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ORIGINAL ARTICLE

Exogenous double-stranded RNA inhibits the infection physiology of rust fungi to reduce symptoms in planta

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Abstract

Rust fungi (Pucciniales) are a diverse group of plant pathogens in natural and agricultural systems. They pose ongoing threats to the diversity of native flora and cause annual crop yield losses. Agricultural rusts are predominantly managed with fungicides and breeding for resistance, but new control strategies are needed on non-agricultural plants and in fragile ecosystems. RNA interference (RNAi) induced by exogenous double-stranded RNA (dsRNA) has promise as a sustainable approach for managing plant-pathogenic fungi, including rust fungi. We investigated the mechanisms and impact of exogenous dsRNA on rust fungi through in vitro and whole-plant assays using two species as models, *Austropuccinia psidii* (the cause of myrtle rust) and *Coleosporium plumeriae* (the cause of frangipani rust). In vitro, dsRNA either associates externally or is internalized by urediniospores during the early stages of germination. The impact of dsRNA on rust infection architecture was examined on artificial leaf surfaces. dsRNA targeting predicted essential genes significantly reduced germination and inhibited development of infection structures, namely appressoria and penetration pegs. Exogenous dsRNA sprayed onto 1-year-old trees significantly reduced myrtle rust symptoms. Furthermore, we used comparative genomics to assess the wide-scale amenability of dsRNA to control rust fungi. We sequenced genomes of six species of rust fungi, including three new families (Araucariomyceaceae, Phragmidiaceae, and Skierkaceae) and identified key genes of the RNAi pathway across 15 species in eight families of Pucciniales. Together, these findings

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indicate that dsRNA targeting essential genes has potential for broad-use management of rust fungi across natural and agricultural systems.

KEYWORDS
dsRNA, fungal genomics, in vitro, myrtle rust, RNAi, rust fungi, spore germination

1 | INTRODUCTION

Rust fungi (Pucciniales) are the largest group of plant pathogens, impacting both agricultural and natural ecosystems (Aime & McTaggart, 2021). Agricultural rusts cause large-scale crop yield losses, while environmentally significant rust diseases, such as myrtle rust (caused by *Austropuccinia psidii*) and white pine blister rust (caused by *Cronartium ribicola*), have caused population decline and localized extinction of endemic flora (Fensham & Radford-Smith, 2021; Pegg et al., 2017; Schoettle & Sniezko, 2007). Common controls of rusts include synthetic fungicides (Miles et al., 2007), deployment of resistant genotypes (Babu et al., 2020; Glen et al., 2007; Periyannan, 2018), and breeding for resistance (Ellis et al., 2014; Varshney et al., 2007). However, these short-term solutions are limited by evolving pathogens (Drenth et al., 2019; McDonald & Linde, 2002), financial and environmental costs associated with agrichemicals (Lykogianni et al., 2021; Makinson, 2018; Makinson & Conn, 2014; Pegg et al., 2014), and the time required to breed improved crop varieties (Almeida et al., 2021). These traditional control strategies are also not viable in the conservation of non-crop species.

RNA interference (RNAi) is a conserved eukaryotic mechanism that regulates posttranscription expression of endogenous genes and defends cells against viruses and transposons (Baulcombe, 2004; Holoch & Moazed, 2015; Nakayashiki & Nguyen, 2008). The RNAi pathway is initiated when Dicer-like (DCL) enzymes recognize and cleave double-stranded RNA (dsRNA) into 20–24 nucleotide (nt) small interfering RNAs (siRNAs) or microRNAs (miRNAs). These small RNAs (sRNAs) associate with Argonaute (AGO) proteins to form the RNA-induced silencing complex (RISC). The RISC recognizes and degrades complementary RNA, repressing transcription or translation of homologous DNA/RNA (Bologna & Voinnet, 2014; Torres-Martinez & Ruiz-Vázquez, 2017). In some cases, the process is amplified by RNA-dependent RNA polymerases (RdRPs) that promote continuous silencing of targeted genes (Dalmay et al., 2000; Sijen et al., 2001). In the context of rust fungi, genes encoding the RNAi pathway, including *AGO*, *DCL*s, and *RdRP*s, have been studied primarily in wheat rusts, where they play roles in fungal growth, development, and pathogenicity (Dubey et al., 2020; Dutta et al., 2019; Kiran et al., 2016; Sperschneider et al., 2021). However, it is unknown whether genes encoding a functional RNAi pathway are present in all species of the Pucciniales.

RNAi has been adapted as a pathogen-specific, non-transgenic, pest and disease control with demonstrated effectiveness against viruses, insects, and fungal pathogens (Fletcher et al., 2020; Taning et al., 2020). RNAi could be used for crop protection either through a transgenic approach, known as host-induced gene silencing (HIGS), or a nontransgenic approach, known as spray-induced gene silencing (SIGS). In HIGS, host plants are genetically engineered to produce pest- or pathogen-specific dsRNA, whereas in SIGS, dsRNA or siRNA are applied exogenously (Fletcher et al., 2020, Taning et al., 2020).

HIGS and SIGS have been used successfully against species in two families of rust fungi, Pucciniaceae and Phakopsoraceae, in both whole-plant and detached leaf assays (Gill et al., 2018; Hu et al., 2020; Panwar et al., 2018; Qi et al., 2018; Saito et al., 2022; Yin et al., 2015; Zhu et al., 2017). Target genes have included those that encode haustorial proteins (Yin et al., 2015), kinases (Panwar et al., 2018; Qi et al., 2018; Zhu et al., 2017), acetyl-transferases (Gill et al., 2018; Hu et al., 2020), and chitin synthases (Saito et al., 2022). Despite this success, the precise molecular mechanisms of HIGS and SIGS are not fully understood. It is hypothesized that dsRNA expressed in transgenic plants, that is, HIGS, is cleaved into siRNAs by plant DCLs and transferred from host to pathogen via extracellular vesicles, a mechanism known as cross-kingdom RNAi (Cai et al., 2018; Rutter & Innes, 2018). In contrast, dsRNA molecules applied in SIGS are likely to be taken up directly by fungi on leaf surfaces (Šečić & Kogel, 2021). This is supported by studies in vitro (Qiao et al., 2021; Wytinck et al., 2020) and one study on soybean leaves by Saito et al. (2022), who found that dsRNA was more effective in reducing lesion formation on soybean leaves when it was co-applied with *Phakospora pachyrhizi* urediniospores than when it was infiltrated into leaves prior to inoculation.

