Research Article

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Fungal effector genes involved in the suppression of chitin signaling as novel targets for the control of powdery mildew disease via a nontransgenic RNA interference approach

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Abstract

BACKGROUND: Chitin is a crucial component of fungal cell walls and an effective elicitor of plant immunity; however, phytopathogenic fungi have developed virulence mechanisms to counteract the activation of this plant defensive response. In this study, the molecular mechanism of chitin-induced suppression through effectors involved in chitin deacetylases (CDAs) and their degradation (EWCAs) was investigated with the idea of developing novel dsRNA-biofungicides to control the cucurbit powdery mildew caused by *Podosphaera xanthii*.

RESULTS: The molecular mechanisms associated with the silencing effect of the *PxCDA* and *PxEWCAs* genes were first studied through dsRNA cotyledon infiltration assays, which revealed a ≈80% reduction in fungal biomass and a 50% decrease in gene expression. To assess the impact on powdery mildew disease control, *in vitro* and *in planta* assays were carried out in growth chamber and glasshouse experiments, with ≈50% reduction in disease symptoms after 8 days postinoculation (dpi) in leaf discs and 12 dpi in plants' leaves, respectively. This control was extended for 21 days when the dsRNAs were protected on the carbon dot nanocarriers. Additionally, the uptake of the dsRNAs by fungal spores was observed 12 h postapplication via confocal microscopy, and efficient processing of dsRNAs into siRNAs by the melon RNAi machinery was observed 24 h postspraying through sRNA-seq.

CONCLUSIONS: This study highlights notable advancements in environmentally friendly disease management, and features the technological potential of RNA-based fungicides together with nanotechnology for cucurbit powdery mildew control.

Supporting information may be found in the online version of this article.

Keywords: biofungicides; carbon dot nanoparticles; chitin signaling suppression; fungal effectors; powdery mildew; RNAi

1 INTRODUCTION

Powdery mildew diseases represent some of the most significant plant diseases globally, affecting major crops, including cereals, grapevines, and numerous economically important vegetables and ornamentals.[1–3] Although many horticultural crops are susceptible to powdery mildew, cucurbits are highly vulnerable, with the biotrophic fungus *Podosphaera xanthii* identified as the primary causal agent.[4] Chemical control and the use of resistant cultivars, represent the primary approaches for managing the disease; however, the rapid emergence of new physiological races and the appearance of fungicide-resistant isolates make disease control challenging for growers.[4,5] Additionally, according to the 'Farm-to-Fork' strategy of the recent European Green Deal, the number of available fungicides will be reduced in the near future.[6] Overall, there is an urgent need for innovative disease management strategies.

A novel crop protection strategy based on the RNA interference (RNAi) mechanism, known as spray-induced gene silencing (SIGS), has recently been developed for the control of fungal diseases.[7,8] RNAi is a conserved mechanism of gene regulation in eukaryotes,

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mediated by small RNAs (sRNAs) that induce gene silencing at transcriptional and post-transcriptional levels.[9] In phytopathogenic fungi, investigations of *Austropuccinia psidii*, *Botrytis cinerea*, *Fusarium graminearum*, *F. oxysporum*, *Mycosphaerella fijiensis*, *Phakopsora pachyrhizi* and *Sclerotinia sclerotiorum* have supported the effectiveness of the exogenous application of dsRNA molecules through SIGS for functional analysis of genes and controlling diseases in their hosts.[10–14] The efficacy of SIGS also has been investigated in cucurbit powdery mildew fungus, revealing high levels of disease control after the application of dsRNAs targeting essential genes for *P. xanthii* development.[15] This highlights the importance of continuing to explore this strategy as a novel tool for disease control in the field.

In order to improve the SIGS strategy under natural conditions, the use of nanocarriers has been proposed.[16] Owing to their small size, nanoparticles (NPs) serve as ideal candidates to increase the penetration and uptake of dsRNA by plant cells, thereby prolonging the duration of their silencing effect.[17–20] Nanoparticles have been classified according to their nature as organic or inorganic. In the case of organic NPs, and to mimic the natural mechanisms by which plants deliver their own siRNAs to pathogens, dsRNAs packaged in liposomes or extracellular synthetic phospholipid bilayers, have been utilized, showing promising results against *B. cinerea*.[21,22] With respect to inorganic NPs, double-layer hydroxide (LDH) NPs have been used to increase dsRNA activity and provide protection against viruses,[23,24] insects[25] and several fungal diseases.[26–28] However, a recent study demonstrated how dsRNA molecules coated with carbon dot (CD) NPs were effectively delivered into cucumber plants, yielding positive results in controlling cucurbit viruses.[29] These examples highlight the successful integration of RNAi and nanotechnology applications in agriculture.

Recently, *P. xanthii* effectors implicated in manipulating chitin-triggered immunity, a natural plant defense against fungal invasion, were identified.[30–32] These effectors include *P. xanthii* chitin deacetylase (CDA), which converts chitin into chitosan by hydrolyzing the *N*-acetamido group in *N*-acetylglucosamine units,[33,34] and effectors with chitinase activity (EWCAs) that degrade immunogenic chitin fragments at pathogen penetration sites.[31] The end products of these effectors, chitosan and degraded chitin oligomers, exhibit reduced affinity for plant chitin receptors, thereby suppressing pathogen-triggered immunity (PTI) and compromising plant defense responses.[31–33] In this work, the potential of the *PxCDA* and *PxEWCAs* genes as targets for cucurbit powdery mildew disease control was evaluated. For this purpose, the molecular mechanisms associated with the silencing effect of both genes were first studied through dsRNA cotyledon infiltration assays. To assess the impact on disease control, *in vitro* and *in planta* SIGS assays were carried out to investigate the effect of the combination of the oligonucleotides with the CD nanocarriers. Finally, the uptake of both dsRNA types by fungal spores was analyzed through confocal laser scanning microscopy, and *PxCDA*-dsRNA uptake and processing into siRNAs by the RNAi melon machinery were investigated via small RNA sequencing (sRNA-seq).

2 MATERIALS AND METHODS

2.1 Plants, microbes and culture conditions

Zucchini cotyledons (*Cucurbita pepo* L.) cv. Negro Belleza (Semillas Fitó, Barcelona, Spain) and melon plants (*Cucumis melo* L.) cv. Rochet (Semillas Fitó) were employed for fungal maintenance and RNAi assays. The plants were cultivated in growth chambers at 24 °C with a 16 h:8 h, light:dark photoperiod.

The *P. xanthii* isolate 2086 was used in this study. For fungal maintenance, 4–5 days disinfected zucchini cotyledons inoculated with fungal spores using an eyelash under binocular magnifying glass and stored *in vitro* in 8-cm Petri dishes with Bertrand medium were utilized following established methods.[35] *Escherichia coli* strain DH5α was routinely used for the maintenance, construction and propagation of RNAi silencing vectors. The bacterial strain was grown at 37 °C in lysogeny broth (LB) supplemented with ampicillin (100 μg mL^{-1}) as needed.

