis. One microgram of total RNA was used for cDNA synthesis with a GoScript Reverse Transcription Kit (Promega). The reactions were performed using GoTaq qPCR Master Mix (Promega), 120 nm of each *CsLOB1*-specific primer pair (CsLOB1_F, 5′-TCCTTCACAATCCTCTCCAAATCT-3′ and CsLOB1_R, 5′-AAGAAGTGCATACCTGCAAGAAC-3′) (Jia et al. 2017), and actin (Actin_F, 5′-CCCTTCCTCATGCCATTCTTC-3′ and Actin_R, 5′-CGGCTGTGGTGGTAAACATGT-3′) as the reference gene (Shimo et al. 2019). The qPCR reaction was performed in QuantStudio 5 (Thermo Fisher Scientific) using the default program. Dissociation curves were analyzed to confirm the primer specificity. The relative gene expression was determined by the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

ASO design and plant absorption

Our pipeline utilized two distinct software tools, Oligowalk and Sfold. Subsequently, the intersection of these lists yielded five common oligos (Supplementary Table S3), which underwent a blast analysis against the citrus genome to verify a potential off-target effect and confirm their presence within coding regions. Three oligos meeting these stringent criteria were selected for utilization in this study, constituting our approach for ASO selection. However, it is worth noting that an alternative approach, utilizing the full set of 20 ASOs from Sfold and the 140 from Oligowalk, could also be considered valid, representing an alternative criterion for selection. Three different sequences were obtained as potential candidates to silence *CsLOB1* (Table 1; Supplementary Fig. S1). The sequences targeted the coding sequence of the *CsLOB1* transcript (citrus genome ID: 18129398) at positions 212 to 230 (ASO1), 304 to 322 (ASO2), and 487 to 505 (ASO3). In addition, a randomly designed ASO was used as a negative control. The ASO absorption by the plant was confirmed by diluting fluorescence-labeled ASO in water and placing the leaf petiole in contact with the solution. The labeled ASO was detected in the xylem region of the leaves 24 h after absorption (Fig. 1A). No signal was detected in untreated leaves (Fig. 1B).

Effect of the ASOs on citrus canker development

To assess the ability of ASO1, ASO2, and ASO3 to reduce citrus canker symptoms, detached leaves were first treated with ASOs through petiole absorption and then infiltrated with *X. citri*_GFP. In general, leaves inoculated with ASO1, ASO2, and ASO3 showed lower symptom severity (Fig. 2A) and bacterial colonization, assessed through GFP fluorescence from infiltrated leaves (Fig. 2B), compared with the water and random controls. The ASO treatments resulted in an average of symptomatic area in leaves between 15 and 17% (ASO1 $P = 0.06$; 0.08), 13 to 16% (ASO2 $P = 0.04$; 0.07), and 15 to 18% (ASO3 $P = 0.008$; 0.04) lower compared with the water and random controls, respectively. Although the symptomatic leaf areas treated with ASO1, ASO2, and ASO3 were smaller compared with the controls, significant differences for both controls were observed only for ASO3 (Fig. 2C). To select the best ASO sequence for further analysis, new experiments were carried out using whole plants instead of detached leaves and ASO infiltration instead of absorption. The controls and ASOs were inoculated in the same leaf and evaluated daily for 14 days (Supplementary Fig. S2). Similarly, as observed for the detached leaves, only leaves treated with ASO3 showed the average of symptomatic lesions significantly reduced compared with both controls (Fig. 3A to C). This reduction led to a 34 to 18% ($P = 0.002$; 0.01) decrease in the symptomatic canker lesions compared with the water and random controls, respectively (Fig. 3C). These results (Figs. 2 and 3) indicated ASO3 as the best sequence to interfere with citrus canker disease, probably to target *CsLOB1*. Additionally, because both controls (water and random) showed the same results, only water was used as a control in the subsequent experiments.

CsLOB1 posttranscriptional gene silencing in *C. sinensis* by ASO3

To verify the effect of ASO3 in silencing *CsLOB1*, the two different ASO3 delivery methods used in previous experiments were

TABLE 1. Parameters of the antisense oligonucleotides (ASOs) used in this study[a]

ASO	Starting target position	Ending target position	Target sequence (5′→3′)	ASO (5′→3′)	GC content (%)	Average unpaired probability for target site nucleotides	Binding site disruption energy (kcal/mol) (ΔG)	Oligo binding energy (kcal/mol)	Probability of being efficient siRNA (Oligowalk)
ASO1	212	230	CUCUCCGCCGCCUAUAGUU	A*A*CTATAGGCGGCGGAG*A*G	57.9	0.621	7.7	−9.7	0.715123
ASO2	304	322	CACCAACCGAACCAUACAA	T*T*GTATGGTTCGGTTGG*T*G	47.4	0.598	7.0	−11.6	0.825929
ASO3	487	505	GAAACAAGUCAGUGAGCUU	A*A*GCTCACTGACTTGTT*T*C	42.1	0.557	5.6	−9.1	0.897697
Random	–	–	–	C*G*CCGACGCAAACGCTT*T*A	57.9	–	–	–	–

[a] Asterisks (*) indicate the positions of the phosphorothioate modifications. siRNA, small interfering RNA.

tested. *X. citri* subsp. *citri* was inoculated in two different ways, by infiltration, when ASO3 delivery was done by absorption, or pin-roller microlesions when ASO3 delivery was carried out by infiltration. Samples were collected for gene expression analysis using RT-qPCR at 1, 2, and 3 days after inoculation with *X. citri*. For both ASO3 delivery methods, a significant reduction of *CsLOB1* transcript levels was observed at all evaluated time points compared with the control (Fig. 4A and B). The significant reduction of *CsLOB1* transcripts was 8.9, 3.8, and 12.8-fold when ASO3 was applied by absorption (Fig. 4A) and 5.4, 15.9, and 10.5 when ASO3 was applied by infiltration (Fig. 4B) in T1, T2, and T3 compared with their respective controls. Altogether, our results demonstrate that ASO3 was able to suppress transcripts in both methods of delivery and bacterial inoculation, suggesting a positive correlation with the reduction of citrus canker symptoms observed in previous experiments.

