Small RNA inhibits infection by downy mildew pathogen 
_Hyaloperonospora arabisidopsis_

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SUMMARY

Gene silencing exists in eukaryotic organisms as a conserved regulation of the gene expression mechanism. In general, small RNAs (sRNAs) are produced within the eukaryotic cells and incorporated into an RNA-induced silencing complex (RISC) within cells. However, exogenous sRNAs, once delivered into cells, can also silence target genes via the same RISC. Here, we explored this concept by targeting the _Cellulose synthase A3_ (CesA3) gene of _Hyaloperonospora arabisidopsis_ (Hpa), the downy mildew pathogen of _Arabidopsis thaliana_. Hpa spore suspensions were mixed with sense or antisense sRNAs and inoculated onto susceptible _Arabidopsis_ seedlings. While sense sRNAs had no obvious effect on Hpa pathogenicity, antisense sRNAs inhibited spore germination and hence infection. Such inhibition of infection was not race-specific, but dependent on the length and capping of sRNAs. Inhibition of infection by double stranded sRNA was more efficient than that obtained with antisense sRNA. Thus, exogenous sRNA targeting conserved CesA3 could suppress Hpa infection in _Arabidopsis_, indicating the potential of this simple and efficient sRNA-based approach for deciphering gene functions in obligate biotrophic pathogens as well as for _R_-gene independent control of diseases in plants.

Keywords: _Arabidopsis_, downy mildew, oomycetes, spray-induced gene silencing (SIGS), sRNA.

INTRODUCTION

Noncoding 20–30 nucleotide (nt)-long small RNAs (sRNAs) have been known to be involved in the regulation of gene expression and defence in eukaryotes (Chen et al., 2018; Qin et al., 2017; Zhang et al., 2019). Different types of RNAs, such as double-stranded RNA (dsRNA) and small interfering RNA (siRNA), can trigger homologous RNA degradation or inhibit mRNA translation (Huang et al., 2016; Nejat and Mantri, 2018). This process is known as RNA silencing and plays a significant role in various biological processes, including innate immunity (Brant and Budak, 2018; Deng et al., 2018) and development (Li et al., 2017; Qin et al., 2017).

In plant–microbe interactions, plants and microbes can exchange RNA molecules, which then integrate into RNA silencing machinery in reciprocal recipient cells. Such cross-kingdom RNA transfer was first demonstrated between fungus and plants (Weiberg et al., 2013). _Botrytis cinerea_, an ascomycete fungus infecting more than 200 plant species, transports its sRNAs that silence both _Arabidopsis_ and tomato genes by hijacking plant cellular gene silencing machinery (Weiberg et al., 2013). On the other hand, a large number of cotton sRNAs were found in _Verticillium dahliae_ hyphae recovered from _V. dahliae_-infected plant tissues and some of the cotton-originated sRNAs can target essential fungal virulence genes (Zhang et al., 2016).

Movement of sRNAs from plant to pathogens has been explored using the host-induced gene silencing (HIGS) technique where the sRNAs are generally made by producing dsRNA in transgenic plants using _Agrobacterium_ or in viruses that replicate through dsRNA. HIGS has been successfully used to suppress essential pathogen genes in various plant–pathogen interaction systems including barley–_Fusarium_ (Koch et al., 2013), _Arabidopsis_– and tomato–_Verticillium_ (Song and Thomma, 2018), barley– and wheat–_Blumeria_ (Nowara et al., 2010) and lettuce–_Bremia_ (Govindarajulu et al., 2015). Similarly, HIGS has also been used against nematodes in _Arabidopsis_ (Huang et al., 2006).