2.2 Isolation of nucleic acids and cDNA synthesis

For DNA and RNA extraction from melon cotyledons infected with *P. xanthii*, the cotyledons were initially frozen in liquid nitrogen and then preserved at −80 °C until use. Genomic DNA extraction was carried out with the MasterPure Yeast DNA Purification Kit (Epicenter Biotechnologies, Madison, WI, USA), whereas total RNA was isolated using TRI Reagent (Sigma-Aldrich, Steinheim, Germany) in accordance with the provided instructions. Total RNA was isolated with TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions, and eluted in diethylpyrocarbonate (DEPC)-treated water. The concentration of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The samples were stored at −80 °C until further use. cDNA synthesis was accomplished using Superscript III reverse transcriptase (Thermo Fisher Scientific) and random primers (Thermo Fisher Scientific) following the manufacturer's recommendations.

2.3 *In vitro* synthesis of dsRNAs

The *P. xanthii* (*Px*) genes *PxCDA* (chitin deacetylase; KX495502, KX495503 and KX495504) *PxEWCA* (nine effectors with chitinase activity; MK078518, MK078519, MK078526, MK078522, MK078520, MK078521, MK078525, MK078524 and MK078523) and *PxTUB2* (β-tubulin, a key component of microtubules; KC333362), along with the melon (*Cucumis melo*) genes *CmMLO* (powdery mildew resistance locus O1; FJ713541) and *CmCERK1* (a chitin elicitor receptor kinase involved in plant immunity; XP_008455461.1), were amplified from cDNA using the Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) with the following conditions: initial denaturation step at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s, with a final elongation at 72 °C for 7 min. The primers used (Table S1) were designed using Primer3 software (http://bioinfo.ut.ee/primer3/ accessed on 12 July 2021).[36] The PCR products were subsequently digested with FastDigest *Nco*I and *Bgl*II restriction enzymes (Thermo Fisher Scientific) and then cloned and inserted into the same pL4440 vector (kindly provided by Andrew Fire, Stanford University) using T4 DNA ligase (Thermo Fisher Scientific) following the manufacturer's guidelines. The resulting plasmids were propagated and maintained in *E. coli* DH5α, and the sequence inserts were confirmed through PCR amplification, digestion and sequencing (Stab Vida, Caparica, Portugal). For dsRNA synthesis, plasmid products were digested with the aforementioned restriction enzymes and used as templates in an *in vitro* transcription reaction with the HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA). dsRNA concentrations were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and integrity was confirmed via 1% agarose gel electrophoresis. For fungal spore uptake studies, fluorescein-labeled dsRNAs were synthesized using the Fluorescein RNA Labeling Mix (Sigma-Aldrich) as per the manufacturer's protocol.

2.4 dsRNA gene silencing

2.4.1 Cotyledon infiltration assay

In order to examine the effects of dsRNAs on fungal development and gene expression, infiltration assays were conducted on melon cotyledons following the methods outlined in.[37] Before each assay, dsRNA solutions (*PxCDA* and *PxEWCAs*) were prepared in infiltration buffer (10 mM MES and 10 mM $MgCl_2$) and adjusted to a final concentration of 100 ng mL^{-1} as used previously.[15] Additionally, *CmCERK1*-dsRNA was used to corroborate the link between the activation of chitin-triggered immunity and disease-reduction phenotypes, as reported previously.[31,37] Positive and negative controls included cotyledons infiltrated with *CmMLO*-dsRNA and dsRNA derived from an empty vector, respectively. The progress of *P. xanthii* infection was assessed through histochemical analysis via the 3,3′-diaminobenzidine (DAB) uptake method, as described previously.[37,38] For molecular quantification of fungal biomass, genomic DNA was isolated from cotyledons at 72 h postinoculation (hpi) and analyzed using qPCR. The *P. xanthii* gene *PxTUB2* and the *C. melo* gene *CmACT7* (actin-7; XM_008462689.2) were quantified, and the *P. xanthii/C. melo* genomic DNA ratio was calculated as described previously.[39] Gene expression was evaluated via quantitative reverse transcription (qRT)-PCR, using total RNA extracted at 72 hpi for cDNA synthesis. The *P. xanthii* gene *PxEF1* (translation elongation Factor 1-alpha; MK249653) and the *C. melo* gene *CmACT7* were used as normalization reference genes.[40,41] The primers utilized in these analyses are described in Table S1. The reactions were conducted on a CFX384 Touch Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using SsoFast EvaGreen Supermix (Bio-Rad) according to the manufacturer's instructions. The cycling conditions consisted of an initial denaturation step at 90 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 5 s. A melt curve analysis was performed by gradually increasing the temperature from 55 to 95 °C at a ramp rate of 0.5 °C s^{-1}nd, with a 5-s hold at each temperature increment. Following amplification, data processing was carried out via CFX Manager Software (Bio-Rad), and amplicon sizes were confirmed through visualization on 2% agarose gels.

2.4.2 In vitro *test: leaf disc assay*

In order to assess the impact of exogenously applied dsRNAs on *P. xanthii* development, a leaf disc assay was employed as described previously.[42] *PxCDA*- and *PxEWCAs*-dsRNA solutions were prepared in DEPC-treated water at 100, 200, 500, and 1000 ng mL^{-1}. The dsRNA-treated and *P. xanthii*-inoculated discs were kept in a growth chamber with a 16 h:8 h, light:dark photoperiod at 24 °C until disease severity evaluation [8 days post-inoculation (dpi)] through image analysis.[43] Disease symptoms were measured by estimating the percentage of the disc surface covered by powdery mildew. Positive controls included discs treated with 100, 200, 500 and 1000 ng mL^{-1} CmMLO-dsRNA, whereas water-treated discs served as negative controls.

2.4.3 In planta *assay: SIGS experiments*

In order to assess the disease control effectiveness of *PxCDA*-dsRNA- and *PxEWCAs*-dsRNA-mediated RNAi *in planta*, SIGS assays were conducted on melon plants in a growth chamber at 24 °C with a 16 h:8 h, light:dark photoperiod. In the first case, the methodology and dsRNA concentration (10 μg mL^{-1}) used were as described previously.[15] Disease symptoms were evaluated via image analysis at 12 dpi as described previously.[43] Melon leaves sprayed with *PxTUB2*-dsRNAs and water were used as positive and negative controls, respectively. A glasshouse experiment was subsequently carried out in the IHSM-UMA-CSIC 'La Mayora' (Algarrobo-Costa, Málaga) with the following treatments applied to the melon leaves: two negative controls (water and empty CD nanocarriers), *PxTUB2*-dsRNA and the commercial fungicide fluopyram at the field dose as positive controls, naked *PxCDA*- and *PxEWCAs*-dsRNAs, *PxCDA*- and *PxEWCAs*-dsRNA-CDs, and untreated and uninoculated plants. *PxCDA*- and *PxEWCAs*-dsRNA solutions were prepared in DEPC-treated water at a concentration of 40 μg mL^{-1}, optimized through prior glasshouse trials (data not shown). The CD NPs were kindly provided by Leonardo Velasco (IFAPA Centro de Churriana, Malaga, Spain), whose synthesis and binding protocols were described previously.[29] To determine the effectiveness of the dsRNAs and the NPs on disease progression over time, images were taken at 14 and 21 dpi. *P. xanthii* gene expression and fungal biomass also were assessed via qRT-PCR and qPCR, respectively, as detailed in Section 2.4.1, at 144 hpi and 168 hpi, respectively.

