Minicell-based fungal RNAi delivery for sustainable crop protection

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Summary

Spray-induced gene silencing (SIGS) using topical dsRNA applications has risen as a promising, target-specific, and environmentally friendly disease management strategy against phytopathogenic fungi. However, dsRNA stability, efficacy, and scalability are still the main constraints facing SIGS broader application. Here we show that Escherichia coli-derived anucleated minicells can be utilized as a cost-effective, scalable platform for dsRNA production and encapsulation. We demonstrated that minicell-encapsulated dsRNA (ME-dsRNA) was shielded from RNase degradation and stabilized on strawberry surfaces, allowing dsRNA persistence in field-like conditions. ME-dsRNAs targeting chitin synthase class III (Chs3a, Chs3b) and DICER-like proteins (DCL1 and DCL2) genes of Botryotinia fuckeliana selectively knocked-down the target genes and led to significant fungal growth inhibition in vitro. We also observed a compensatory relationship between DCL1 and DCL2 gene transcripts, where the silencing of one gene upregulated the expression of the other. Contrary to naked-dsRNAs, ME-dsRNAs halted disease progression in strawberries for 12 days under greenhouse conditions. These results elucidate the potential of ME-dsRNAs to enable the commercial application of RNAi-based, species-specific biocontrols comparable in efficacy to conventional synthetics. ME-dsRNAs offer a platform that can readily be translated to large-scale production and deployed in open-field applications to control grey mould in strawberries.

Introduction

Each year, grey mould disease caused by the fungal pathogen Botryotinia fuckeliana (anamorph: Botrytis cinerea) leads to $10 to $100 billion of global agricultural losses (Petrash et al., 2019). Predominant control strategies for grey mould disease heavily rely on chemical fungicidal products (Fernández-Ortuno et al., 2015; Rupp et al., 2017). The frequent and prophylactic use of these chemical fungicides has led to ecotoxicological harm, human health risk and resistance development in fungal pathogens (Weber, 2011; Amiri et al., 2013; Konstantinou et al., 2015; Panebianco et al., 2015). These repercussions evidence the urgent need for alternative biocontrols that are effective and environmentally sustainable.

RNAi is a post-transcription gene regulation mechanism that is present in all known eukaryotes. The cellular RNAi machinery is initiated by double-stranded RNAs (dsRNAs) that are initially processed into small interfering RNAs (siRNAs) by Dicer and Dicer-Like (DCL) proteins and eventually leads to the degradation of target mRNAs through the action of the RNA-induced silencing complex (RISC). RNAi-based genetic transformation technology has widely been utilized to control several insect pests, and diseases, in what is collectively coined as ‘host-induced gene silencing’ (HIGS) (Fire et al., 1998; Baulcombe, 2015; Wang et al., 2016). In HIGS, gene(s) essential for the development, survival, and virulence of the pathogen or the host plant’s susceptibility is being inactivated by gene silencing, leading to improved...
resistance against invading pathogens. For instance, the expression of dsRNAs targeting DCL1/2 or Target of the Rapamycin (TOR) (Xiong et al., 2019) genes of B. fuckeliana significantly suppressed grey mould disease progression in transgenic Arabidopsis, potato and tomato plants. However, technical limitations of generating stable transformed plants and the growing public concerns surrounding the cultivation of genetically modified (GM) crops restrict the broad application of HIGS technology to a majority of horticultural crops. Recently, spray-induced gene silencing (SIGS), which involves the exogenous application of dsRNAs and siRNAs has emerged as an appealing alternative to HIGS, as it does not incorporate foreign genes in the treated species and transgenerational inheritance of silencing constructs is unlikely (Wang et al., 2017; Mcloughlin et al., 2018a; Islam and Sherif, 2020). The exogenous application of dsRNA or siRNA to reduce B. fuckeliana infection has already been reported in many pathobiological systems (Wang et al., 2016; Mcloughlin et al., 2018b). However, due to limited field stability and high production cost, sprayable dsRNA biofungicides remain only conceptually conceivable, and not yet practically feasible at a large scale (Wang et al., 2016; Mitter et al., 2017). To address the applicability issues associated with sprayable dsRNAs, layered double hydroxides (LDH) based nanoparticle, termed “BioClay” has been suggested and utilized as a carrier for dsRNAs to improve their field stability and effectiveness against plant virus (Mitter et al., 2016). This unique feature has led to the development of minicells as human therapeutic delivery vesicles for drugs (MacDiarmid et al., 2007), vaccines (Carleton et al., 2013), and siRNAs (MacDiarmid et al., 2009). Deletions of the E. coli cell cycle-related genes minCDE produce a large number of intact and stable minicells (Hale et al., 1983; MacDiarmid et al., 2007). We generated an E. coli mutant (minCDE and mc) that produces a large number of minicells with compromised RNase-III activity (Fig. 1A), thus allowing the expression of dsRNAs (Takiff et al., 1989). We transformed this E. coli mutant with DNA constructs to express dsRNAs targeting B. fuckeliana genes that are essential for pathogenicity. The first group of genes we used are the B. fuckeliana RNAi- machinery related genes, DCL1 (ME-DCL1) and DCL2 (ME-DCL2). Recently it has been demonstrated that DCL1 and DCL2 are involved in the synthesis of fungal siRNA-effectors to suppress plant immunity and facilitate grey mould disease progression (Wang et al., 2016). The second group of genes we targeted are cell wall integrity-related genes, including two isoforms of chitin synthase class III; Chs3a (ME-CHS3a) and Chs3b (ME-CHS3b1 and ME-CHS3b2). Chitin is the rigid carbohydrate polymer that constitutes a main structural element of the cell wall of fungi. In B. fuckeliana, seven different classes of the chitin synthase genes have been identified, with duplications present in class III (Chs3a and Chs3b). Deletions of Chs3a significantly reduce the virulence and radial growth of B. fuckeliana (Soulie et al., 2006).