Several recent studies have shown that exogenously applied sRNAs can be taken up by fungal or plant cells and trigger RNA silencing. The exogenous application of RNA by spraying it directly onto plants has been referred to as spray-induced gene
silencing (SIGS) (Koch et al., 2016). The induction of gene silencing by spraying, or otherwise applying RNA, avoids the need to develop transgenic plants (Wang and Jin, 2017). This method has been tested against fungal pathogens, including Fusarium graminearum (Koch et al., 2016) and Fusarium culmorum (Koch et al., 2018). Several different methods have been used to deliver siRNAs onto plants. For example, high-pressure spraying siRNAs was found to efficiently silence transgenic GFP gene expression in Nicotiana tabacum (Dalakouras et al., 2016). In a different approach, Mitter et al. (2017) used clay nanosheets to deliver dsRNA onto plants for silencing homologous viral RNA. For insect pests, an ingestion method seems to be another way to deliver RNAs. Insects were fed an artificial diet containing dsRNAs in order to induce RNA silencing. This strategy was successfully exploited to target against coleopteran species such as western corn rootworm Diabrotica virgifera virgifera (Baum et al., 2007).

The oomycetes include a unique group of biotrophic and hemibiotrophic plant pathogens and are distinct from fungi (Kamoun et al., 2014). The cell walls of oomycetes have been reported to be primarily β-1,3-glucans and cellulose with little or no chitin (Kamoun, 2003). Oomycete hyphae are coenocytic (multinucleate with no division by septa) and their vegetative nuclei are in a diploid state (Fugelstad, 2008; Coates and Beynon, 2010). Genetic manipulation has been developed for some oomycete pathogens. For example, stable transformations using protoplast uptake and regeneration have been reported for the culturable oomycetes including Phytophthora infestans, Phytophthora sojae and Phytophthora citricola (Kamoun, 2003; Mcleod et al., 2008). Using a DNA-directed RNAi system, efficient gene silencing has been achieved in P. infestans (Abrahamian et al., 2016). Saraiva et al. (2014) used uptake of dsRNA into protoplasts of Saprolegnia parasitica and reported the efficient silencing of the tyrosinase gene. However, routine genetic transformations or gene silencing studies in obligate oomycete species have been hampered by the lack of efficient reliable methods.

Hyaloperonospora arabidopsidis (Hpa) is an obligate biotrophic oomycete pathogen that causes downy mildew disease on Arabidopsis thaliana. The Hpa–Arabidopsis system has been tested against fungal pathogens, including Fusarium graminearum (Koch et al., 2016) and Fusarium culmorum (Koch et al., 2018). Several different methods have been used to deliver siRNAs onto plants. For example, high-pressure spraying siRNAs was found to efficiently silence transgenic GFP gene expression in Nicotiana tabacum (Dalakouras et al., 2016). In a different approach, Mitter et al. (2017) used clay nanosheets to deliver dsRNA onto plants for silencing homologous viral RNA. For insect pests, an ingestion method seems to be another way to deliver RNAs. Insects were fed an artificial diet containing dsRNAs in order to induce RNA silencing. This strategy was successfully exploited to target against coleopteran species such as western corn rootworm Diabrotica virgifera virgifera (Baum et al., 2007).

The main cell wall components of oomycetes are β-glucans and cellulose (Fugelstad, 2008; Raaymakers and Van den Ackerveken, 2016). We focused on a cellulose synthase gene as a target for sRNA-mediated silencing. Using Pfam (Punta et al., 2012), we identified M4BU64 of Hpa belonging to the cellulose synthase gene family. In silico analysis revealed that this gene corresponds to HpaG810051 in the Emoy2 genome and exists as a single copy gene. EnsemblProtists gene annotation revealed HpaG810051 does not have an intron and the open reading frame encodes a predicted protein of 1144 amino acids (molecular mass 127.028 kDa). A BLASTX search against the database revealed that HpaG810051 has a high similarity to CesA3 proteins from other oomycetes, thus we designated HpaG810051 as Hpa-CesA3. We then obtained the nucleotide and amino acid sequences of the CesA3 genes of Albugo candida, Albago laibachii, Bremia lactucae, Phytophthora capsici, P. infestans and Plasmopara viticola and aligned them with those of Hpa-CesA3. Alignment of amino acid sequences revealed a 93% identity of Hpa-CesA3 with P. capsici-CesA3, 92% with P. infestans-CesA3 and P. viticola-CesA3, 91% with B. lactucae-CesA3, 77% with A. candida-CesA3 and 76% with A. laibachii-CesA3 (Fig. S1). Domain and motif searches of the Hpa-CesA3 revealed a nucleotide-diphospho-sugar transferase domain (W545-E891) and a cellulose synthase domain (F814-Y1122), as well as 15 transmembrane domains (Fig. 1). Alignment of nucleotide sequences of these seven genes showed that Hpa-CesA3 had 65–84% identity to its orthologues (Fig. S2). Interestingly, nucleotide alignment showed no region with 100% identity that would allow designing of a common sRNA for CesA3 gene silencing across these oomycete species.