2.5 Visualization of dsRNA uptake by the fungus

In order to visualize dsRNA uptake by *P. xanthii* conidia, fungal spores were deposited onto a 1% water–agar pad on a glass slide. Then, a 5-μL drop of sterile distilled water (negative control) or fluorescein-labeled *PxCDA*-dsRNA (5000 ng) was added. The mixture was incubated overnight in darkness at room temperature. One hour before imaging, 2 μL micrococcal nuclease (75 U) was added, followed by incubation for 2 h in darkness at 37 °C to eliminate any remaining dsRNA in the media.[44] The samples were analyzed via a confocal laser scanning microscope (LSM880; Zeiss, Oberkochen, Germany).

2.6 Small RNA-seq and bioinformatics analysis of melon leaves

In order to investigate dsRNA processing into siRNAs by melon plants without the presence of the fungal pathogen, total RNA from water- and *PxCDA*-dsRNA-treated plants was extracted 24 h postapplication from a second true leaf. To study the movement of the oligonucleotides through the plant, total RNA was extracted 72 h postapplication from a non-dsRNA-treated leaf (third true leaf). Twelve sequencing libraries were generated following the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (New England Biolabs), and single-end (SE) sequencing was performed on the Illumina NextSeq 550 platform. Adapter trimming, cleaning and size filtering (removal of sequences >24 nt and <18 nt long) of the raw reads were performed via Trimmomatic.[45] The sequence data quality was analyzed with FastQC.[46] Next, contaminant noncoding RNAs were removed by aligning the datasets against the Rfam database via Bowtie 2.[47,48] The siRNA population was mapped with Bowtie 2 to the *PxCDA*-dsRNA sequence. The resulting sam and bam files were processed with SAMtools,[49] and the study of the siRNAs was performed with in-house scripts. si-Fi21 and i-Score Designer software were used to provide insights into *PxCDA*-dsRNA-derived siRNAs.[50,51]

2.7 Statistical analysis

Statistical analysis of the data was conducted via IBM Spss v25 software (SPSS, Chicago, IL, USA) via Fisher's least significant difference test (LSD).

3 RESULTS

3.1 Cotyledon infiltration of *CDA*- and *EWCAs*-dsRNAs mediates gene silencing in *Podosphaera xanthii*

The roles of the *PxCDA* and *PxEWCAs* genes in fungal development and disease establishment were investigated through cotyledon infiltration assays. To evaluate fungal growth, two complementary methods were employed: haustorial counting via light microscopy and molecular estimation of fungal biomass via qPCR. As shown in Fig. 1(a),(b), both genes were crucial for fungal development. Gene silencing markedly reduced haustorial counts