The transformed E. coli mutant was cultured in batch phase, induced for dsRNA expression and processed via differential centrifugation. Minicell budding from transformed bacteria are depicted in a SEM micrograph in Fig. 1A. The recovery of purified minicells by
centrifugation was verified with dynamic light scattering, where centrifugation yields a single peak of approximately 400nm, representing the minicells as illustrated in Fig. 1B and C. The dsRNA from the pure minicell solution was extracted and size-verified by gel electrophoresis (Fig. 1D). Using this platform, we were able to produce more than 100 mg/l minicell-encapsulated dsRNA in four different bioreactors (Table S1). The ability of minicells to protect dsRNAs from RNase A degradation was confirmed in vitro (Fig. 1E). Compared to naked-dsRNAs, minicells were resistant to RNase A treatment and yielded an intact dsRNA band on a native agarose gel, consistent with untreated (no RNase) ME-dsRNA. These results indicate that the RNase cannot access dsRNA that is minicell-encapsulated. The naked-dsRNA was degraded beyond the limit of detection given the same RNase A treatment, while the non-treated naked-dsRNA band was visualized using the same method indicating that the RNase A was responsible for the degradation. Of note, there was a slight and unexpected increase in the intensity of the dsRNA band with RNase treatment (Fig. 1E), possibly reflecting the non-linearity of the native agarose gel or could be a simple variation of RNA amounts during gel loading, or increased RNA recovery during extraction.

The potential of ME-dsRNAs to function as an effective biofungicide was examined in vitro by exposing actively growing fungal mycelia of B. fuckeliana to ME-dsRNAs. The treatment of ME-dsRNA individually targeting DCL1 (ME-DCL1) or DCL2 (ME-DCL2) did not lead to any significant mycelial growth inhibition (Fig. 2A). These results are in agreement with what has previously been reported by Wang et al., 2016, where mutations in DCL1 or DCL2 did not compromise the virulence or growth of B. fuckeliana, whereas the fungus double mutant dcl1 Δ dcl2 showed reduced virulence. In line with this finding, the combined application of ME-dsRNA targeting DCL1 and DCL2 at 1000 ng/ml inhibited fungal growth at 72 h post-treatment (hpt) (Fig. 2B, and Fig. S1). Our results further support their finding that silencing of both DCL1 and DCL2 is required to inhibit B. fuckeliana mycelial growth. In contrast, when naked-dsRNAs targeting both DCL1 and DCL2 were used, no significant mycelial growth inhibition was observed (Fig. S2). A previous research by Nerva et al. (2020) indicated that 500 ng naked-dsRNA should be applied every 12 h to the B. fuckeliana culture media to achieve mycelial growth inhibition, otherwise dsRNA degradation by the nuclease and/or the availability of the dsRNA for siRNA biogenesis could limit the long-lasting effects of naked-dsRNAs. In the present study, we showed that a
single ME-dsRNA application (1000 ng/ml) was significantly effective compared to empty minicells for as long as 72 hpt. This remarkable efficacy of ME-dsRNA could be explained, at least partially, by the ability of minicells to protect dsRNAs from enzymatic degradation (Mac-Diarmid et al., 2009) as demonstrated above (Fig. 1E).