Spores of fungal foliar pathogens anchor themselves, germinate, and concentrate their cytoplasm in appressoria to build force and penetrate leaf surfaces through stomata (Wynn, 1975). The interface between leaves and fungal spores in plant–pathogen interactions has been examined on artificial leaf-like surfaces made from polystyrene (Bernstein & Jones, 1969; Collins & Read, 1997; Dickinson, 1949; Wynn, 1975), silicone (Lee & Dean, 1994), and cellulose nanofibre (Saito et al., 2021). Artificial leaf-like surfaces were used to assess the effect of environmental factors on pathogen infection (Doan & Leveau, 2015) and to study how dsRNA-induced RNAi impacts the infection process of rust fungi (Saito et al., 2022).

We investigated the mechanisms, conservation, and functionality of RNAi in rust fungi to assess its utility in the management of plant disease. We aimed to determine (i) whether genes encoding the RNAi pathway are conserved across the Pucciniales, (ii) which life cycle stages of rust fungi could take up exogenous dsRNA, (iii) whether dsRNA treatment affects the physiology of spore germination and infection by rust fungi, and (iv) whether essential genes

are suitable as universal targets for exogenous dsRNA-mediated management of rust fungi. We provide experimental evidence of the internalization/external association of dsRNA with infective spore stages, evidence for a functional RNAi pathway in two rust species, and genomic analyses to support a hypothesis that the RNAi pathway is conserved across rust fungi. The outcomes of this research will inform the design and implementation of next-generation management strategies to control plant diseases in agricultural systems as well as natural environments.

2 | RESULTS

2.1 | dsRNA either associates externally or is internalized by urediniospores

To determine whether rust spores can take up dsRNA from the environment, urediniospores and teliospores of *A. psidii*, and urediniospores of *Coleosporium plumeriae* were incubated with in vitro-synthesized Cy3-labelled *green fluorescent protein* (*GFP*) dsRNA on water agar or glass slides, and then viewed under a confocal fluorescence microscope. Micrococcal nuclease was used to remove excess external Cy3-dsRNA, as in Qiao et al. (2021). Germinating urediniospores of *A. psidii* and *C. plumeriae* displayed Cy3 fluorescence 4–8 h after dsRNA application, indicating that dsRNA is either internalized or associates with the surface of the spore, which may protect it from micrococcal nuclease activity (Figures 1a,b,d–f and S1). Cy3 fluorescence was seen across multiple *Z*-stacks, showing that the Cy3-dsRNA was present at different focal planes within the spores, which supports that dsRNA is internalized rather than external (Figure S2). Germinating urediniospores of *A. psidii* only displayed Cy3 fluorescence in their germ tubes (Figure 1a,b), whereas Cy3 fluoresced in the urediniospores and germ tubes of *C. plumeriae* (Figure 1d–f). Cy3-CTP-treated *C. plumeriae* urediniospores, which acted as a negative control, only displayed a background fluorescence in their germ tubes that was differentiated from Cy3-dsRNA fluorescence with fluorescence lifetime imaging microscopy (FLIM; Datta et al., 2020; Figure S1). Fluorescence was seen in urediniospores at very early stages of germination, indicating that dsRNA associates with urediniospores as soon as they germinate (Figure 1e,f). Teliospores were fluorescent before micrococcal nuclease treatment (Figure 1a) but not after (Figure 1c), indicating that dsRNA was not internalized or externally associated.

2.2 | *A. psidii*-specific dsRNA decreases infection on detached leaves

We used detached leaves of *Syzygium jambos* to examine the impact of dsRNAs that targeted 10 different *A. psidii* genes: *β-tubulin* (*β-TUB*), *translation elongation factor 1a* (*EF1-a*), *acetyl-CoA transferase* (*ATC*), *cytochrome P450* (*CYP450*), *mitogen activated protein kinase* (*MAPK*), *glycine cleavage system H* (*GCS-H*), *28S* ribosomal RNA, and three haustorial targets (*HAUS01136*, *HAUS01215*, and *HAUS12890*) (Figure 2a,b). These genes were chosen based on their broad spectrum of roles as predicted essential genes, genes used successfully in past studies of RNAi, or targets expressed by haustoria (Geiser et al., 2004; Kurtzman & Robnett, 1998; Seifert et al., 2007; Yin et al., 2015). *GFP* dsRNA was included as a nonspecific control. The number of pustules per leaf decreased significantly in eight of our treatments: *EF1-a*, *28S rRNA*-1, *β-TUB*, *MAPK*, *ATC*, *GCS-H*, *CYP450*, and *H01215* (Figure 2a,b,d). There was no difference in pustules per leaf between the −dsRNA control and the nonspecific control (Figure 2c).

Predicted essential genes were more effective targets than genes up-regulated in the haustoria (*H01215*, *H01136*, *H12890*). Two separate dsRNAs were designed to target the *28S rRNA* ribosomal large subunit region. The overlapping *28S rRNA*-1 and *28S rRNA*-2 dsRNA were 340 bp and 685 bp in length, respectively, and we observed a significant inhibition of *A. psidii* germination in *28S rRNA*-1 treatments compared to *28S rRNA*-2 treatments (Figure 2). This observation was in line with Höfle et al. (2020), who found that efficiency of inducing RNAi decreased with increasing dsRNA length.

2.3 | dsRNA inhibits urediniospore germination and formation of appressoria in vitro

We used polyvinylpyrrolidone (PVP)-treated polydimethylsiloxane (PDMS) discs as artificial leaf-like surfaces and the three most effective targets from the detached leaf assay, *β-TUB*, *EF1-a*, and *28S rRNA*-1, to determine the effect of dsRNA treatment on urediniospores of *A. psidii* and *C. plumeriae* (Figure 3). The germination rate of *A. psidii* urediniospores on PDMS decreased significantly when treated with dsRNA that targeted *β-TUB* (43% germination), *EF1-a* (23% germination), and *28S rRNA*-1 (9% germination) compared to the nonspecific dsRNA (*GFP*) and -dsRNA controls (75% and 78% germination, respectively) (Figure 3a). Germ tubes formed appressoria and infection pegs, with visible cytoplasmic streaming in the *GFP* and dsRNA controls. Germ tubes of *β-TUB* or *EF1-A* dsRNA-treated urediniospores were withered, lacked cytoplasmic streaming, and did not form appressoria and infection pegs. The germination rate was significantly reduced in urediniospores treated with *28S rRNA*-1 dsRNA, and germ tubes were short, straight, and did not produce appressoria (Figure 3c–f).