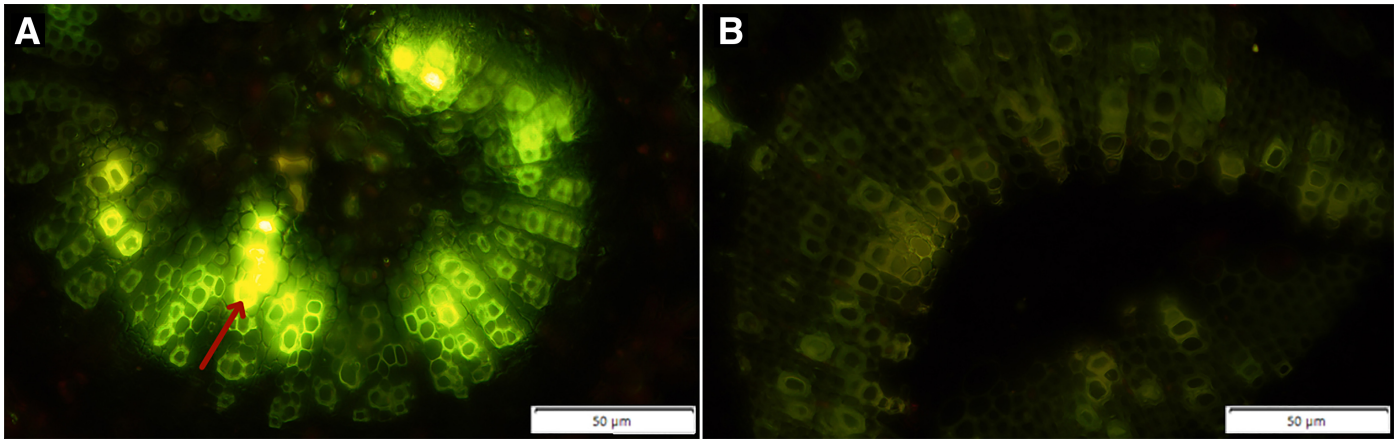

Fig. 1. Fluorescence-labeled antisense oligonucleotide (ASO) localization in *Citrus sinensis* cells. Images were acquired using fluorescence microscopy with the WB3 filter (495 nm excitation, 520 nm emission). **A,** Cells after absorbing 10 µM of ASO-5′6-FAM solution. Red arrow indicates the presence of a fluorescent ASO in the xylem vessels. **B,** Untreated cells showing absence of fluorescence.