The robust efficacy of ME-DCL1 and ME-DCL2 was also confirmed at the molecular level using qRT-PCR (Fig. 2C and D). Of note, our data not only showed a significant reduction in DCL1 and DCL2 transcript levels when the respective minicells were used, but we also observed a potential compensatory relationship between DCL1 and DCL2 expression. The downregulation of DCL1 transcripts, via ME-DCL1 treatment, was paralleled with an upregulation of DCL2 transcripts, and vice versa (Fig. 2C and D). Although this is the first study to report such expression patterns in the Botrytis species, the functional redundancy of DICERs and DCLs has previously been reported in Arabidopsis, Drosophila and Neurospora crassa (Catalanotto et al., 2004; Czech et al., 2008; Wang et al., 2015). The potential compensatory relationship between DCL1 and DCL2 genes and

![Image](image_url)

**Fig. 2.** Effects of the minicell-encapsulated dsRNAs (ME-dsRNAs) on mycelial growth and silencing of respective RNAi-machinery related-genes in *Botryotinia fuckeliana* at different time points. A. Inhibition of fungal growth in response to ME-dsRNAs targeting RNAi-machinery related-genes: dicer-like protein 1 (DCL1), and dicer-like protein 2 (DCL2) of *B. fuckeliana* at different concentrations. B. Mycelial growth inhibition rate in response to ME-DCL1 and ME-DCL2. C and D. Relative normalized expressions of the *B. fuckeliana* genes in response to ME-DCL1 and ME-DCL2 (E); DCL1, (F); DCL2. Data represents the mean ± SEM. Bars labelled with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test. Asterisks indicate significant differences between minicells and ME-dsRNA-treated sample at each time point according to t-test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. © 2020 The Authors. Microbial Biotechnology published by John Wiley & Sons Ltd and Society for Applied Microbiology, *Microbial Biotechnology*, 14, 1847–1856.
its implications in dsRNA-based management of *B. fuckeliana* will be the focus of our future studies. The combination of ME-DCL1 + 2 treatment, resulted in a spike in expression of both genes at the 24 hpt, followed by a gradual, yet significant, decline in transcript levels of both genes at 48 and 72 hpt (Fig. 2C and D). This finding is consistent with the results in Fig. 2A and B, where the combined, but not the single application, of ME-DCL1 and 2 led to a complete inhibition of the mycelial growth of *B. fuckeliana*.

As shown in Fig. 3A and B, ME-dsRNAs targeting the chitin synthase 3a (*Chs3a*) and 3b (*Chs3b*) genes showed significant antifungal activity, comparable to efficacy observed using DCL1/2 gene targets, as discussed...
above. Both ME-CHS3a and ME-CHS3b2, applied at 500 ng/ml, showed significant antifungal activity, and the combination of the two constructs targeting \( \text{Chs3b} \) (\( \text{CHS3b1} \times 2 \)) at 1000 ng/ml completely inhibited the mycelial growth (Fig. 2B, Fig. S3). To further validate these findings at the molecular level, the relative expression of target genes was evaluated by qRT-PCR, using \( \text{B. fuckeliana} \) \( -\)-actin and Tubulin genes as housekeeping genes and an empty minicell treatment as a negative control. Similar to \( \text{DCL1} & 2 \), the application of ME-CHS3a led to an initial induction of \( \text{Chs3a} \) transcription at 24 hpt, before being suppressed at later times (Fig. 3C), suggesting the existence of a feedback loop to confront potential exposures to dsRNA molecules such as those of mycoviruses; however, further studies are needed to support this conclusion. Treatments ME-CHS3b1 \( +\) 2 significantly reduced the relative transcript level of \( \text{Chs3a} \) and \( \text{Chs3b} \) in fungal mycelia, at 72 hpt. The reduction