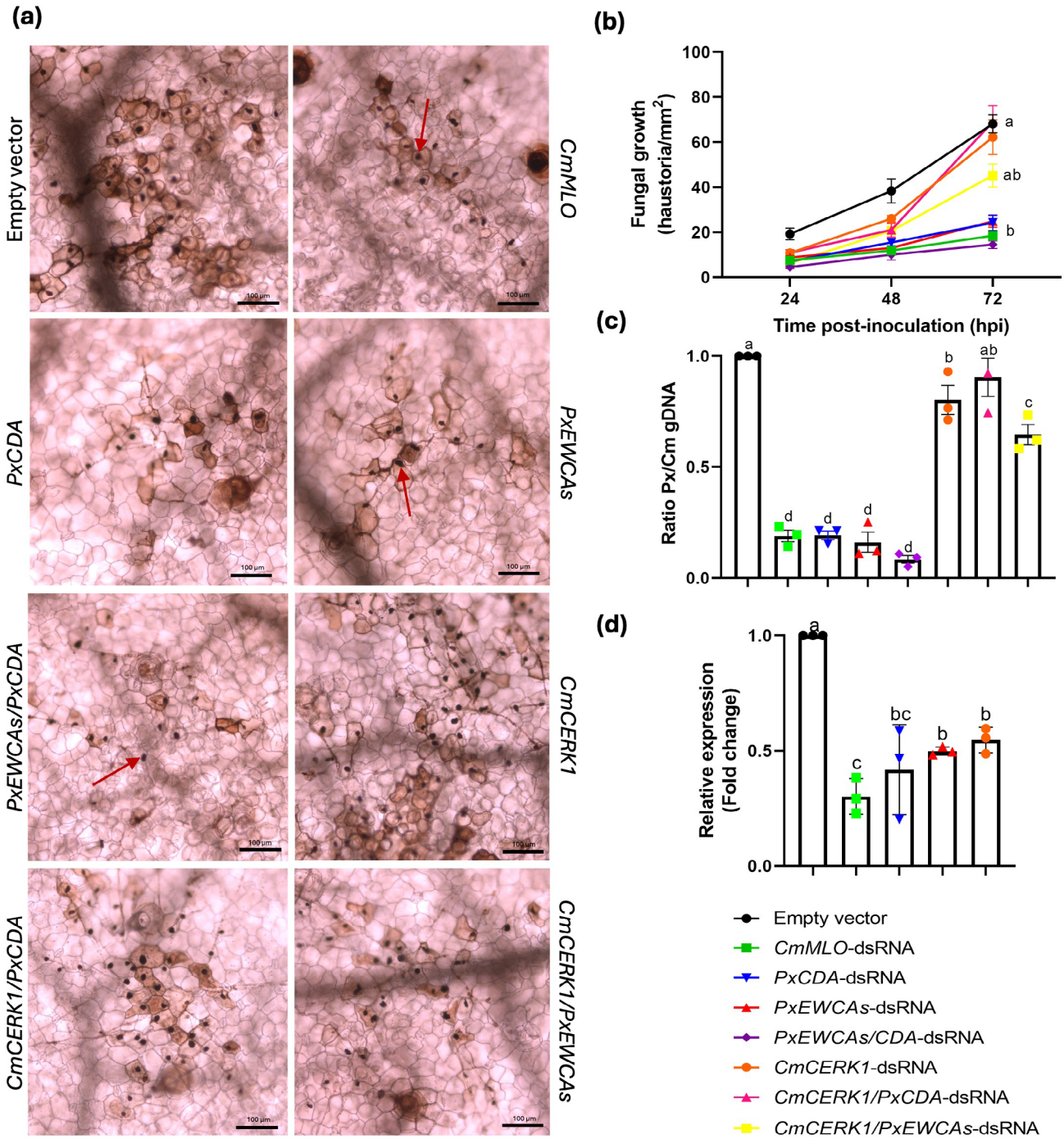

Figure 1. Effect of dsRNA application on *Podosphaera xanthii* growth and disease development. (a) Visualization of fungal structures. The detection of haustoria was performed by the 3,3′-diaminobenzidine (DAB) uptake method. Pictures were taken at 72 h postinoculation with *P. xanthii*. Arrowheads indicate *P. xanthii* haustoria. Bars = 100 µm. (b) Time-course analysis of *P. xanthii* haustorium formation per unit area (mm^2) on melon cotyledons post-silencing of *P. xanthii* and melon genes. The data represent the means of 30 samples from three independent experiments ± SEs. (c) Molecular assessment of *P. xanthii* biomass in dsRNA-infiltrated melon cotyledons. The bars represent the means ± SEs of three technical replicates from three different samples. Data points with the same letter are not significantly different at $P \leq 0.05$, as determined by Fisher's least significant difference (LSD) test. (d) Evaluation of the efficacy of dsRNA-induced gene silencing of *P. xanthii* and melon genes. The bars represent the means ± SEs of three technical replicates from three different samples. Data points with the same letter are not significantly different at $P \leq 0.05$, as determined by Fisher's LSD test.

to levels comparable to those of the *CmMLO* gene; additionally, in tissues cosilenced with the *CmCERK1* gene, fungal growth was fully restored [Fig. 1(a),(b)]. Fungal biomass also was quantified at 72 hpi using qPCR, corroborating the haustorial count results [Fig. 1(c)]. The reduction in fungal growth was attributed to a ≈50% decrease in the expression of all target genes, as estimated by qRT-PCR [Fig. 1(d)].

3.2 PxCDA and PxEWCAs are potential targets for cucurbit powdery mildew control

In order to investigate the impact of these two *P. xanthii* effectors as new targets for the development of dsRNA-based fungicides for cucurbit powdery mildew disease control, the exogenous application of dsRNA was carried out via *in vitro* and *in planta* assays following protocols described previously.[15,42] Cotyledon leaf discs treated with different concentrations of *PxCDA*-dsRNA exhibited significantly reduced disease symptoms, with 59.93, 57.84, 52.58, and 51.59% at concentrations of 100, 200, 500 and 1000 ng mL^{-1}, respectively [Fig. 2(a),(b)]. For the application of *PxEWCAs*-dsRNA, reductions of 46.56, 42.40, 40.94, and 40.70% were observed at the same concentrations [Fig. 2(a),(b)]. The dsRNAs were sprayed onto the second leaves of melon plants in growth chamber assays as described previously.[15] As outlined in Fig. 2(c),(d), the application of dsRNA resulted in a reduction in fungal growth, leading to a significantly fewer disease symptoms, as measured in terms of the leaf surface covered by powdery mildew. In the case of individual silencing of *PxCDA*-dsRNA and *PxEWCAs*-dsRNA, disease severity decreased by 51.91% and 52.13%, respectively. The cosilencing of both genes improved disease control by ≤60.96%, which was not statistically significant compared with the positive control *PxTUB2*-dsRNA.

3.3 SIGS control of cucurbit powdery mildew under glasshouse conditions is improved with CD nanocarriers

In order to assess the potential of the studied *P. xanthii* genes as a sustainable approach for disease management under glasshouse conditions, SIGS assays were conducted by applying oligonucleotides either naked or encapsulated in CD NPs for delivery and protection. *PxCDA*-dsRNA and *PxEWCAs*-dsRNA were tested at 40 μg mL^{-1}, a concentration that was greater than that used in growth chamber assays owing to uncontrolled glasshouse conditions, which resulted in lower concentrations being less efficient (data not shown). As depicted in Fig. 3, the application of dsRNAs of the *PxCDA* and *PxEWCAs* genes resulted in a ≈50% reduction in disease symptoms, quantified in terms of the leaf surface covered by powdery mildew [Fig. 3(a),(b)] on melon leaves treated with dsRNA naked or dsRNA-CDs. However, after an additional week (21 dpi), an increase in disease severity in the nonencapsulated dsRNA treatment group was observed, with 20 and 10% disease reduction in the *PxCDA*-dsRNA and *PxEWCAs*-dsRNA treatment groups, respectively; however, disease severity remained stable in the dsRNA-CD treatment groups, extending the suppression of the disease compared with that in the naked oligonucleotide group [Fig. 3(a),(b)]. Gene expression and fungal biomass also were molecularly quantified at 144 and 168 hpi, respectively, revealing similar reductions to those reported previously in infiltration assays [Figs 1(c),(d) and 3(c),(d)].

3.4 *Podosphaera xanthii* spores can take up dsRNAs from media

In order to determine whether *P. xanthii* spores can take up both *PxCDA*-dsRNA and *PxEWCAs*-dsRNA directly from their environment, conidia were incubated with *in vitro*–transcribed fluorescein-labeled dsRNAs of both genes, as described previously.[44] The fluorescence signals started accumulating within fungal cells at 12 h postincubation (Fig. 4). Because no signal was detected at the previous time points (data not shown), observation was continued until 18 h postincubation, with no decrease in the signal, demonstrating that a powdery mildew fungus can take up dsRNAs independent of its host plant.

3.5 Uptake and processing of sprayed dsRNA into siRNAs by melon plants

In order to explore the ability of the plant's silencing machinery to take up and process the applied dsRNA into siRNAs that could be taken up by the fungus during infection, an sRNA-seq experiment was conducted. For this purpose, water or *PxCDA*-dsRNA treatments were applied to selected sprayed (local) leaves. Leaf segments from local and nonsprayed (distal) leaves were subsequently harvested at 24 and 72 h postspraying, respectively. Twelve libraries with three biological replicates were constructed and sequenced via Illumina technology. A total of 9 526 851–18 016 171 raw reads were obtained from each library, resulting in 3 623 912–5 111 616 sequences following the first round of filtering and 1 016 725–2 197 651 siRNAs after the second filtering (Fig. S1). The detailed bioinformatics workflow is shown in Fig. 5(a). To determine the length of the siRNA populations enriched in melon leaves, the presence or absence of dsRNA-derived siRNAs in both local and distal leaves was examined. The length distribution revealed that the amount of the general population of siRNAs was uniform and consistent, with 21-nt and 22-nt siRNA size class profiles being the most abundant [Fig. 5(b)]. Mapping of the siRNA population to the *PxCDA*-dsRNA sequence revealed a significant abundance of dsRNA-derived siRNAs only in local leaves at 24 h postspraying [Fig. 5(c)]. These data show that plants treated with dsRNA contained siRNAs corresponding to the *PxCDA* gene, demonstrating that the topically applied dsRNA molecules were introduced into the plant cells and processed into siRNAs by the endogenous RNAi plant machinery. However, the performance of two common software programs for the design of RNAi strategies, si-Fi21 and i-Score Designer, was evaluated.[50,51] For this purpose, *PxCDA*-dsRNA was used as a query to predict the siRNA-rich regions in its sequence via si-Fi21 and was compared with the experimental data from the treated local leaves. According to the results, three of the five experimental regions matched the tool's output, which allowed us to determine its partial reliability for that task [Fig. 5(d)]. Moreover, the *PxCDA*-dsRNA sequence was entered into i-Score Designer to obtain the best probable siRNAs. The five most frequent sequences in the dsRNA-derived siRNA files were selected and complemented with the information generated by the i-Score Designer prediction (Table 1).