We then looked at the expression pattern of Hpa-CesA3 using the available published transcriptome data in Arabidopsis Col-0 inoculated with the avirulent or virulent Hpa isolates Emoy2 or Waco9, respectively (Asai et al., 2018). It is clear that Hpa-CesA3 is expressed highly in the spores and the level of expression drops significantly in the mycelia during development (Fig. S3).

**Hpa-CesA3 antisense sRNA inhibits Hpa sporulation**

Since Hpa is an obligate biotrophic pathogen and grows on Arabidopsis, we checked whether Hpa-CesA3 had any homology to Arabidopsis CesA genes (Burn et al., 2002). BLASTN searches
Figure 1: Domain structure of Hpa-CesA3. The mature protein has 15 transmembrane domains, a nucleotide-diphosphoglucone transferase domain (amino acid residues 545-891) and a cellulose synthase domain (amino acid residues 814-1122). sRNA is the region where sRNAs were designed.
against the *Arabidopsis* database revealed no significant similarity. Subsequently, we designed 25-nt sense and antisense RNA oligonucleotides from the 5′ region of the gene that does not have any homology in other genes in the *Hpa* genome. The sense or antisense sRNAs were mixed with *Hpa* spores at 5, 10 and 20 µM concentrations and 7-day-old *Arabidopsis* seedlings were drop inoculated. At 7 days post-inoculation (dpi), *Hpa* sporulation was checked and no visible difference in sporulation was observed between control plants (Fig. 2a) and those inoculated with spore suspensions containing 5, 10 or 20 µM sense sRNA. However, sporulation was visibly reduced on plants inoculated with spore suspensions containing 5 and 10 µM antisense RNA (Fig. 2b,c). Interestingly, there was no sporulation on plants inoculated with a spore suspension containing 20 µM antisense RNA (Fig. 2d). Quantitative data analysis further demonstrated the significant reduction or no sporulation in plants inoculated with *Hpa* spores mixed with antisense sRNAs (Fig. 3). The experiment was repeated at least five times, each time with a minimum of three replicates, and similar results were obtained. We also designed 25-nt sense and antisense DNA oligonucleotides from the same region of the gene and carried out similar inoculation experiments with 20 µM DNA oligonucleotides. There was no difference in the sporulation between control plants and those inoculated with sense or antisense DNA oligonucleotides (Fig. S4).

**Hpa-CesA3 antisense sRNA inhibits spore germination**

To investigate how *Hpa-CesA3* antisense sRNA inhibits sporulation, we inoculated 7-day-old *Arabidopsis* seedlings with a spore suspension containing 20 µM antisense sRNA. Trypan blue

![Fig. 2](application_of_antisense_srna_targeting_hpa_cesa3_inhibits_sporulation_.jpg)

**Fig. 2** Application of antisense sRNA targeting *Hpa-CesA3* inhibits sporulation. *Hpa-Emoy2* spores were mixed with antisense sRNA at different concentrations and 7-day-old *Arabidopsis* seedlings were drop inoculated. Seedlings were examined for sporulation at 7 days post-inoculation. (a) Control (no antisense sRNA), (b) seedlings inoculated with spores mixed with 5 µM antisense sRNA, (c) seedlings inoculated with spores mixed with 10 µM antisense sRNA and (d) seedlings inoculated with spores mixed with 20 µM antisense sRNA. The inoculation experiments were repeated five times and similar observations were made. There was no sporulation in seedlings inoculated with spores mixed with 20 µM antisense sRNA.