![Fig. 4. The effectiveness of topically applied minicells-encapsulated dsRNAs (ME-dsRNAs) and naked-dsRNAs in inhibiting grey mould diseases under greenhouse conditions. A. Schematic representation of the time points of ME-dsRNAs and naked-dsRNA applications on strawberry fruit 1 h prior to inoculation, and B, 7 days prior to \( \text{B. fuckeliana} \) inoculation and the sampling time of the strawberry fruits from the strawberry plants for testing disease severity. C and E. Images showing ME-dsRNAs and naked-dsRNA sprayed onto fruits 1 h prior to \( \text{B. fuckeliana} \) inoculations reducing grey mould disease symptoms and lesion diameter when compared with the application of empty minicells. D and F. Images showing ME-dsRNA sustained protections against grey mould disease compared to naked-dsRNAs and the disease lesion diameter on treated fruits at 12 days post-treatment of ME-dsRNA. Data represent the mean ± SEM. Bars labelled with the different letters are significantly different at \( P < 0.05 \) according to Duncan’s multiple range test.](image-url)

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in Chs3a observed in response to ME-CHS3b2 and ME-CHS3b1 + 2 at 72 hpt (Fig. 3C and D), could be explained by the high degree of similarities (60.5%) between Chs3a and Chs3b gene homologs (Choquer et al., 2004). Interestingly, it has previously been reported that Chs3b transcripts were not detected in the mycelium of B. fuckeliana using the semi-quantitative RT-PCR approach (Choquer et al., 2003). Likewise, we observed significant reduction in Chs3b transcript levels in responses to ME-CHS3b1 + 2 treatment, however, the level of the Chs3b transcripts was generally low as indicated by the high Cq values (i.e. 35 out of 40 PCR cycles). These datasets together suggest that ME-dsRNA targeting the Chs3b gene of B. fuckeliana suppressed both Chs3a and Chs3b, and the simultaneous suppression of both genes of chitin synthase class III might be crucial for the complete inhibition of B. fuckeliana mycelial growth.

ME-dsRNAs, targeting different B. fuckeliana genes, were next tested for their efficacy as a topical spray application on fruit-bearing strawberries under greenhouse conditions. The initial in vivo experiment investigated the bio-fungicidal capacity of naked- or ME-dsRNA when applied 1h before B. fuckeliana inoculation (Fig. 4A, and Figs S4–S6). ME-CHS3b1 + 2, ME-DCL1 + 2 and naked-CHS3b1 + 2 completely inhibited disease progression, while ME-CHS3a and naked-DCL1 + 2 showed minimal fungal growth (Fig. 4C and E). The second experimental strategy investigated the short-term efficacy (3–7 days post-treatment) of exogenous dsRNAs (Fig. 4B), as field stability is reported as a major limitation for RNAi-based fungicides (Gan et al., 2010; Wang et al., 2016; Mitter et al., 2017). For this experiment, ME-, naked-dsRNAs or empty minicells were applied 7 days prior to inoculation of B. fuckeliana, and disease progression was analysed at 5 days post-inoculation (DPI) (Fig. 4B, and Figs S7 and S8). After the analysis of two separate experiments (Fig. 4B, D, E, and Figs S7 and S8), we can conclude that while naked-dsRNAs failed to provide disease protection, showing a similar level of disease progression as the negative control, ME-dsRNAs provided complete protection, with no visible signs of grey mould (Fig. 4D and F). These results demonstrate that ME-dsRNAs can extend the protection window against grey mould diseases by 5–12 days compared to naked-dsRNAs.

The target-specific inhibition by ME-dsRNAs was confirmed in vitro, by experimental results showing that ME-
dsRNAs for *B. fuckeliana* failed to inhibit the mycelial growth of two other fruit-rotting fungal pathogens, *Alternaria alternata* (Fig. 5A–D), and *Penicillium expansum* (Fig. 5E–H), which represents a major advantage of the ME-dsRNA as a promising new class of selective biofungicides. Interestingly, there is more than 77% similarity between the dsRNA sequence of *B. fuckeliana*’s chitin synthase 3a gene and its nearest homolog in *P. expansum* (XM_016744173.1), yet no mycelial growth inhibition was observed when *P. expansum* was treated with ME-CHS3a. Taken together with Figs 2 and 3, these results validate the efficacy of ME-dsRNAs for specific gene silencing of essential *B. fuckeliana* genes and in vitro fungal inhibition.

This study presents a robust, scalable platform for producing ME-dsRNAs using a prokaryotic expression system that sufficiently addresses the major shortcomings of exogenous dsRNA-based biofungicides; especially those related to stability, efficacy and scalability. This synthetic biology platform has the potential to be incorporated into commercial disease management programs against *B. fuckeliana* and other economically important phytopathogenic fungi. Investigating and understanding the mechanisms of ME-dsRNA interactions with pathogenic fungi is a core focus of our ongoing current and future research. We envision the use of minicell-based RNAi technology in integrated pest/disease management programs for controlling pests, viruses, and other fungal pathogens, in a sustainable way that addresses public concerns around GMOs and overuse of synthetic chemicals.