4 DISCUSSION

The declining effectiveness of commonly employed fungicides against powdery mildew has spurred an urgent demand for alternative methods to reduce environmental contamination and the risk of fungicide resistance.[5] Recently, interest in SIGS as a viable approach for plant disease management has increased. SIGS, which is based on RNAi technology, can suppress pathogen activity and, therefore, disease progression, providing efficient and sustainable crop protection.[12,52,53] The results of our study suggest that PxCDA and PxEWCAs are pivotal proteins for powdery mildew virulence, as silencing these genes suppressed fungal

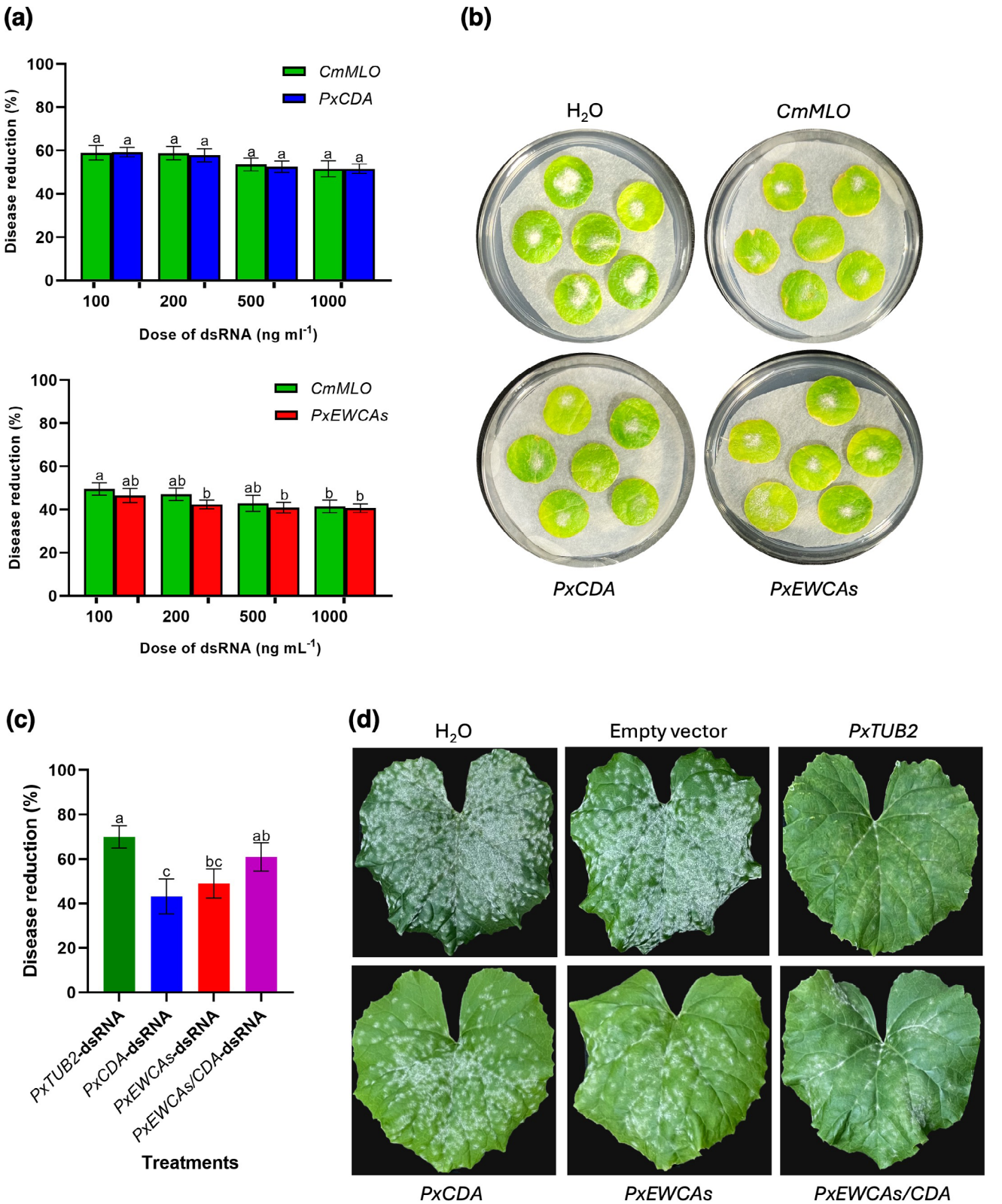

Figure 2. Impact of the application of dsRNA on the growth of *Podosphaera xanthii* and the development of associated diseases. (a) Powdery mildew disease reduction after placing zucchini cotyledon discs in contact with several doses of dsRNA targeting the *CmMLO*, *PxCDA* and *PxEWCA* genes, followed by inoculation with fresh suspensions of *P. xanthii* conidia. The data represent the means of 36 samples from three independent experiments, with error bars indicating the standard error. Data points sharing the same letter are not significantly different at $P = 0.05$, as determined by Fisher's least significant difference (LSD) test. (b) Representative images of powdery mildew symptoms in zucchini cotyledon discs treated with a 100 ng mL^{-1} dose of dsRNAs. (c) Disease reduction after SIGS assays on melon plants sprayed with 10 μg mL^{-1} dsRNAs of the *P. xanthii CDA* and *EWCAs* genes. Photographs of the fungal pathogen were analyzed at 12 days postinoculation (dpi). (d) Representative images of melon leaves sprayed with H_2O or empty vector as a negative control or *PxTUB2* as a positive control, revealing a significant reduction in powdery mildew symptoms. Leaves from plants sprayed with *PxCDA*-dsRNA, *PxEWCAs*-dsRNA and *PxCDA-PxEWCAs*-dsRNAs, presented reductions in disease symptoms similar to those of the positive control.