![Fig. 3](antisense_but_not_sense_srna_inhibits_sporulation_.jpg)

**Fig. 3** Antisense but not sense sRNA inhibits sporulation. *Arabidopsis* seedlings were drop inoculated with *Hpa-Emoy2* spores containing 0, 5, 10 and 20 µM sense or antisense sRNA. Ten inoculated seedlings from each sample were collected at 7 days post-inoculation and placed in 250 µL H2O. The number of spores was counted using a haemocytometer. The average and standard error of three replicates are shown. The experiment was repeated five times with similar results. Asterisks (*) indicate significant difference to control inoculation at the corresponding sRNA (*P* < 0.05, paired Student’s *t*-tests).
staining of inoculated leaves was carried out at 7 dpi to reveal the extent of Hpa development in the tissues. Although normal pathogen development was observed in the control leaf tissues (Fig. 4a), there were no hyphae in, or pathogen spores on, the cotyledons inoculated with the spore suspension containing 20 µM sRNA (Fig. 4b,c). This indicates that antisense sRNA may have inhibited spore germination, thus preventing infection. Non-germinating spores may have been washed away during trypan blue staining. To investigate this further, we set up germination assays using cellophane strips. After 48 h spores were examined for germination under a light microscope. Untreated, control spores were bright and produced germ tubes at various lengths within the 2 days (Fig. 5a,b). However, spores treated with antisense sRNA became dark brown and germination tubes were mainly absent or, in rare cases, were arrested (Fig. 5c,d). We repeated this assay five times and observed 100% inhibition of germination in all experiments (Table S1).

Hpa-CesA3 antisense sRNA-mediated suppression of Hpa infection is not race specific

Hpa-CesA3 antisense sRNA-mediated suppression of Hpa infection was carried out using the Hpa-Emoy2 isolate. To determine whether or not the suppression of infection we observed was isolate-specific, we carried out a similar study using Hpa-Cala2 isolate. Using BLASTN, we determined that the Emoy2 Hpa-CesA3 gene had 99.97% identity to that of Cala2 and 100% identity at the sRNA target region (Fig. S5). Subsequently, we inoculated 7-day-old Arabidopsis seedlings with Hpa-Cala2 spores mixed with and without 20 µM antisense sRNA. As expected, we observed normal sporulation in control seedlings while seedlings inoculated with spore suspension containing 20 µM antisense sRNA did not show any sporulation (data not shown), indicating Hpa-CesA3 antisense sRNA-mediated suppression of Hpa infection was not race-specific.

Capping antisense sRNA is essential for suppression of Hpa infection

The experiments described above were carried out with 25 nt capped antisense sRNAs. To determine whether capping influenced the silencing of Hpa-CesA3, we obtained an uncapped version of the same antisense sRNA and carried out similar inoculation studies. After 7 dpi, the control Arabidopsis seedlings and those seedlings inoculated with spores mixed with uncapped antisense 25 nt sRNA developed typical Hpa infection, resulting from normal sporulation and germination (Fig. 6a,b). In the same experiments, inoculations with spores mixed with capped antisense sRNA showed neither germination nor sporulation (Fig. 6c). These results reveal that sRNA capping is essential for sRNA biological activity in suppressing Hpa infection of plants.

sRNA length has an impact on Hpa-CesA3 antisense sRNA-mediated suppression of Hpa infection

The length of sRNA could influence the gene silencing (Vargason et al., 2003). In addition to the 25 nt sRNA, we tested the effect of 24 and 30 nt antisense Hpa-CesA3 sRNAs on Hpa infection. Using 24 nt sRNAs, normal sporulation was observed on the seedlings inoculated with 20 µM sRNAs and plants became
infected with *Hpa*. However, there was no sporulation on seedlings inoculated with spores containing 20 µM 30 nt antisense sRNAs and the inoculated *Arabidopsis* plants remained healthy (Fig. 7).