Urediniospores of *C. plumeriae* treated with *β-TUB*, *EF1-a*, and *28S rRNA*-1 dsRNA also had decreased germination rates (Figure 3b) and inhibited development of germ tubes and appressoria (Figure 3g–j). The germination rate of *C. plumeriae* urediniospores was significantly reduced when treated with dsRNA targeting *β-TUB* (8.3% germination), *EF1-a* (5% germination), or *28S rRNA*-1 (5% germination) compared to the *GFP* and −dsRNA controls (75% and 73.3% germination, respectively). For all three targets, germ tubes of urediniospores that germinated when treated with dsRNA lacked cytoplasmic streaming and did not form

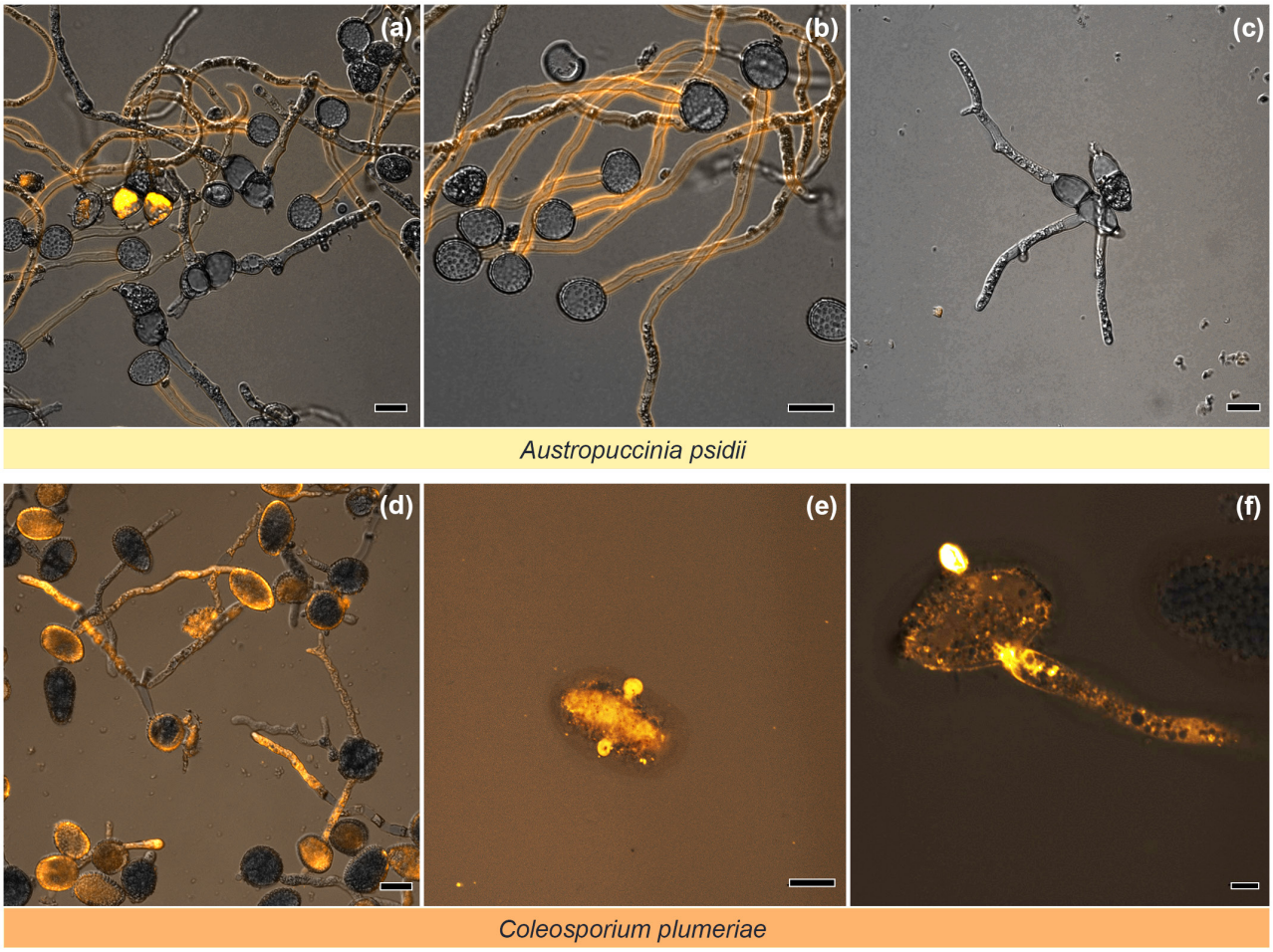
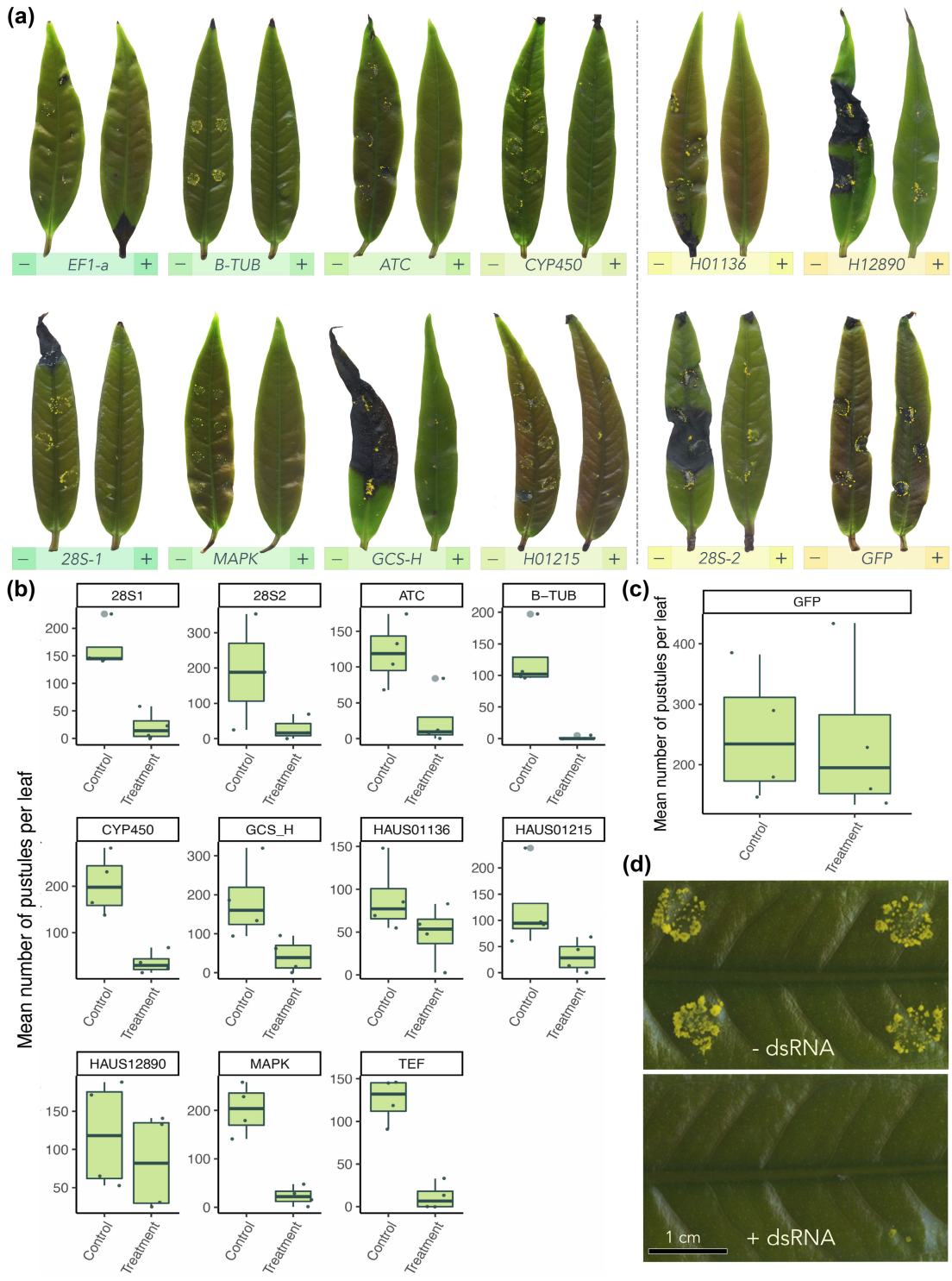