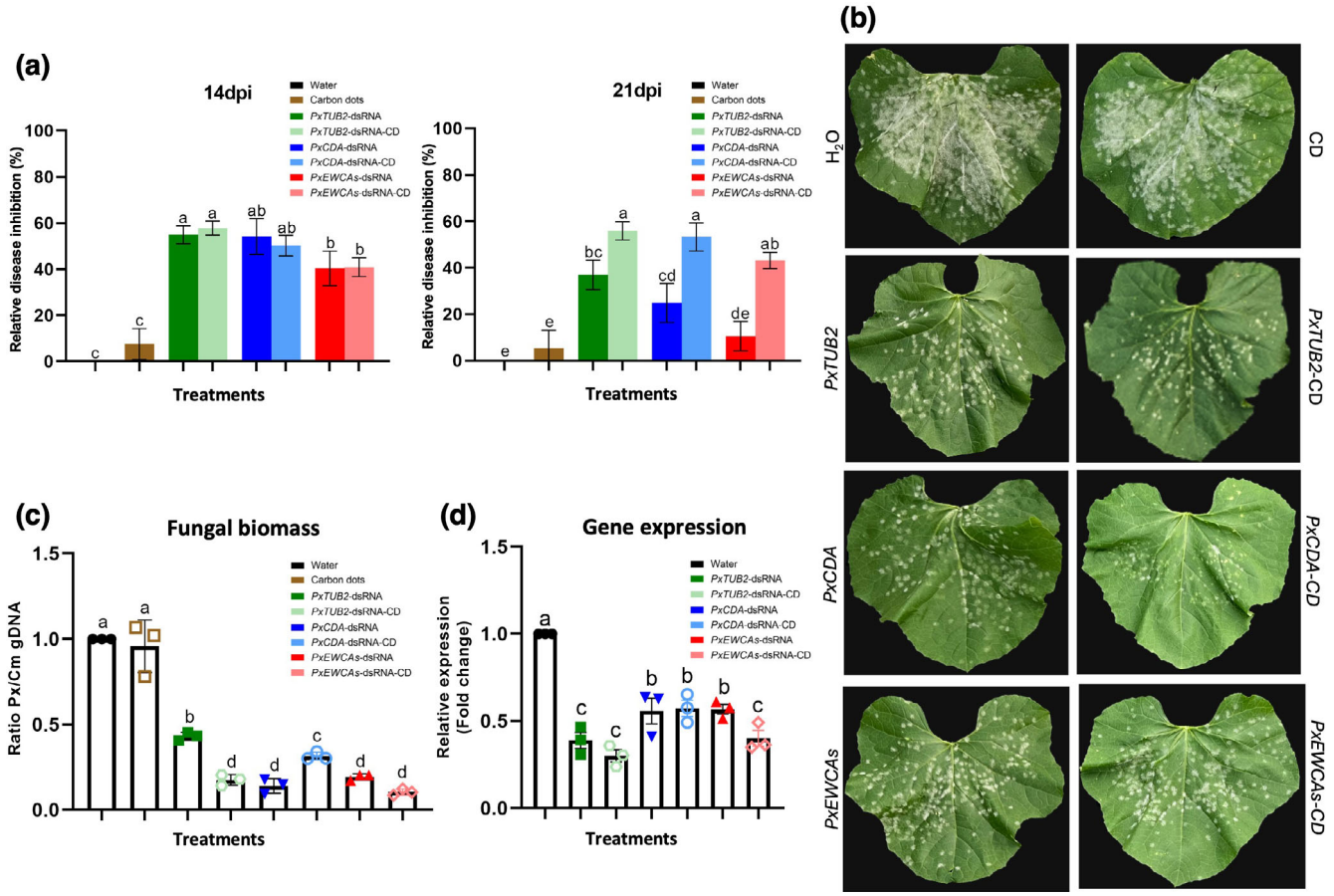

Figure 3. (a) Powdery mildew symptoms in melon plants following the application of naked and nanoencapsulated CD-dsRNAs. Photographs of the fungal pathogen were analyzed at 14 days postinoculation (dpi) and 21 dpi. (b) Representative images of the experiment. The images captured at 14 dpi included H_2O and a leaf from a plant sprayed with an empty CD nanocomposite as negative controls, a leaf sprayed with *PxTUB2*-dsRNA naked and nanoencapsulated as positive controls, and leaves from plants sprayed with naked or nanoencapsulated dsRNAs in CD. (c) Fungal biomass analysis in glasshouse assays at 168 h postinoculation (hpi). The bars represent the means ± SEs of three technical replicates from three different samples. Data points with the same letter are not significantly different at $P \leq 0.05$, as determined by Fisher's least significant difference (LSD) test. (d) Gene expression analysis in glasshouse assays at 144 hpi. The bars represent the means ± SEs of three technical replicates from three different samples. Data points with the same letter are not significantly different at $P \leq 0.05$, as determined by Fisher's LSD test.

growth and rapidly activated plant defense responses mediated by the cmCERK1 plant receptor. Moreover, the cosilencing of both genes along with the plant chitin receptor restored fungal growth, indicating that the observed suppression of fungal development upon gene silencing was caused by the activation of chitin-triggered immunity.[34] In accordance with these results, the foliar application of these dsRNAs on melon leaves led to a ≈50% disease reduction, which is in accordance with previous studies from other authors that aimed to control several phytopathogens.[12,54–57] However, the simultaneous silencing of both the *PxCDA* and *PxEWCAs* genes did not result in significantly greater efficiency than the application of each treatment separately. This may be a consequence of the saturation of the RNAi machinery, as described previously,[58] who reported no improvement when mixtures of two or three dsRNA constructs were sprayed on wheat leaves compared with the protection provided by each construct individually against *F. graminearum*.

Additionally, this silencing approach was tested in combination with nanotechnology under glasshouse conditions, and for this purpose, CD NPs were employed. This nanomaterial has been used in biomedicine for drug delivery and bioimaging.[59–61] Our results revealed ≈50% disease inhibition in both the dsRNA-nanoencapsulated and nonnanoencapsulated groups at 14 dpi, which was consistent with the results obtained from the growth chamber assay; however, after 21 dpi, this percentage remained stable only in the dsRNA-CD-nanoencapsulated groups. These results are in accordance with those of previous studies using inorganic nanocarriers. For example, Delgado-Martin and collaborators sprayed naked virus-derived dsRNA or coated cucumber plant leaves with CDs, and 50-fold more oligonucleotides protected with CDs than with uncoated dsRNA were detected in the leaves, with 13-fold more abundant siRNAs generated when the dsRNA was coated with CDs.[29] However, delivering the dsRNA-LDH complex (BioClay) spray instead of naked dsRNA extended the virus protection window from 57 days to ≥20 days,[23] provided Fusarium crown and root rot protection for ≥60 days,[26] and prolonged protection against *B. cinerea* on tomato leaves from 1 to 3 weeks, on tomato fruit from 5 to 10 days, and on mature chickpea plants up to 4 weeks, covering the critical period of podding, whereas naked dsRNA provided limited protection.[27] However, artificial vesicles also provide strong protection from nuclease degradation and removal by leaf washing, which leads to prolonged RNAi-mediated protection against *B. cinerea* on both pre- and postharvest plant material,