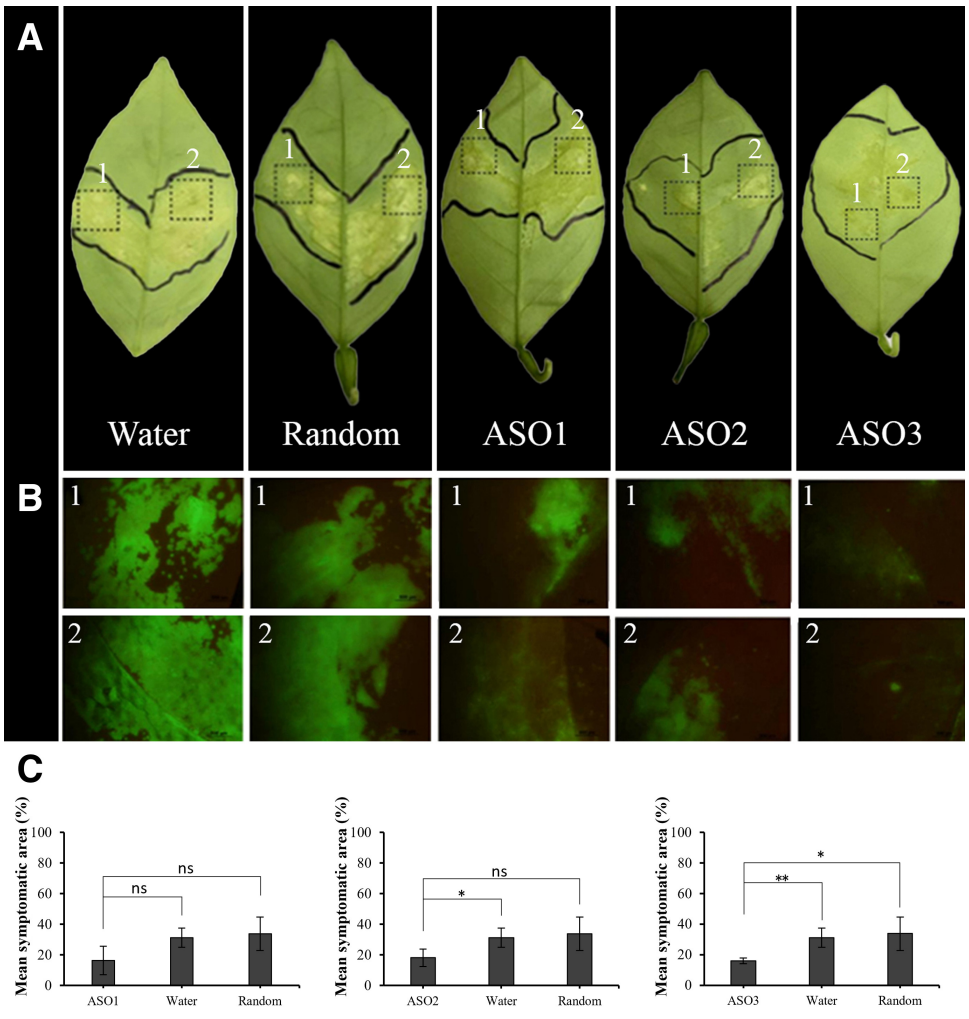

Fig. 2. Analysis of citrus canker and bacterial colonization after antisense oligonucleotide (ASO) absorption and *Xanthomonas citri*_GFP infiltration. **A,** Representative leaves infiltrated with *X. citri*_GFP in the area circled in black. **B,** Images of *X. citri*_GFP-infiltrated areas using a GFP filter in different treatments. Numbers 1 and 2 in B correspond to the respective squad area in A. Water indicates *Citrus sinensis* leaf inoculated with water as a control. Random indicates *C. sinensis* leaf inoculated with random ASO control. ASO1, ASO2, and ASO3 indicate *C. sinensis* leaves inoculated with respective ASOs. **C,** Symptomatic area analysis performed in Quant software to determine the percentage of the area exhibiting citrus canker symptoms. Asterisks (*) indicate significant differences based on Student's *t* test (*, $P < 0.05$) (**, $P < 0.01$) comparing ASO1, ASO2, and ASO3 treatments with controls (water and random ASO). Error bars represent the standard deviation of the means. Three independent experiments were performed with five biological repeats. ns, not significant.

ASO3 treatment reduces citrus canker symptoms

To confirm the potential of ASO3 to decrease citrus canker symptoms, new experiments using whole plants were performed. In agreement with previous results, all plants treated with ASO3 exhibited a substantial reduction in canker symptoms at all evaluated time points compared with the control (Fig. 5A). Notably, canker lesions in ASO3-treated leaves remained restricted and did not expand over time, in contrast to control leaves, where symptoms developed into large spongy pustules (Fig. 5A). Canker lesions were quantified by analyzing four areas of each leaf ($n = 3$) at each time point (Fig. 5B; Supplementary Fig. S3). An increase in canker pustule size was only observed in leaves treated with water, whereas no such increase occurred in leaves treated with ASO3. Indeed, the reduction of the pustule size induced by ASO3 was of 60.3% ($P = 0.033$), 80.1% ($P = 0.002$), 50.6% ($P = 0.020$), and 82.7% ($P < 0.0001$), respectively, for 7, 9, 11, and 14 days after inoculation, demonstrating a significant decrease in citrus canker symptoms. Furthermore, the *X. citri* subsp. *citri* bacterial population was significantly lower in leaves treated with ASO3 compared with the untreated control at 14 days after inoculation (Fig. 5C). The decrease in symptom development, together with the delay in bacterial growth and the reduction of *CsLOB1* transcript level, indicates that ASO3 silences *CsLOB1*, thereby affecting the interaction of *X. citri* subsp. *citri* and *C. sinensis*, resulting in attenuated disease symptoms.

Discussion

Citrus is one of the most important fruit crops worldwide (Neves et al. 2020). However, citrus production is facing many diseases, such as citrus canker caused by *X. citri* subsp. *citri* (Martins et al. 2020), that increase economic losses and environmental impact. Due to the importance of promoting sustainable agricultural production, the development of new technologies to control phytopathogens is urgently needed. In this study, we applied a pipeline to design ASO sequences to silence the susceptibility gene *CsLOB1* in *C. sinensis*. In our pipeline, three ASOs were selected for functional validation, among which ASO1 and ASO2 exhibited lower effectiveness compared with ASO3 in reducing citrus canker symptoms. This could be attributed to the ASO sequence prediction by bioinformatics tools, which generate sequences along with a probability percentage of their efficacy (Lu and Mathews 2008). In agreement with this result, ASO3 showed the highest probability of being efficient, as indicated by Oligowalk (Table 1). In any event, functional experiments are imperative to validate the efficacy of the designed ASOs in targeting mRNA sequences (José de Sousa et al. 2023; Mizuta and Higashiyama 2014; Sandoval-Mojica et al. 2021; Távora et al. 2021).

In our study, different methods of ASO delivery and bacteria inoculation demonstrated the efficiency of ASO3 in reducing the level of *CsLOB1* transcripts, reducing citrus canker disease. The symptom reduction ranged from approximately 15 to 83%, and it is likely attributed to the method of ASO delivery. Curiously, despite the reduction in the canker pustule sizes, we still observed bacterial growth in the plant tissues even with ASO3, which agrees with the previous studies where *CsLOB1* was edited using CRISPR-Cas (Duan et al. 2018; Su et al. 2023).