**Double-stranded sRNA is more effective than single-stranded sRNA in inhibiting infection**

Since application of 5 and 10 µM antisense sRNA allowed reduced sporulation in infected plants (Fig. 2), we investigated...
DISCUSSION

Using the Arabidopsis–Hyaloperonospora model system, we showed that targeting the Hpa-CesA3 gene in Emoy2 and Cala2 isolates by exogenously applying gene-specific sRNAs inhibits germination and hence infection of Arabidopsis.

We chose the Hpa-CesA3 gene because cellulose is an important structural component of the cell wall of oomycetes (Raaymakers and Van den Ackerveken, 2016). Blum et al. (2012) investigated CesA3 genes in a total of 25 different oomycete species originating from the six ‘crown’ oomycete orders (Albuginales, Leptomitales, Peronosporales, Pythiales, Rhipidiales and Saprolegniales) for their sensitivity to the fungicide mandipropamid (MPD). Interestingly, those authors reported that only species belonging to the order of Peronosporales, of which Hpa is a member, were inhibited by this fungicide. Furthermore, Grenville-Briggs et al. (2008) reported CesA3 to be the most strongly expressed gene during mycelial growth of Phytophthora, Saprolegnia and Pythium species. Working with P. infestans using a protoplast transformation strategy, Grenville-Briggs et al. (2008) used in vitro-generated long dsRNA to silence CesA genes. From their studies, they concluded that silencing these genes leads to disruption of cell walls surrounding appressoria and the inability to form functional appressoria. Interestingly, they reported that CesA3 is either less important or other CesA genes may be compensating for its loss of function (Grenville-Briggs et al., 2008).

Being an obligate plant pathogen, Hpa is not amenable to the genetic transformation and manipulation reported for P. infestans (Zheng et al., 2014). Previously, we have tested several different methods, including electroporation, to obtain stable transgenic isolates and study gene functions. Although we observed marker gene expression in a few individual spores, we were unable to generate transgenic isolates or obtain uniformly transformed spore lines (N. Holton and M. Tör, unpublished data). Here, we used synthesized 25 nt sRNAs to silence the CesA3 gene in Hpa.
by simply mixing the sRNAs with spores and inoculating cotyledons of seedlings or applying to cellophane strips. In inoculation experiments with antisense sRNAs, we showed a dose-dependent reduction in sporulation on the inoculated cotyledons. This clearly indicates that (a) exogenously applied single-stranded and ds sRNAs can be taken up by the spores without electroporation or any other transfection method, (b) the single-stranded sRNAs somehow bind to the native CesA3 RNA, forming a dsRNA and triggering the gene silencing machinery within the Hpa spores, (c) ds sRNA seems to be more effective in inhibiting infection than single-stranded sRNA, (d) the silencing seems to be very effective, as Hpa is diploid and multinucleate, and (e) the CesA3 gene is essential for pathogenicity of Hpa. Use of sense and antisense DNA oligonucleotides served as controls.

Trypan blue staining of seedlings inoculated with a sporule suspension containing 20 µM antisense sRNAs revealed a lack of infection, indicating the inhibition of pathogen development at the germination stage; this was confirmed by the germination assays, which showed 100% inhibition of germination. Our results are in contrast to the findings of Grenville-Briggs et al. (2008) where silencing CesA genes in P. infestans resulted in abnormal appressorium development rather than the inhibition of germination. This may well have been due to the redundancy factor as P. infestans has a larger genome than Hpa (Haas et al., 2009).