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Conflict of interest

The authors declare the following competing interests: AgroSpheres, INC., an early stage AgTech company that is active in developing commercial minicell-encapsulated products, contributed to sponsoring this research.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. RNA production data from four different bioreactors.

Table S2. Specific primers used for qRT-PCR analysis.

Fig. S1. The Botryotinia fuckeliana mycelial growth inhibitory activity of the minicells-encapsulated dsRNAs targeting DCL1 and DCL2 genes of B. fuckeliana at 72 hours after treatment. (a) ME-DCL1, (b) ME-DCL2, and (c) ME-DCL1+2: R: Biological replication; ME: empty-Minicells.

Fig. S2. The Botryotinia fuckeliana mycelial growth inhibitory activity of the minicells-encapsulated dsRNAs (ME-dsRNAs; ME-DCL1 and ME-DCL2) targeting both DCL1 and DCL2 genes of B. fuckeliana at 72 hours after treatment. R: Biological replication.

Fig. S3. The Botryotinia fuckeliana mycelial growth inhibitory activity of the minicells-encapsulated dsRNAs targeting Chs3a and Chs3b genes of B. fuckeliana at 72 hours after treatment. (a) ME-Chs3a, (b) ME-Chs3b1, (c) ME-Chs3b2, and (d) ME-Chs3b1+2: R: Biological replication; ME: empty-Minicells.

Fig. S4. Topically applied minicells-encapsulated dsRNAs (ME-dsRNAs; ME-Chs3a) targeting chitin synthase 3a (Chs3a) and combination of two ME-dsRNAs (ME-Chs3b1+ME-Chs3b2) targeting chitin synthase 3b (Chs3b) of Botryotinia fuckeliana on the strawberry fruits inhibited the gray mold disease progression. (a) Schematic representation of the ME-dsRNAs on strawberry fruit 1 hour prior to inoculation. The gray mold diseases symptom development at (b) 4 days post inoculation (dpi), (c) 5dpi, and (d) 7dpi. In order to determine the gray mold disease symptom development following ME-dsRNAs treatment. ME-Chs3a applied at dose of 500 ng/mL and the combination of ME-Chs3b1 and 2 used at 1000 ng/mL (500 µL/fruit).

Fig. S5. Gray mold diseases symptom development on minicells-encapsulated dsRNAs (ME-dsRNAs) or naked-dsRNA targeting Botryotinia fuckeliana genes sprayed fruits when challenged with the B. fuckeliana 1h post treatment (1st trial). In first trial we used the minicells, ME-Chs3a targeting Chs3a, ME-Chs3b1-2 targeting Chs3b, ME-DCL1+2 targeting DCL1 and DCL2, and naked-Chs3b1+2 (Chs3b).

Fig. S6. Gray mold diseases symptom development on minicells-encapsulated dsRNAs (ME-dsRNAs) or naked-dsRNA targeting Botryotinia fuckeliana genes sprayed fruits when challenged with the B. fuckeliana 1h post treatment (2nd trial). In 2nd trial we used the minicells, minicells-encapsulated dsRNAs (ME-DCL1+2) and naked-DCL1+2 (targeting DCL1 and DCL2) treatments.
Fig. S7. Minicells-encapsulated dsRNAs (ME-dsRNAs) for sustained protection against *Botryotinia fuckeliana* (1st trial). Plants were sprayed on day 0 with minicells, ME-CHS3b1+2 or naked-CHS3b1+2 (targeting *B. cinaeral* Chs3b gene) and inoculated with *B. fuckeliana* on sprayed fruits at 7 days post treatment. Gray mold disease severity was observed at 5 days post inoculation (dpi).

Fig. S8. Minicells-encapsulated dsRNAs (ME-dsRNAs) for sustained protection against *B. cinerea* (2nd trial). Plants were sprayed on day 0 with minicells, ME-DCL1+2 or naked-DCL1+2 (targeting *B. fuckeliana* DCL1 and DCL2 genes) and inoculated with *B. fuckeliana* on sprayed fruits at 7 days post treatment. Gray mold disease severity was observed at 5 days post inoculation (dpi).

Data S1. Experimental procedures.