FIGURE 1 Double-stranded RNA (dsRNA) either associates externally or is taken up by *Austropuccinia psidii* and *Coleosporium plumeriae* urediniospores but not teliospores. Spores were incubated with 1 μg of Cy3-labelled *green fluorescent protein* dsRNA on 1% water agar for 6 h (d) or 8 h (a–c,e,f) and were then treated with 75 U of micrococcal nuclease and Cy3 fluorescence observed using a Zeiss LSM700 confocal microscope. *A. psidii* urediniospores and teliospores are shown before (a) and after (b, c) micrococcal nuclease treatment. (a) A mixture of *A. psidii* urediniospores and teliospores, (b) *A. psidii* urediniospores and (c) *A. psidii* teliospores. *C. plumeriae* urediniospores at different stages of germination are shown after micrococcal nuclease treatment (d–f). Scale bars represent 20 μm (a–d), 5 μm (e), and 20 μm (f), respectively.

FIGURE 2 Exogenous application of *Austropuccinia psidii*-specific double-stranded RNA (dsRNA) decreases the incidence of myrtle rust (MR) on *Syzygium jambos* in detached-leaf assays. *S. jambos* leaves ($n = 4$) infected with *A. psidii* and treated with 11 *A. psidii*-specific dsRNAs were assessed 2 weeks postinoculation. Treatments consisted of 10^6 *A. psidii* spores/ml in sterile distilled water containing 0.05% Tween 20 mixed with 100 ng/μl dsRNA. −dsRNA (control) was *A. psidii* spores in sterile distilled water and 0.05% Tween 20. *Green fluorescent protein* (*GFP*) dsRNA was included as a nonspecific control. (a) Boxplot with superimposed scatter representing the mean number of *A. psidii* pustules per *S. jambos* leaf ($n = 3$) treated with 11 *A. psidii*-specific dsRNAs. Significance as compared to the control (−dsRNA) is represented by grey asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student's *t* test). This figure was made in R v. 4.0.3 (R Core Team, 2020). (b) Photo comparisons of all dsRNA treatments and controls on *S. jambos* leaves. For each gene target a control (−) and treatment (+) leaf is shown. Each treatment leaf had a respective control leaf from the sample plant and of the same age, size, and growth stage to ensure accurate comparison. Colours from green to orange represent most to least significant reduction in spore number from control to treatment, respectively. All treatments to the left of the dotted line (ribosomal RNA *28S*-1 (*28S*-1), *β-tubulin* (*β-TUB*), *translation elongation factor 1-α* (*EF1-a*), *mitogen activated protein kinase* (*MAPK*), *acetyl CoA-transferase* (*ATC*), *glycine cleavage system-H* (*GCS-H*), *cytochrome P450* (*CYP450*) and haustorial target (*H01215*)) resulted in a significant reduction in pustule number compared to control leaves. All treatments to the right of the dotted line (haustorial target *H01136*, ribosomal RNA *28S*-2 (*28S*-2) and haustorial target *H12890*) did not result in a significant reduction in pustule number from control to treatment leaves. (c) Boxplot with superimposed scatter representing *GFP* (nonspecific control) mean number of *A. psidii* pustules per *S. jambos* leaf ($n = 3$) treated with 11 *A. psidii*-specific dsRNAs. Significance as compared to the control (−dsRNA) is represented by grey asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student's *t* test). This figure was made in R v. 4.0.3 (R Core Team, 2020). (d) Close-up photo comparison of control (*A. psidii* only) and treatment (*A. psidii* + *β-TUB* dsRNA) on *S. jambos* leaves, taken 2 weeks postinoculation.



appressoria and infection pegs (Figure 3g–j). Interestingly, Saito et al. (2022) saw a significant reduction in appressoria formation, but not in urediniospore germination, when targeting chitin synthases in *P. pachyrhizi*.

We attempted to quantify relative gene expression in several different experiments but had inconsistent results, including for controls. Ultimately these experiments will need to use a dsRNA target that does not inhibit spore germination or virulence, and is expressed in germinating rust spores.