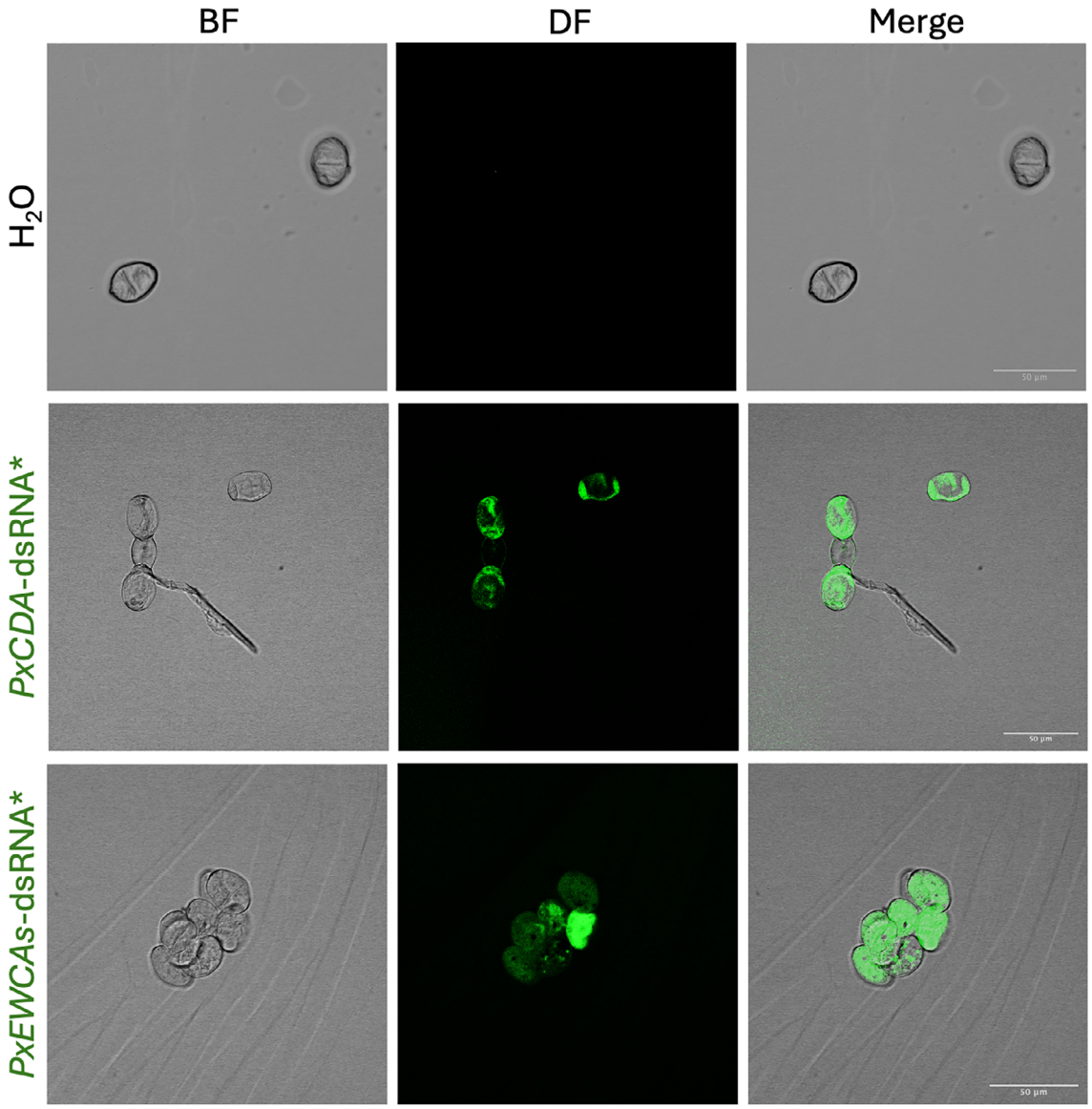

Figure 4. Representative images of *Podosphaera xanthii* spores taking up extracellular dsRNAs. BF, brightfield; DF, darkfield. Scale bar = 50 µm.

extending the protection period conferred by dsRNA to 10 days on tomato and grape leaves and 21 days on grape leaves.[22]

Our study also demonstrated that the fungal pathogen *P. xanthii* can absorb external dsRNAs 12 h postapplication. Likewise, fungal species from various classes of Ascomycota presented varying rates and efficiencies of dsRNA uptake. *In vitro* fluorescence signals were detected as early as 6 h after dsRNA application in *B. cinerea*, *S. sclerotiorum* and *Rhizoctonia solani*. In *Aspergillus niger*, a strong fluorescence signal was observed 10 h post-treatment (hpt), and in *Verticillium dahlia*, a strong fluorescence signal was observed 12 hpt.[62] Thus, the efficiency of dsRNA uptake clearly differs among different fungal species. However, further research is warranted to pinpoint the pathways responsible for dsRNA trafficking between pathogens and hosts, and to elucidate the mechanisms underlying RNA uptake in fungi.

Finally, the uptake and processing of dsRNA molecules targeting the *PxCDA* gene by the RNAi melon plant machinery were examined via sRNA-seq. The results revealed no large differences in the length of the whole population of siRNAs between local and distal leaves under either condition. Notably, a siRNA length of 22 nt was the most abundant profile in all the samples. This type of siRNA has been characterized as efficient in translational repression but less effective in target cleavage.[63] They are produced by the DICER-LIKE 2 (DCL2) protein in plants and may play an important role in adapting to environmental stresses.[63] The DCL4 protein is responsible for the biogenesis of 21-nt siRNAs, the second most abundant size in our datasets, which direct mRNA cleavage.[64] These enriched length profiles are similar to those from previous results in tomato plants treated with dsRNA against tomato mosaic virus.[65] Furthermore, our results proved that the uptake of the applied dsRNA was successful in local leaves at 24 hpt. The long-distance movement of *PxCDA*-dsRNA-derived siRNAs was not significant at 72 hpt, but the possibility that systemic translocation could still occur further in time cannot be ruled out. Nevertheless, dsRNA-derived siRNAs have been detected in distal leaves at 3 dpi[66] but also at 6 or even 21 dpi.[65,67] Therefore, it is plausible that their detection in distal leaves could increase the period of sampling after spraying. Additionally, sRNA-