CsLOB1 is a transcriptional factor that is known to play a key role as a susceptibility gene in *C. sinensis*–*X. citri* subsp. *citri*

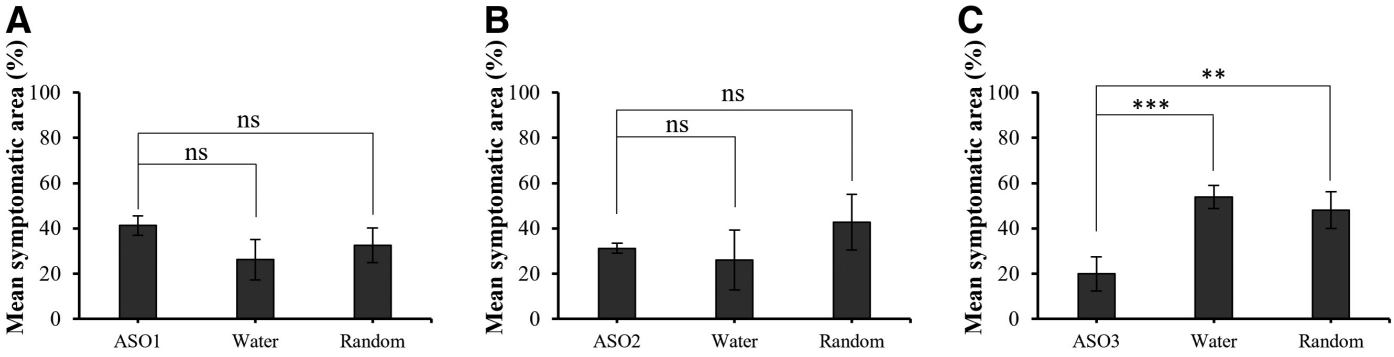

Fig. 3. Quantification of the citrus canker symptomatic area (%) in planta. Symptomatic area analysis was performed using the Quant software to determine the percentage of leaf area exhibiting citrus canker symptoms. Results are the mean of triplicate experiments ± SD. Asterisks (*) indicate significant differences based on Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$) comparing the antisense oligonucleotide (ASO) treatment with controls (water and random). Error bars represent the standard deviation of the means. **A to C,** Leaves infiltrated with *Xanthomonas citri*_GFP followed by infiltration with ASO1, ASO2, and ASO3, respectively. Three independent experiments were performed with three biological repeats. ns, not significant.

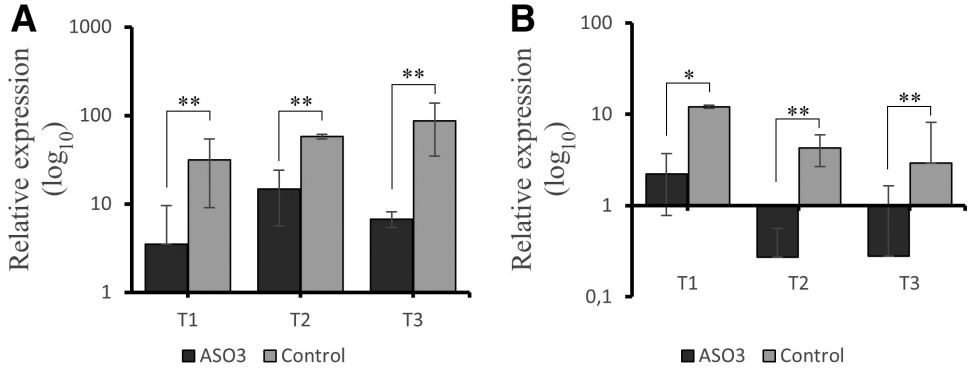

Fig. 4. Transcript level of *CsLOB1* in leaves treated or not with antisense oligonucleotide (ASO) 3 at 1, 2, and 3 days after inoculation with *Xanthomonas citri.* **A,** ASO3 delivery by petiole absorption and *X. citri* subsp. *citri* inoculated by infiltration. **B,** ASO3 delivery by leaf infiltration and *X. citri* subsp. *citri* inoculated by pin-roller. Results are the mean of duplicate experiments with three replicates compared with Time 0 (before inoculation). Asterisks (*) indicate significant differences based on Student's *t* test (*, $P < 0.05$; and **, $P < 0.01$). Error bars represent the standard deviation of the means. T1, T2, and T3 indicate 1, 2, and 3 days after *X. citri* subsp. *citri* inoculation.

interaction, where the bacterial effector positively regulates *CsLOB1* gene expression, which induces morphological modification favoring a compatible interaction and bacterial colonization (Barnes and Anderson 2018; Zou et al. 2021). Recent studies employing CRISPR-mediated *LOB1* gene editing have produced citrus plants with increased resistance to citrus canker disease (Huang et al. 2022a; Jia and Wang 2020; Jia et al. 2017, 2022; Peng et al. 2017; Su et al. 2023). Our data align with these findings, demonstrating that enhanced citrus canker resistance can be achieved by silencing *CsLOB1*. Notably, to achieve such results, we used ASO technology to target *CsLOB1*, which has the advantage of inducing gene silencing without transgeny or the use of plant transformation and regeneration techniques, known to be time consuming and laborious (Krasnodębski et al. 2023; Turnbull et al. 2021). In addition, ASOs can be used for different citrus species or cultivars. The use of ASOs for gene silencing in plants is relatively recent (Dinç et al. 2011; Krasnodębski et al. 2023; Sandoval-Mojica et al. 2021; Távora et al. 2021) compared with their use as biotherapeutics (Krasnodębski et al. 2023). As pointed out by Krasnodębski et al. (2023), this technology is a low-cost and easy-to-implement method for direct manipulation of gene activity, and although predominantly applied in animal cells, its utilization in plants seems to be even more straightforward and should be better explored. Moreover, ASOs have been used to increase the basic knowledge regarding gene function in plant−pathogen interactions. Recently, the use of ASOs was applied for assessing the behavior of susceptibility genes in tomato triggered by *Xanthomonas euvesicatoria* pv. *perforans* during the plant−pathogen interaction (José de Sousa et al. 2023). According to the authors, screening these genes is essential to implement alternative management strategies capable of silencing target genes that play a key role in symptom induction. Consequently, these applications can be expanded to other pathosystems.