Using the Hpa-Cala2 genomic sequences (Woods-Tör et al., 2018), we found that the CesA3 gene of Emoy2 has 99.97% identity to CesA3 from Cala2 and the designed sRNA was gene-specific. In addition, the inhibition of sporulation of the Cala2 isolate by the sRNA confirmed that the method is effective and that this gene is necessary for infection.

Ideally, we would design an sRNA molecule that could inhibit infection by several oomycete species. However, gene silencing relies on a conserved nucleotide sequence and, although the domains of CesA3 are conserved at the amino acid level across some of the important oomycete species, this is not so at the nucleotide level; unfortunately, there is not a conserved region of the nucleotide sequence that is long enough to design a common sRNA. Nevertheless, the method we developed here can easily be adapted to other oomycete species using newly designed gene-specific sRNAs.

It is well known that all eukaryotic mRNA contains a cap structure, an N7-methylated guanosine linked to the first nucleotide of the RNA (Ramanathan et al., 2016). The cap has been reported to have several roles in cell viability, including promoting gene expression, mRNA stability and degradation, nuclear export of RNA and initiation of protein synthesis (Cowling, 2010). Recent studies also reported various cap structures in sRNAs in human cells (Abdelhamid et al., 2014), indicating that sRNAs can also go through a modification at their 5’ ends. Our inoculation studies clearly showed that the capping of sRNA was necessary for effective gene silencing. When Hpa spores are collected from infected seedlings, they are not derived from a sterile environment. Spore suspensions usually contain bacteria, tiny plant materials such as trichomes and other small substances. As the stability of exogenously applied sRNA in spore suspensions is very important, the cap structure of the sRNA may provide this required stability both outside and inside the spores.

In general, two classes of small non-coding RNAs exist in plant cells: miRNAs (encoded by the genome) and sRNAs (derived from dsRNA produced by multiple sources) (Khraiwesh et al., 2012). In addition, the size of sRNAs in organisms can be different (Derbyshire et al., 2018). Several studies revealed that, in plants and animals, each sRNA (acting as a guide) binds to an Argonaute family protein and a sequence-specific gene silencing ribonucleoprotein (RNP) is formed by base pairing between the sRNA and its target mRNA; this is known as the RNA-induced silencing complex (RISC) in miRNA and siRNA pathways (Budak and Akpinar, 2015; Vargason et al., 2003; Wilson and Doudna, 2013).

In addition to the presence of a cap at the 5’ end of the sRNA, our results also showed the importance of the length of the sRNAs. We observed that 24 nt antisense sRNA did not inhibit infection, whereas it was completely inhibited by 25 or 30 nt antisense sRNAs. In a recent study, Åsman et al. (2016) co-immunoprecipitated sRNAs with Argonaute proteins of the oomycete pathogen P. infestans and identified high enrichment of 24–26 nt sRNAs. In a similar study, Jia et al. (2017) sequenced sRNAs in another oomycete pathogen Phytophthora parasitica and reported that 25–26 nt sRNAs associate with efficient gene silencing in this pathogen. Although we do not know the exact reason of why 25 and 30 nt sRNAs silence Hpa-CesA3 but not 24 nt, it is tempting to speculate that the 24 nt sRNAs may not be binding to the Hpa-CesA3 transcript or may not be guiding the RISC complex to degrade CesA3 mRNA.

Application of sRNA is more advantageous than a transgenic approach. Using this system, it should be possible to study pathogen development and pathogenicity in obligate pathogens. Till now, effectors from obligate oomycetes such as Hpa have been studied either by bombardment or via a bacterial delivery system to plants (Bailey et al., 2011). An ideal method would be also to use reverse genetics to silence an effector gene within the pathogen and investigate whether this would alter pathogenicity. In addition, in some cases, effector genes in obligate pathogens are mapped to a locus where there are several genes within the interval. This system would allow rapid identification of the candidate gene. Using this simple method, we should now be able to study genes that are involved in pathogenicity and dissect different biological pathways of otherwise inaccessible obligate pathogens.