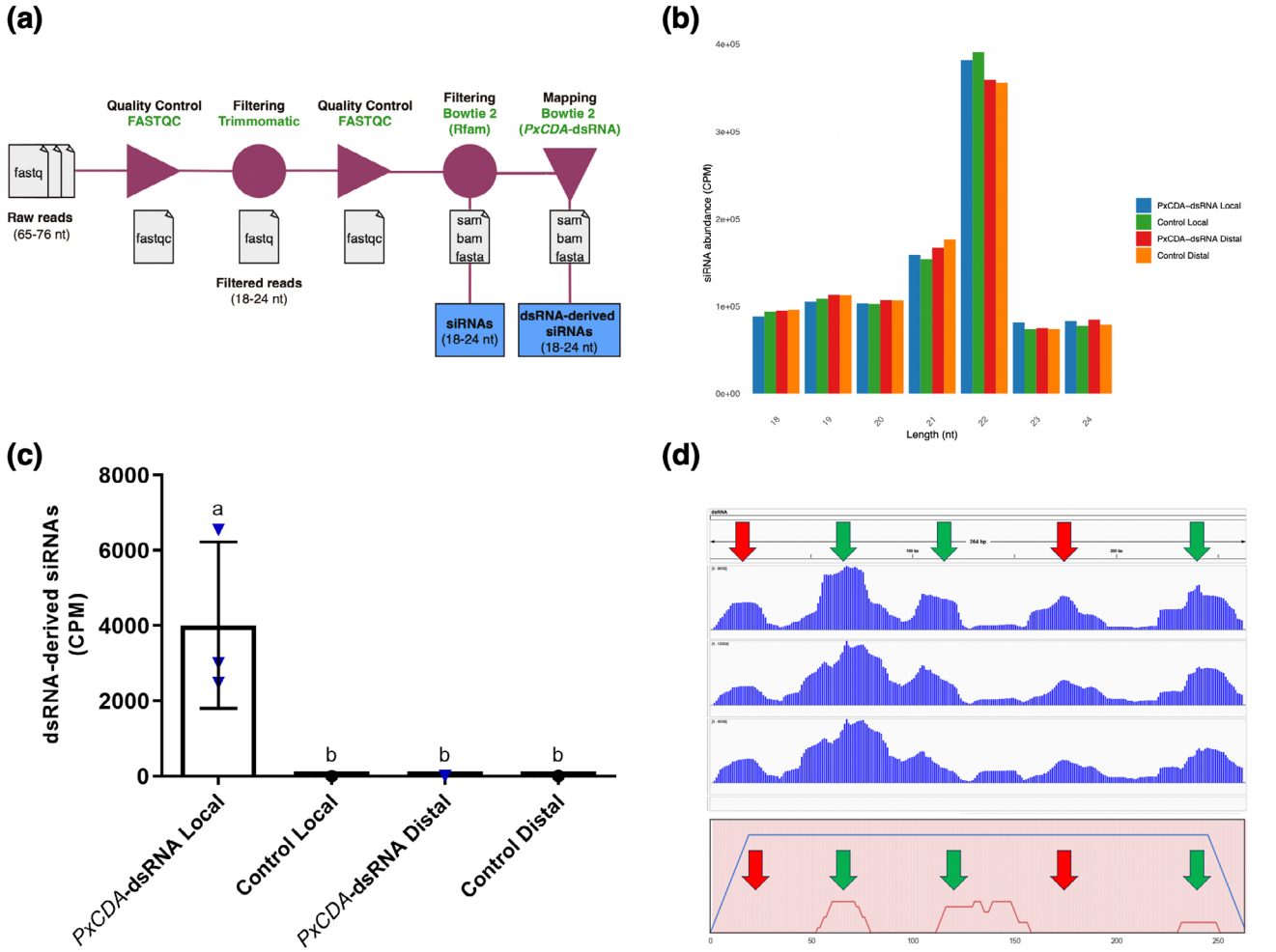

Figure 5. Summary of the sRNA-seq data. (a) Bioinformatics workflow for sRNA-seq data analysis. (b) Length distribution abundance (CPM, counts per million) of siRNA populations in melon leaves. The resulting clean reads were counted for each sRNA class according to their size. The average values from three replicates per condition are shown. (c) Abundance (CPM) of *PxCDA*-dsRNA-derived siRNAs. The siRNA populations were mapped to the *PxCDA*-dsRNA sequence. The bars represent the means ± SEs of three biological replicates. Data points with the same letter are not significantly different at $P \leq 0.05$, as determined by Fisher's least significant difference (LSD) test. (d) Experimental and predicted dsRNA-derived siRNAs. Three regions identified via *in silico* prediction matched the results of the sRNA-seq experiment. The blue tracks show the coverage of the mapping from the three replicates of *PxCDA*-dsRNA of local leaves against the dsRNA. The red track represents the RNAi design mode resulting from si-Fi21,[50] and the red line indicates the siRNAs that match the default criteria for efficiency along the dsRNA sequence.

Table 1. Top five over-represented *PxCDA*-dsRNA-derived siRNAs

			i-Score designer				
Sequence	Length (nt)	Abundance (CPM)	Sequence position	ΔG (kcal mol^{-1})	i-Score (score)	*s-Biopredsi* (score)	DSIR (score)
CAAGCGCUGGAAACUGUG	18	58 723.63202	41	−40*	44.9*	0.556*	67.8*
GAAAGCUUGGCUCUGUCCU	19	57 501.00296	167	−40.2	49.4	0.416	64.8
CUUCUGAUCAUUGCGCUGC	19	54 288.86246	65	−38.5	30.8	0.336	54.1
CAACUUCUGAUCAUUGCGC	19	51 750.64941	62	−36.1	35.7	0.445	61
AAAGCUUGGCUCUGUCCU	18	47 535.58748	168	−40.2*	41.2*	0.355*	56.5*

Note: Abundance (CPM) was calculated as the mean of three replicates of *PxCDA*-dsRNA-sprayed leaves at 24 h. The main metrics of the i-Score designer[51] are presented: the position in the sequence, the ΔG value throughout the siRNA stretch and the design scores based on different algorithms.
*Results were predicted for the corresponding sequence of 19 nt, as the software only generates 19-mer siRNA sequences.

seq validated the si-Fi21 prediction of three siRNA-rich regions of the *PxCDA*-dsRNA sequence and allowed us to identify two more regions. This forecast derives from the integrated analysis of siRNA sequences and their target sites, enabling the identification of siRNAs with potentially high efficacy. The tool's usage is recommended because of its reliability in selecting siRNAs with

significant silencing capability, utilizing both high-sensitivity (HS) and high-efficiency (HE) modes.[50] The most abundant *PxCDA*-dsRNA-derived siRNA sequences from our datasets were subsequently compared with those generated by i-Score Designer. It could be interesting to assess the efficiency of these plant-generated siRNAs in future SIGS assays for disease control, comparing them with the top bioinformatically predicted ones according to different design scores. A similar approach was tested to suppress *Hyaloperonospora arabidopsidis* infection in *Arabidopsis thaliana* by applying exogenous sRNA targeting the *CesA3* gene.[68] In this case, antisense and double-stranded sRNAs inhibited spore germination, with double-stranded sRNAs being more effective, showing potential for gene function analysis and disease control in plants.

In conclusion, SIGS represents a state-of-the-art method with distinct benefits against plant pathogens. A significant advantage is its straightforward spray application, facilitating the widespread and effective distribution of dsRNA molecules across extensive field areas. This scalable approach effectively combats a variety of pathogens, making it a promising solution for disease management as the production of dsRNA becomes increasingly cost-effective.[69] This technique's specificity is another key benefit, allowing for the selective silencing of pathogen genes without affecting nontarget organisms. This selectivity helps maintain ecological balance, unlike fungicides, which pose ecological risks, such as groundwater contamination and harm to a wide range of organisms. However, the use of SIGS for disease control faces challenges owing to the vast areas needing coverage, its instability and the significant quantities of dsRNA required for comprehensive protection. Future research should prioritize the identification of additional target genes for the use of dsRNAs or siRNAs for powdery mildew management and evaluate the commercial potential of SIGS, which combines different metabolic pathways, possibly in conjunction with lower doses of commercial fungicides.

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DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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