The application of ASOs in citrus was recently shown to control '*Candidatus* Liberibacter asiaticus', a bacterium transmitted by *Diaphorina citri* and the causal agent of citrus greening or HLB, considered the worst disease affecting citrus groves worldwide (Sandoval-Mojica et al. 2021). In this work, the authors modified the ASO sequences by incorporating 2′-deoxy-2′-fluoro-d-arabinonucleic acid (FANA). According to the authors, these chemical modifications increased the resistance of the FANA-ASO to degradation and enhanced its binding to targeted mRNA. The study demonstrated that FANA-ASOs silence bacterial essential genes, indicating the potential utility of FANA-ASOs in current HLB management strategies.

In other studies using ASOs for plant silencing approaches, it was necessary to dilute the ASOs in a sugar solution (Krasnodębski et al. 2023; Sun et al. 2007; Xie et al. 2014) for absorption by the leaf vascular system. However, we were able to observe the presence of the labeled random ASO in the vascular tissue using a dilution in water. It is likely that the efficiency of absorption might vary depending on many aspects, such as plant species, tissues, age, and method of ASO delivery. Additionally, using ASOs with the established phosphorothioate modification to improve stability and avoid molecule degradation, we were able to confirm their uptake and effect. Taken together, as the use of ASO application in plants is still little explored for biotechnology purposes, improvements in the technique can be made to optimize its absorption, stability, and efficiency.