**EXPERIMENTAL PROCEDURES**

**Plant lines, pathogen isolates and propagation**

Hyaloperonospora arabidopsis isolates Emoy2 and Cala2 were maintained on A. thaliana Ws-eds1 (Parker et al., 1996).
Preparation of inoculum for experiments was performed as described previously (Tör et al., 2002). Sporulation was assessed 7 dpi, when the Hpa life cycle had been completed. To quantify sporulation, ten infected seedlings from each replicate were taken and placed into an Eppendorf tube containing 250 µl H₂O. Samples were vortexed and conidiospores were counted using a haemocytometer.

sRNA and DNA oligonucleotide synthesis

The Hpa-CesA3 gene (HpaG810051) was used as the target gene in this method. Sense and antisense sRNAs designed at various lengths were Hpa_CesA3_RNA_AS_24 5'-GGGCAUCGCA CGUACCUCAGUAC-3', Hpa_CesA3_RNA_AS_25 5'-GCGCAUC GCACGGACCACGUACAGC-3', Hpa_CesA3_RNA_S_25, 5'-CGUACUG AGGUACUGCGAUGCAGCACG-3' and Hpa_CesA3_RNA_S_30 5'-GUC GUACUGAGGUACGUGCGACGC-3'. Hpa_CesA3_RNA_AS_24 5'-AGUGCCCGCAUCGCAUGCAGUAC GAC-3' and Hpa_CesA3_RNA_AS_30 5'-AGGUACGUGCGAUGCAGCACGACU-3'. Hpa_CesA3_RNA_AS_24 5'-AGUGCCCGCAUCGCAUGCAGUAC GAC-3' and Hpa_CesA3_RNA_AS_30 5'-AGGUACGUGCGAUGCAGCACGACU-3'. Double-stranded sRNA were generated by mixing equal volume of Hpa_CesA3_RNA_S_30 and Hpa_CesA3_RNA_AS_30 oligos, heating at 95 °C for 5 min and allowing to anneal at room temperature for 20 min.

Similarly, sense and antisense DNA oligonucleotides were also designed to the same region. These were Hpa_CesA3_DNA_S_25 5'-GCCGCATCGCACGTACCTCAGTACG-3' and Hpa_CesA3_DNA_AS_25 5'-CGTACTGAGGTACGTGCGATGCGGC-3'. These were obtained as synthesized deoxyribonucleotides or ribonucleotides from Sigma (Gillingham, UK) or Eurofin (Ebersberg, Germany).

Application of sRNAs to pathogen spores and plant inoculations

Hpa spores were collected from infected A. thaliana Ws-eds1 seedlings, washed twice in sterile distilled water and the spore concentration was adjusted to 5 × 10⁴/mL using a haemocytometer. sRNAs were added to the spore suspension at a final concentration of 5, 10 and 20 µM and spores were subsequently drop inoculated onto 7-day-old seedlings. As a control, seedlings were also inoculated with spores in the same way with 20 µM antisense sRNA, without sRNAs or DNA oligonucleotides. Seedlings were inspected from 3 dpi for sporulation. Sporulation was quantified as described above.

Spore germination assays

MS medium (Murashige and Skoog, 1962) was prepared with 4.3 g/L MS basal salt mixture powder (Sigma, M5524), agar (1.5%) (Sigma, A1296), sucrose (10 g/L) (Sigma, 84100), and distilled water. MS powder was dissolved in sterile distilled H₂O, the pH was adjusted to 5.7 using 1 M NaOH/HCl, and agar and sugar were added. The medium was sterilized by autoclaving at 15 psi and 121 °C for 15 min. Approximately 20 µL of the medium was aliquoted into each sterile Petri dish in a laminar airflow unit.

Cellophane strips, 1.5 cm in length, were cut from plain transparent florists’ cellophane and autoclaved in distilled water. After autoclaving, cellophane strips were placed onto the MS medium in Petri dishes under a laminar airflow and dishes were kept in the fridge for long-term storage.