2.4 | Impact of exogenous dsRNA is concentration-dependent

To determine whether the effect of dsRNA was proportional to the amount applied, we tested three concentrations (1 ng/μl, 10 ng/μl, and 100 ng/μl) of *GFP*, *β-TUB*, *EF1-a*, and *28S rRNA*-1 dsRNA on the germination rate of *A. psidii* and *C. plumeriae* urediniospores (Figure 4). Higher concentrations of pathogen-specific dsRNAs caused significantly lower rates of urediniospore germination in

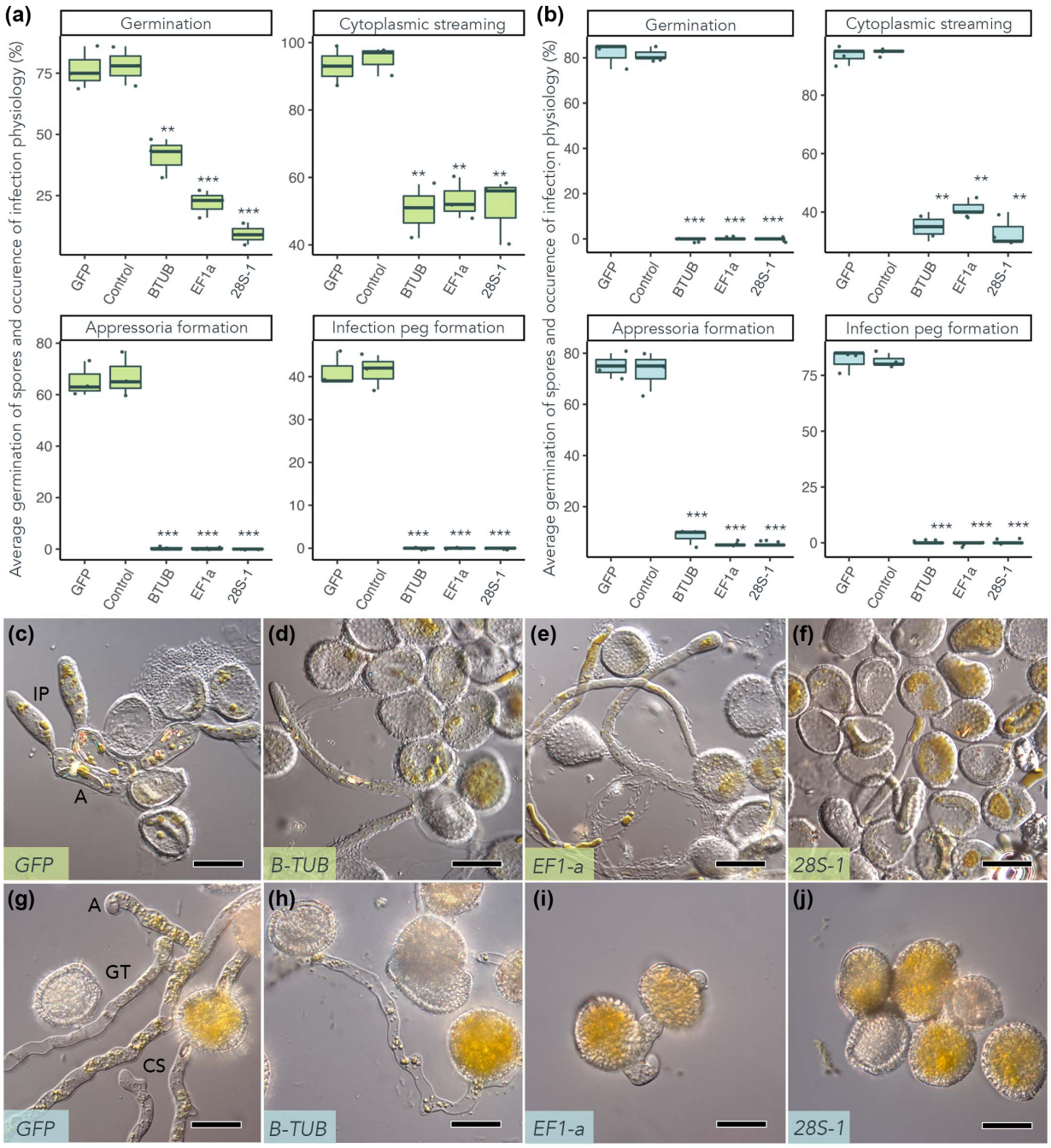


FIGURE 3 Double-stranded RNA (dsRNA) targeting essential rust fungi genes significantly alters growth of *Austropuccinia psidii* and *Coleosporium plumeriae* urediniospores on polyvinylpyrrolidone (PVP)-treated polydimethylsiloxane (PDMS) discs. Substrates were inoculated with *A. psidii* or *C. plumeriae* and simultaneously treated with *β-tubulin* (*β-TUB*), *translation elongation factor 1-α* (*EF1-a*), or ribosomal *28S* dsRNA. Treatments contained 10^6 *A. psidii* urediniospores/ml mixed with 1 μg/10 μl of pathogen-specific dsRNA. Control (−dsRNA) is *A. psidii* or *C. plumeriae* urediniospores in 0.05% Tween 20. *Green fluorescent protein* (*GFP*) dsRNA was included as a nonspecific control. (a, b) Boxplots with scatter representing spore germination rate ($n = 3$ biological replicates) (as % germination), presence of cytoplasmic streaming, and appressoria and infection peg formation (as % occurrence in germinated spores) in (a) *A. psidii* and (b) *C. plumeriae* urediniospores. Significance (as compared to the −dsRNA control group) is represented by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student's *t* test). One hundred urediniospores were counted per biological replicate and counts were repeated three times on separate substrates. This figure was made in R v. 4.0.3 (R Core Team, 2020). (c–f) Comparison of *A. psidii* (green) germ-tube morphology between (c) *GFP* control and (d) *EF1-a*, (e) *β-TUB*, and (f) *28S* dsRNA treatment groups at 100× magnification. Scale bars represent 20 μm. (g–j) Comparison of *C. plumeriae* (blue) germ-tube morphology between (g) *GFP* control and (h) *EF1-a*, (i) *β-TUB*, and (j) *28S* dsRNA treatment groups at 100× magnification. Scale bars represent 20 μm. A, appressoria; CS, cytoplasmic streaming; GT, germ tube; IP, infection peg. Photos were taken 24 h postinoculation. As no significant difference between −dsRNA (negative control) and *GFP* dsRNA (nonspecific control) was observed in either species, only *GFP* dsRNA-treated urediniospores are shown.

both *A. psidii* and *C. plumeriae* (as compared to the −dsRNA control). Germination rates were significantly lower at dsRNA concentrations of 100 ng/μl, as compared to at 10 and 1 ng/μl, for which only two treatments inhibited germination of *A. psidii*. We also observed uniform germination rates in our nonspecific control group (*GFP* dsRNA) across all concentrations and in both rusts.