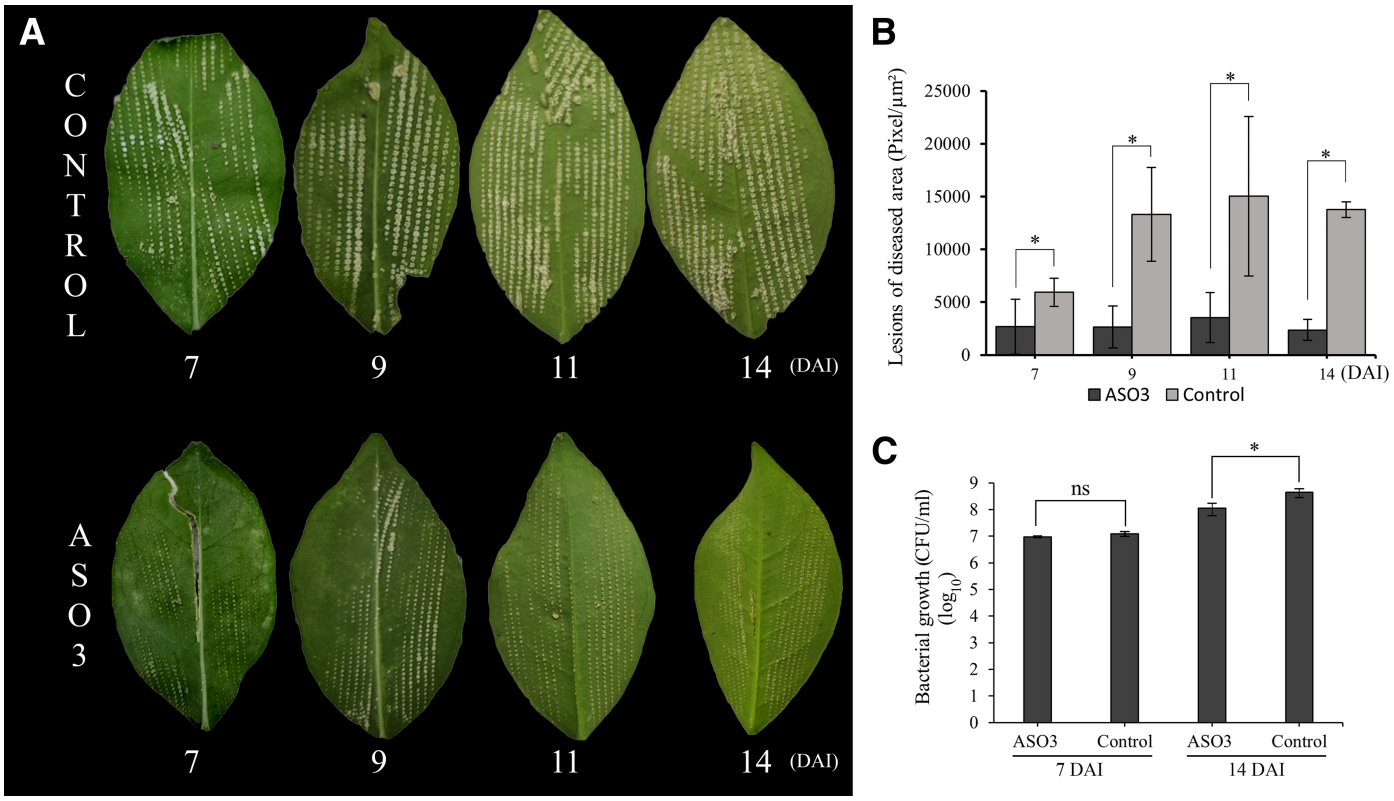

Fig. 5. Effect of antisense oligonucleotide (ASO) 3 on citrus canker disease in *Citrus sinensis*. **A,** Representative images of citrus canker in leaves treated with ASO3 or water control followed by *Xanthomonas citri* subsp. *citri* inoculation using the pin-roller technique. **B,** Quantitative evaluation of diseased area in leaves treated with ASO3 and water control. Each leaf was divided into four quadrants, and the symptomatic area was quantified using ImageJ software. The numbers 7, 9, 11, and 14 correspond to days after inoculation (DAI). The experiment was repeated three times with three replicates. **C,** Bacterial populations (CFU/ml) were evaluated from leaves infiltrated with ASO3 and water control followed by *X. citri* subsp. *citri* inoculation by pin-roller and collected at 7 and 14 DAI. Asterisks (*) indicate significant differences based on Student's *t* test (*, $P < 0.05$) comparing ASO3 treatment and the respective control at each time point. Error bars represent the standard deviation of the means. The experiment was repeated twice with three internal replicates used for each experiment. ns, not significant.

Based on our results, we assume that the stability of ASO3 probably persists for at least 14 days after bacterial inoculation, as a significant decrease of citrus canker symptoms is still observed in treated plants during this period. This finding is noteworthy, considering that citrus canker symptoms typically manifest shortly after bacterial infection, and considering that the application of copper in a shorter spray interval (7, 14, 21, or 28 days) is required to significantly reduce yield loss of citrus canker diseases (Behlau et al. 2010), the stability of ASOs during this critical period could potentially prevent the development of citrus canker. Furthermore, we verified that the efficiency of the ASO silencing mechanism greatly relies on the level of the target mRNA transcripts and the ASO concentration in the cell. The efficacy of ASOs is predominantly hampered by challenges in cellular delivery. The primary molecular and cellular hurdles to maximizing antisense effectiveness lie in intracellular trafficking (Roth 2005). Given that, exploring other ASO targets in both plant and bacteria, along with formulations incorporating adjuvants and/or nanotechnology to enhance ASO penetration into plant cells, holds promise for improving its application and ushering in new biotechnological approaches to control plant pathogens.

Literature Cited

Abe, V. Y., and Benedetti, C. E. 2016. Additive roles of PthAs in bacterial growth and pathogenicity associated with nucleotide polymorphisms in effector-binding elements of citrus canker susceptibility genes. Mol. Plant Pathol. 17:1223-1236.

An, S.-Q., Potnis, N., Dow, M., Vorhölter, F.-J., He, Y.-Q., Becker, A., Teper, D., Li, Y., Wang, N., Bleris, L., and Tang, J.-L. 2020. Mechanistic insights into host adaptation, virulence and epidemiology of the phytopathogen *Xanthomonas*. FEMS Microbiol. Rev. 44:1-32.

Barnes, W. J., and Anderson, C. T. 2018. Release, recycle, rebuild: Cell-wall remodeling, autodegradation, and sugar salvage for new wall biosynthesis during plant development. Mol. Plant 11:31-46.

Behlau, F., Belasque, J., Jr., Graham, J. H., and Leite, R. P., Jr. 2010. Effect of frequency of copper applications on control of citrus canker and the yield of young bearing sweet orange trees. Crop Prot. 29:300-305.

Behlau, F., Gochez, A. M., and Jones, J. B. 2020. Diversity and copper resistance of *Xanthomonas* affecting citrus. Trop. Plant Pathol. 45: 200-212.

Benizri, S., Gissot, A., Martin, A., Vialet, B., Grinstaff, M. W., and Barthélémy, P. 2019. Bioconjugated oligonucleotides: Recent developments and therapeutic applications. Bioconjug. Chem. 30:366-383.

Caserta, R., Teixeira-Silva, N. S., Granato, L. M., Dorta, S. O., Rodrigues, C. M., Mitre, L. K., Yochikawa, J. T. H., Fischer, E. R., Nascimento, C. A., Souza-Neto, R. R., Takita, M. A., Boscariol-Camargo, R. L., Machado, M. A., and De Souza, A. A. 2019. Citrus biotechnology: What has been done to improve disease resistance in such an important crop? Biotechnol. Res. Innov. 3:95-109.

Chan, J. H. P., Lim, S., and Wong, W. F. 2006. Antisense oligonucleotides: From design to therapeutic application. Clin. Exp. Pharmacol. Physiol. 33: 533-540.

Dhuri, K., Bechtold, C., Quijano, E., Pham, H., Gupta, A., Vikram, A., and Bahal, R. 2020. Antisense oligonucleotides: An emerging area in drug discovery and development. J. Clin. Med. 9:2004.