Hpa spores were collected, washed twice in sterile distilled water and the spore concentration was adjusted to 5 × 10⁴ spores/mL using a haemocytometer. Approximately 10 µL spore suspension, with 0 or 20 µM antisense sRNA, was dropped onto each piece of cellophane. Plates were incubated with a 12 h light/12 h dark regime at 16 °C. Spores were examined under a light microscope 48 h after incubation and germinated spores were counted.

Staining plant tissues

Seedlings of infected and non-inoculated controls were stained with a solution of phenol, lactic acid, glycerol and water (1:1:1:1) supplemented with 1 mg/mL trypan blue, decolorized in chloral hydrate and visualized under a compound microscope as described in Woods-Tör et al. (2018).

Statistical analysis

For statistical analysis, paired Student’s t-tests were performed on data obtained from plant infection assays.

Bioinformatics

IICB Genomics and Transcriptomics Resources (http://eumicrobedb.org) and the EnsemblProtist (http://prots.ensembl.org) database were used for information on Hpa. Web servers including InterPro (Quevillon et al., 2005) (http://www.ebi.ac.uk/inter pro/) and Pfam (Punta et al., 2012) ((http://pfam.wustl.edu/) were used for the analysis of Hpa-CesA3. BLAST (Altschul et al., 1997) was used to perform similarity-search of nucleotide and amino acid sequences of Hpa-CesA3 against oomycete and Arabidopsis sequences. Primer design was performed using Geneious v. 10.0 (Kearse et al., 2012).

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AUTHOR CONTRIBUTIONS
M.T. and Y.H. planned and designed the research. Ö.B., O.T., C.N. and M.T. conducted the laboratory work. M.T., Y.H. and H.B. analysed and interpreted the data and wrote the manuscript.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Comparison of CesA3 amino acid sequences from different oomycete pathogens. Amino acid sequences of CesA3 proteins from Hyaloperonospora arabidopsis (Hpa, M4B6U4), Albugo candida (AFB77612), Albugo laibachii (CCAA23182), Bremia lactucae (AFB20351), Phytophthora capsici (AFB20353), Phytophthora infestans (ABP9690), Plasmodora viticola (ADD84672) were aligned using Geneious v. 10. Black or dark grey boxes with white letters indicate identity or similarity to Hpa-CesA3, respectively.

Fig. S2 Comparison of CesA3 nucleotide sequences from different oomycete pathogens. Nucleotide sequences of CesA3 gene from Hyaloperonospora arabidopsis (Hpa), Albugo candida, Albugo laibachii, Bremia lactucae, Phytophthora capsici, Phytophthora infestans, Plasmodora viticola were aligned using Geneious v. 10. Black or dark grey boxes with white letters indicate identity or similarity to Hpa-CesA3, respectively.
Fig. S3 Expression pattern of Hpa-CesA3. Expression levels were represented as TPM (tags per million) of total reads mapped to *Hyaloperonospora arabidopsidis* genome. Data was acquired from Asai et al. (2018). Cs, conidiospore, dpi, days post-inoculation.

Fig. S4 Sense and antisense DNA oligonucleotides do not inhibit sporulation. *Arabidopsis* seedlings were drop inoculated with *Hpa-Emoy2* spores containing 20 µM sense or antisense DNA oligonucleotides. Inoculated 10 seedlings from each sample were collected 7 days post-inoculation and placed in 250 µL H$_2$O. The number of spores was counted using a hemocytometer. Averages and standard errors of three replicates are shown. Experiment was repeated three times with similar results.

Fig. S5 Nucleotide sequence alignment of Hpa-CesA3 from Emoy2 and Cala2 isolates. Sequences were aligned using Geneious v. 10. Black or dark grey boxes with white letters indicate identity or similarity to Hpa-CesA3 from Emoy2, respectively. sRNA indicates the sequences where sRNAs were designed from.

Table S1 Spore germination assay using antisense sRNA.

Table S2 Number of spores detected in seedlings inoculated with dsRNA.