2.5 | *A. psidii*-specific dsRNA decreases infection in planta

We tested whether exogenous dsRNA could prevent infection of *A. psidii* on whole *S. jambos* plants (Figures 5 and S3). Disease incidence (based on the mean diseased portion of new leaves [%]) decreased significantly when inoculum was treated with dsRNA targeting *EF1-a* (1.3%) (Figure 4d), *β-TUB* (0%) (Figure 4e), or *28S rRNA*-1 (4.7%) (Figure 4f) compared to the −dsRNA (Figure 5b) and *GFP* (Figure 5c) controls (27% and 25.1%, respectively). dsRNA was stable on *S. jambos* leaves for at least 1 week after application (Figure 6).

2.6 | RNAi machinery is conserved across rust fungi

We examined conservation of genes in the RNAi pathway for 15 species in eight families of rust fungi to predict whether they may be amenable to control with exogenous RNAi. We found at least one homologue each of *Argonaute* (*AGO*), *Dicer-like* (*DCL*), and *RNA-dependent RNA polymerase* (*RdRP*) genes in each species (Figure 7). We did not find evidence of orthology of RNAi pathway genes of the Pucciniomycotina (Basidiomycota) with those identified in *Fusarium graminearum*, *Neurospora crassa* (Ascomycota), or *Cryptococcus neoformans* var. *neoformans* (Agaricomycotina, Basidiomycota) based on a phylogenetic hypothesis. Most of the examined rust fungi had one homologue of *DCL*, with the exception of duplicated, orthologous copies or alleles in *A. psidii*, *C. plumeriae*, and *Skierka agallochae* (Figure 7a). Two paralogs of *RdRP* were recovered in four species of *Puccinia* and *C. plumeriae* (Figure 7b), and *Araucariomyces fragiformis* and *A. psidii* had either duplicated orthologous copies or two alleles in a dikaryotic genome. All species of rust fungi contained two paralogs of *AGO*, except *A. fragiformis* and *Hemileia vastatrix*, which had one copy each (Figure 7c).

3 | DISCUSSION

Exogenous dsRNA treatment is a potential management solution for rust fungi, and we aimed to determine its utility across the Pucciniales and its mode of action following application of dsRNA on the leaf surface. RNAi has broad potential to manage rust fungi as genes in the RNAi pathway are conserved across the order with evidence of activity in four families. We showed that dsRNA either associates externally or is internalized by germinating urediniospores of rust fungi. dsRNA targeting essential genes inhibited urediniospore germination and appressorium formation on PDMS artificial leaf-like surfaces and also significantly reduced pustule number on detached leaves and whole plants.

We used two species of rust fungi, *A. psidii* and *C. plumeriae*, and demonstrated that exogenous dsRNA prevented urediniospore germination and formation of infection structures. Exogenous application of dsRNA targeting genes with a range of functions (*EF1-a*, *28S rRNA*-1, *β-TUB*, *MAPK*, *ATC*, *GCS-H*, *CYP450*, and *H01215*) significantly reduced pustule development of *A. psidii* on detached *S. jambos* leaves. Urediniospore germination was reduced within 24 h after inoculation on PDMS and treatment with dsRNA that targeted the essential protein-coding genes *β-TUB*, *EF1-a*, and *28S rRNA*-1. Urediniospores that germinated had withered germ tubes, lacked cytoplasmic streaming, and did not produce appressoria. This supports our hypothesis that unprocessed dsRNA on the leaf surface can be taken up by the fungus and prevent urediniospore germination and infection.

As biotrophs, in vitro assays are challenging for rust fungi; our urediniospore germination experiments established that PVP-treated PDMS discs are suited to investigation of the potential of exogenous dsRNA for pathogen control. This innovation of PDMS discs to determine the impact of dsRNA on a leaf-like surface builds on the use of artificial surfaces in earlier studies of spore germination and formation of appressoria (Bronkhorst et al., 2021; Collins, 1996; Saito et al., 2022; Stone et al., 2012). Our study is the first to use these PDMS leaf-like surfaces to visualize the impact of dsRNA on the formation of infection structures, such as appressoria and infection pegs, in rust fungi.

The most effective inhibition of *A. psidii* and *C. plumeriae* urediniospore germination was observed at dsRNA concentrations of 100 ng/μl. The significant decrease in spore germination with increased dsRNA concentration suggests that the optimal working concentration is higher than in other RNAi studies that have used a concentration of 20 ng/μl (Hu et al., 2020; Koch et al., 2016; Ruiz-Jiménez et al., 2021). Based on the successful inhibition of disease in our assays, the ideal dsRNA concentration (to maximize impact and minimize cost) is closer to 100 ng/μl, which we plan to optimize for applied uses in agriculture and conservation.

Exogenously applied dsRNA prevented or significantly reduced infection by *A. psidii* on whole plants, and highlights its potential to manage rust fungi in environmental or agricultural systems. Combined with our finding that dsRNA either associates externally or is internalized by germinating urediniospores, exogenous dsRNA treatment may be used to manage outbreaks of rust fungi caused by these clonal spores. A limitation of disease management using this approach is our finding that dsRNA is not internalized by teliospores and therefore may not prevent meiosis and overwintering, although this finding requires testing in teliospores of additional taxa. Urediniospores of rust fungi germinate through germ pores, which may provide a point of entry and an explanation for the difference between association of dsRNA with urediniospores and teliospores. *Sclerotinia sclerotiorum* takes up dsRNA via clathrin-mediated