Dinç, E., Tóth, S. Z., Schansker, G., Ayaydin, F., Kovács, L., Dudits, D., Garab, G., and Bottka, S. 2011. Synthetic antisense oligodeoxynucleotides to transiently suppress different nucleus- and chloroplast-encoded proteins of higher plant chloroplasts. Plant Physiol. 157:1628-1641.

Duan, S., Jia, H., Pang, Z., Teper, D., White, F., Jones, J., Zhou, C., and Wang, N. 2018. Functional characterization of the citrus canker susceptibility gene *CsLOB1*. Mol. Plant Pathol. 19:1908-1916.

Heydarpanah, S., Rezaei, R., Taghavi, S. M., and Charehgani, H. 2020. Efficacy of different copper compounds in the control of *Xanthomonas citri* subsp. *citri* pathotypes A and A*. J. Phytopathol. 168:73-80.

Horwich, M. D., and Zamore, P. D. 2008. Design and delivery of antisense oligonucleotides to block microRNA function in cultured *Drosophila* and human cells. Nat. Protoc. 3:1537-1549.

Hu, Y., Zhang, J., Jia, H., Sosso, D., Li, T., Frommer, W. B., Yang, B., White, F. F., Wang, N., and Jones, J. B. 2014. *Lateral organ boundaries 1* is a disease susceptibility gene for citrus bacterial canker disease. Proc. Natl. Acad. Sci. U.S.A. 111:E521-E529.

Huang, X., Wang, Y., and Wang, N. 2022a. Base editors for citrus gene editing. Front. Genome Ed. 4:852867.

Huang, X., Wang, Y., and Wang, N. 2022b. Highly efficient generation of canker-resistant sweet orange enabled by an improved CRISPR/Cas9 system. Front. Plant Sci. 12:769907.

Jia, H., Omar, A. A., Orbović, V., and Wang, N. 2022. Biallelic editing of the *LOB1* promoter via CRISPR/Cas9 creates canker-resistant 'Duncan' grapefruit. Phytopathology 112:308-314.

Jia, H., and Wang, N. 2020. Generation of homozygous canker-resistant citrus in the T0 generation using CRISPR-SpCas9p. Plant Biotechnol. J. 18: 1990-1992.

Jia, H., Zhang, Y., Orbović, V., Xu, J., White, F. F., Jones, J. B., and Wang, N. 2017. Genome editing of the disease susceptibility gene *CsLOB1* in citrus confers resistance to citrus canker. Plant Biotechnol. J. 15:817-823.

José de Sousa, L., Carolina Mendes Bezerra, A., Rocha Maximiano, M., Távora, F. T. P. K., Faustino Cury, N., Brilhante de Oliveira Neto, O., Véronique Bindschedler, L., Luiz Franco, O., Blum, L. E. B., and Mehta, A. 2023. Expression of tomato susceptibility genes triggered by *Xanthomonas euvesicatoria* pv. *perforans* and disease control by short antisense oligodeoxynucleotide gene silencing. Physiol. Mol. Plant Pathol. 128:102163.

Korobeynikov, V. A., Lyashchenko, A. K., Blanco-Redondo, B., Jafar-Nejad, P., and Shneider, N. A. 2022. Antisense oligonucleotide silencing of FUS expression as a therapeutic approach in amyotrophic lateral sclerosis. Nat. Med. 28:104-116.

Krasnodębski, C., Sawuła, A., Kaźmierczak, U., and Żuk, M. 2023. Oligo—Not only for silencing: Overlooked potential for multidirectional action in plants. Int. J. Mol. Sci. 24:4466.

Lamichhane, J. R., Osdaghi, E., Behlau, F., Köhl, J., Jones, J. B., and Aubertot, J.-N. 2018. Thirteen decades of antimicrobial copper compounds applied in agriculture. A review. Agron. Sustain. Dev. 38:1-18.

Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25:402-408.

Lu, Z. J., and Mathews, D. H. 2008. OligoWalk: An online siRNA design tool utilizing hybridization thermodynamics. Nucleic Acids Res. 36: W104-W108.

Lusser, M., Parisi, C., Rodriguez Cerezo, E., and Plan, D. 2011. New plant breeding techniques. State-of-the-art and prospects for commercial development. Publications Office of the European Union, Luxembourg.

Martins, P. M. M., de Oliveira Andrade, M., Benedetti, C. E., and de Souza, A. A. 2020. *Xanthomonas citri* subsp. *citri*: Host interaction and control strategies. Trop. Plant Pathol. 45:213-236.

McMahon, M. A., Rahdar, M., Mukhopadhyay, S., Bui, H.-H., Hart, C., Damle, S., Courtney, M., Baughn, M. W., Cleveland, D. W., and Bennett, C. F. 2023. GOLGA8 increases bulk antisense oligonucleotide uptake and activity in mammalian cells. Mol. Ther. Nucleic Acids 32:289-301.

Mizuta, Y., and Higashiyama, T. 2014. Antisense gene inhibition by phosphorothioate antisense oligonucleotide in *Arabidopsis* pollen tubes. Plant J. 78:516-526.

Nascimento, C. A., Teixeira-Silva, N. S., Caserta, R., Marques, M. O. M., Takita, M. A., and de Souza, A. A. 2022. Overexpression of *CsSAMT* in *Citrus sinensis* induces defense response and increases resistance to *Xanthomonas citri* subsp. *citri*. Front. Plant Sci. 13:836582.

Neves, M. F., Trombin, V. G., Marques, V. N., and Martinez, L. F. 2020. Global orange juice market: A 16-year summary and opportunities for creating value. Trop. Plant Pathol. 45:166-174.