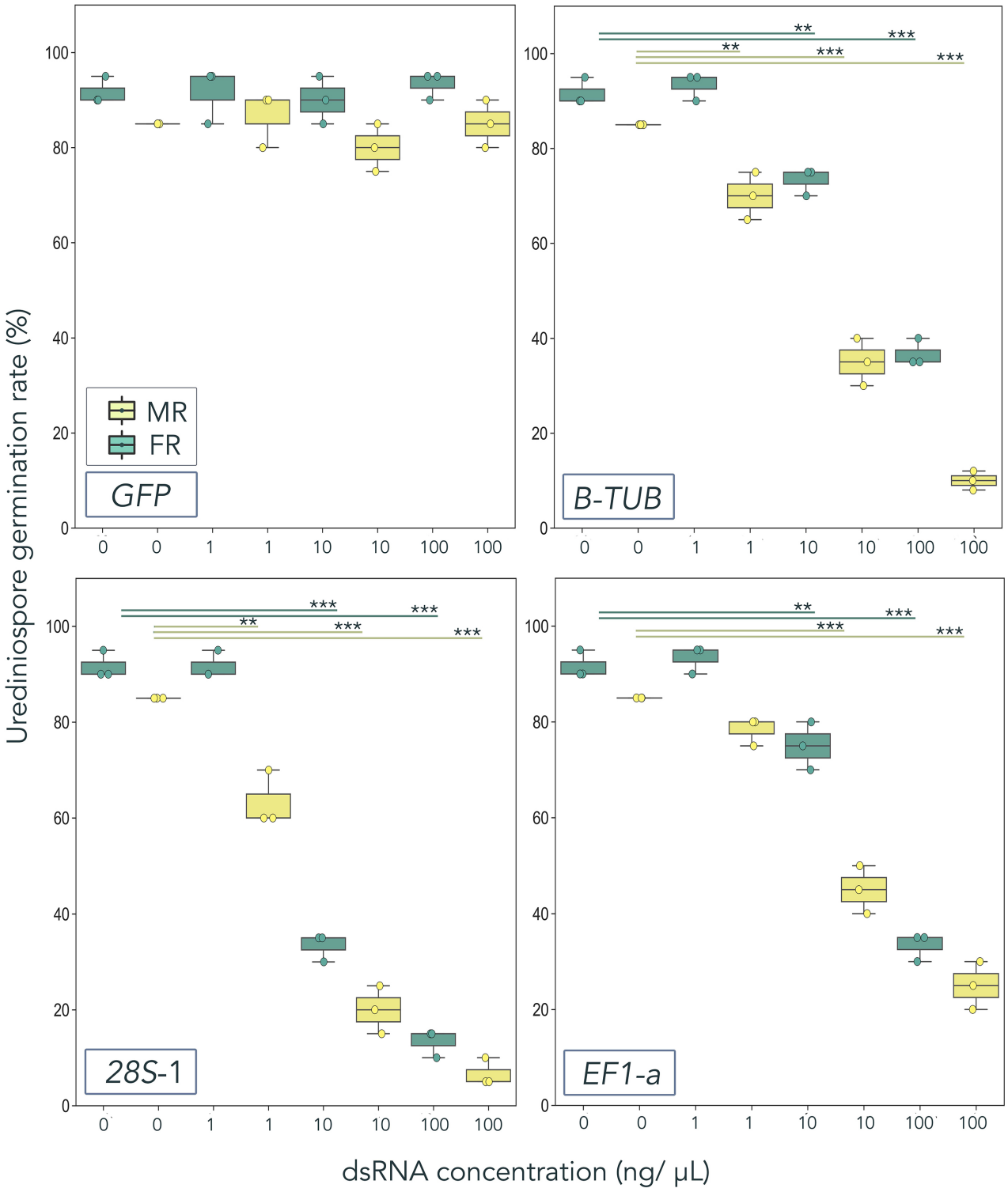


FIGURE 4 Impacts of exogenously applied dsRNA on *Austropuccinia psidii* and *Coleosporium plumeriae* urediniospore germination are concentration-dependent. Urediniospores were germinated in vitro on polyvinylpyrrolidone (PVP)-treated polydimethylsiloxane (PDMS) discs. Substrates were inoculated with *A. psidii* or *C. plumeriae* and simultaneously treated with *β-tubulin* (*β-TUB*), *translation elongation factor 1-α* (*EF1-a*), or ribosomal *28S* (*28S*-1) dsRNA. Treatments contained 10^6 *A. psidii* urediniospores/ml mixed with 100 ng/µl, 10 ng/µl, or 1 ng/µl of pathogen-specific dsRNA. Boxplots with scatter representing spore germination rate ($n = 3$ biological replicates) (as % germination) in *A. psidii* (teal, MR) and *C. plumeriae* (light green, FR). Significance (as compared to the 0 ng/µl control group) is represented by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student's *t* test). One hundred urediniospores were counted per biological replicate and counts were repeated three times on separate substrates.