Oberemok, V., Puzanova, Y., and Novikov, I. 2022. Controlled lavender field fragrance: The biotechnological "Provence" of the future provided by antisense oligoilators and olinscides. Preprints 120334.

Peng, A., Chen, S., Lei, T., Xu, L., He, Y., Wu, L., Yao, L., and Zou, X. 2017. Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene *CsLOB1* promoter in citrus. Plant Biotechnol. J. 15:1509-1519.

Prado, G. S., Rocha, D. C., dos Santos, L. N., Contiliani, D. F., Nobile, P. M., Martinati-Schenk, J. C., Padilha, L., Maluf, M. P., Lubini, G., Pereira, T. C., Monteiro-Vitorello, C. B., Creste, S., Boscariol-Camargo, R. L., Takita, M. A., Cristofani-Yaly, M., and de Souza, A. A. 2024. CRISPR technology towards genome editing of the perennial and semi-perennial crops citrus, coffee and sugarcane. Front. Plant Sci. 14:1331258.

Rigano, L. A., Siciliano, F., Enrique, R., Sendín, L., Filippone, P., Torres, P. S., Qüesta, J., Dow, J. M., Castagnaro, A. P., Vojnov, A. A., and Marano, M. R. 2007. Biofilm formation, epiphytic fitness, and canker development in *Xanthomonas axonopodis* pv. *citri*. Mol. Plant-Microbe Interact. 20:1222-1230.

Rinaldi, C., and Wood, M. J. A. 2018. Antisense oligonucleotides: The next frontier for treatment of neurological disorders. Nat. Rev. Neurol. 14:9-21.

Roeschlin, R. A., Uviedo, F., García, L., Molina, M. C., Favaro, M. A., Chiesa, M. A., Tasselli, S., Franco-Zorrilla, J. M., Forment, J., Gadea, J., and Marano, M. R. 2019. PthA4^{AT}, a 7.5-repeats transcription activator-like (TAL) effector from *Xanthomonas citri* ssp. *citri*, triggers citrus canker resistance. Mol. Plant Pathol. 20:1394-1407.

Roth, C. M. 2005. Molecular and cellular barriers limiting the effectiveness of antisense oligonucleotides. Biophys. J. 89:2286-2295.
Sandoval-Mojica, A. F., Hunter, W. B., Aishwarya, V., Bonilla, S., and Pelz-Stelinski, K. S. 2021. Antibacterial FANA oligonucleotides as a novel approach for managing the Huanglongbing pathosystem. Sci. Rep. 11:2760.
Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9:671-675.
Shao, Y., Wu, Y., Chan, C. Y., McDonough, K., and Ding, Y. 2006. Rational design and rapid screening of antisense oligonucleotides for prokaryotic gene modulation. Nucleic Acids Res. 34:5660-5669.
Shimo, H. M., Terassi, C., Lima Silva, C. C., de Lima Zanella, J., Mercaldi, G. F., Rocco, S. A., and Benedetti, C. E. 2019. Role of the *Citrus sinensis* RNA deadenylase CsCAF1 in citrus canker resistance. Mol. Plant Pathol. 20:1105-1118.
Su, H., Wang, Y., Xu, J., Omar, A. A., Grosser, J. W., Calovic, M., Zhang, L., Feng, Y., Vakulskas, C. A., and Wang, N. 2023. Generation of the transgene-free canker-resistant *Citrus sinensis* using Cas12a/crRNA ribonucleoprotein in the T0 generation. Nat. Commun. 14:3957.
Sun, C., Ridderstråle, K., Höglund, A.-S., Larsson, L.-G., and Jansson, C. 2007. Sweet delivery – sugar translocators as ports of entry for antisense oligodeoxynucleotides in plant cells. Plant J. 52:1192-1198.
Távora, F. T. P. K., Bevitori, R., Mello, R. N., Cintra, M. M. D. F., Oliveira-Neto, O. B., Fontes, W., Castro, M. S., Sousa, M. V., Franco, O. L., and Mehta, A. 2021. Shotgun proteomics coupled to transient-inducible gene silencing reveal rice susceptibility genes as new sources for blast disease resistance. J. Proteomics 241:104223.
Turnbull, C., Lillemo, M., and Hvoslef-Eide, T. A. K. 2021. Global regulation of genetically modified crops amid the gene edited crop boom–A review. Front. Plant Sci. 12:630396.
Vale, F., Fernandes-Filho, E., and Liberato, J. 2003. Quant–A Software for Plant Disease Severity Assessment. 8th International Congress of Plant Pathology, Christchurch, New Zealand.
Xie, Z., Sundström, J. F., Jin, Y., Liu, C., Jansson, C., and Sun, C. 2014. A selection strategy in plant transformation based on antisense oligodeoxynucleotide inhibition. Plant J. 77:954-961.
Yan, Q., and Wang, N. 2012. High-throughput screening and analysis of genes of *Xanthomonas citri* subsp. *citri* involved in citrus canker symptom development. Mol. Plant-Microbe Interact. 25:69-84.
Zou, X., Du, M., Liu, Y., Wu, L., Xu, L., Long, Q., Peng, A., He, Y., Andrade, M., and Chen, S. 2021. CsLOB1 regulates susceptibility to citrus canker through promoting cell proliferation in citrus. Plant J. 106:1039-1057.