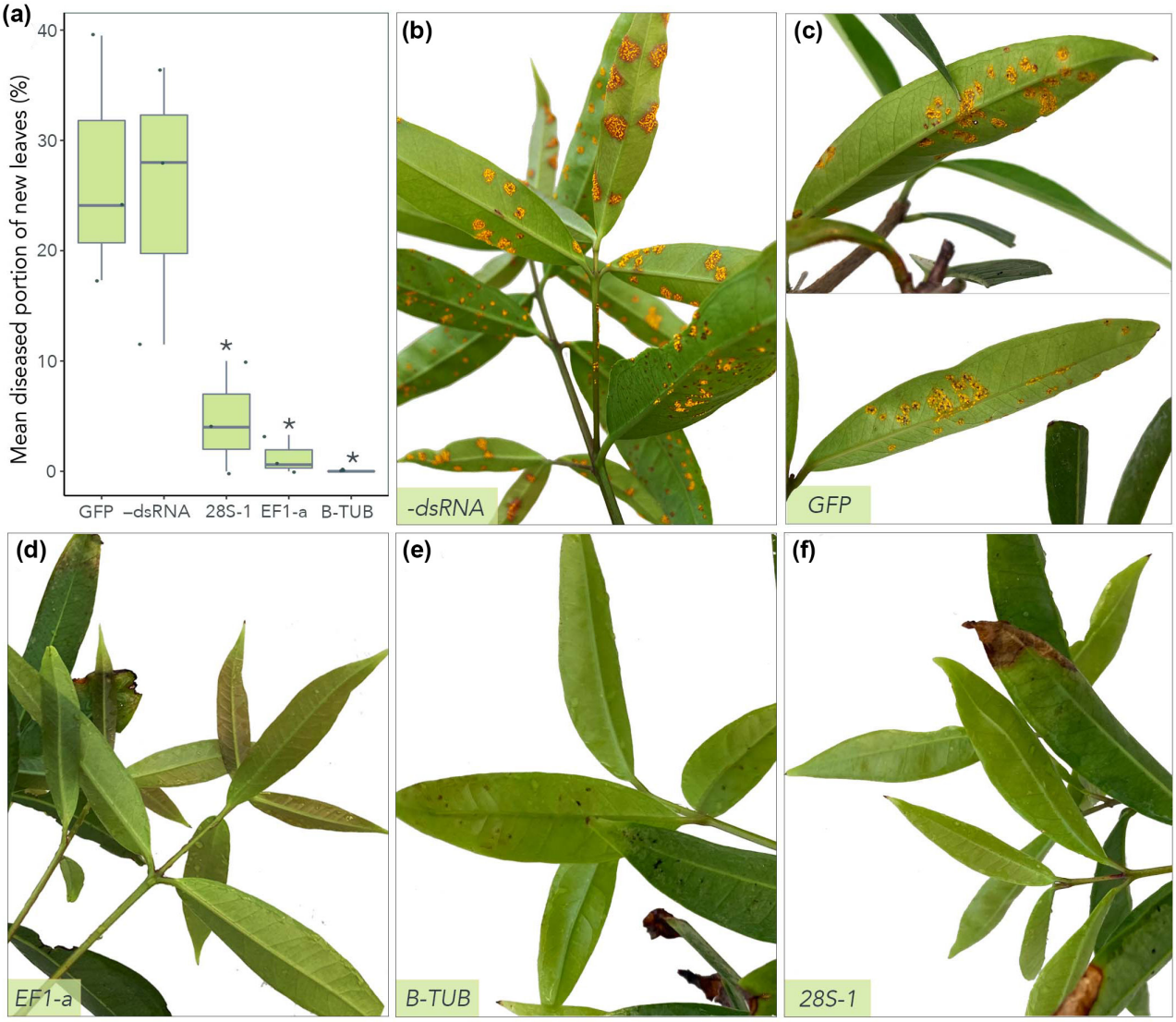


FIGURE 5 Exogenous application of *Austropuccinia psidii*-specific double-stranded RNA (dsRNA) decreases incidence of myrtle rust (MR) on *Syzygium jambos* in whole-plant assays. *S. jambos* plants were infected with *A. psidii* and spray treated with three *A. psidii*-specific dsRNAs. Treatments contained 10^6 *A. psidii* urediniospores/ml mixed with 1 μg/10 μl of *β-tubulin* (*β-TUB*), *translation elongation factor 1-α* (*EF1-a*), or ribosomal *28S* RNA (*28S*-1) dsRNA. Control (−dsRNA) is *A. psidii* urediniospores in 0.05% Tween 20. *Green fluorescent protein* (*GFP*) dsRNA was included as a nonspecific control. (a) Bar plot of diseased tissue as mean ($n = 3$ biological replicates) percentage coverage of leaf. Each biological replicate represents a separate plant and includes all young (susceptible) growth in the disease assessment (usually four to eight leaves). Bars represent standard error of the mean. Significance (as compared to the −dsRNA control group) is represented by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student's *t* test). Disease area was measured using the Leaf Doctor application (Pethybridge & Nelson, 2015). This figure was made in R v. 4.0.3 (R Core Team, 2020). (b–f) Photo comparison of *S. jambos* plants inoculated with *A. psidii* ((b) −dsRNA control) and treated with (c) *GFP*, (d) *EF1-a*, (e) *β-TUB*, or (f) *28S* dsRNA. Plants were photographed 2 weeks postinoculation.

endocytosis (Wytinck et al., 2020) and this mechanism may resolve whether dsRNA is actively taken up by germinating rust spores. Knowledge of whether basidiospores, produced by meiosis, take up dsRNA may help coordinate dsRNA application and management of sexual reproduction in life/disease cycles, with the potential to prevent outbreaks of new strains of rust fungi.

Genes encoding the RNAi pathway are conserved in eight families of rust fungi examined in the present study, and Hu et al. (2020) demonstrated activity of this pathway in a ninth family, Phakopsoraceae (*P. pachyrhizi*). Based on conservation of genes in the RNAi pathway across taxa in the Pucciniales, we hypothesize that RNAi could be used to manage all species of rust fungi.

Treatment with exogenous dsRNA targeting essential genes is a potential management option for rust fungi beyond the studied species, as dsRNA can be readily made using universal primers without knowledge of the gene sequence in target taxa. Off-target effects outside of Pucciniales are unlikely based on the variability of these genes as molecular barcodes, short target sequence length, and high degree of sequence homology required for silencing (Seifert et al., 2007). Our findings challenge hypotheses that highly

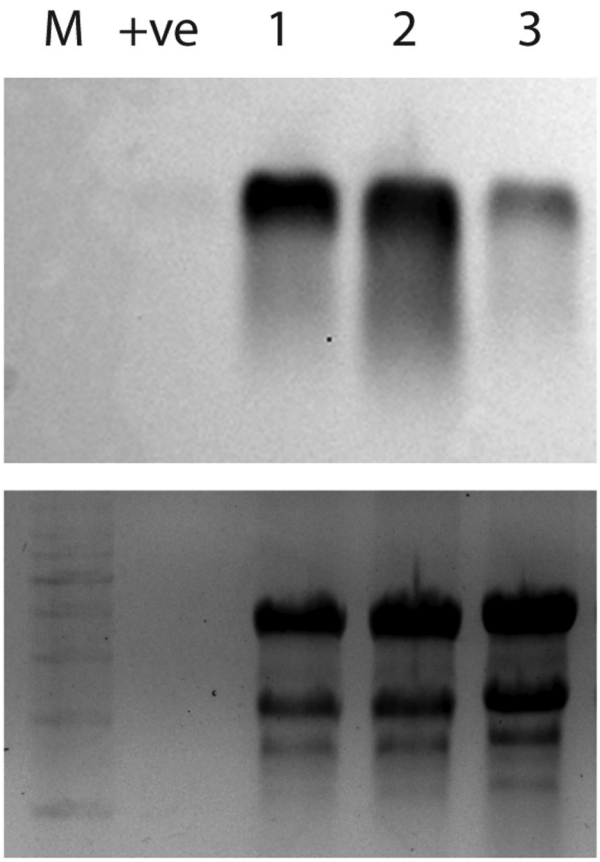


FIGURE 6 Northern blot showing dsRNA stability on *Syzygium jambos* leaves 1 week after application. *GFP* dsRNA (20–40 µl of 250 ng/µl) (Genolution Inc.) in 0.01% Pulse penetrant (Nufarm Australia) was pipetted onto the upper surface of *S. jambos* leaves ($n = 3$). Full-length *GFP* dsRNA was detected using a digoxygenin-labelled *GFP* DNA probe and chemiluminescent detection. 1 ng of *GFP* dsRNA was run as a positive control (+ve). Upper image is the blot, lower image is ribosomal RNA bands on the gel with 1 kb+ DNA ladder (M) before transfer (loading control).

transcribed targets are less suited to RNAi (Larsson et al., 2010; McLoughlin et al., 2018) and support similar findings in *Fusarium* sp., *Botrytis cinerea*, *Magnaporthe oryzae*, and *Colletotrichum truncatum*, where RNAi against *β-tubulin* and *translation elongation factor 2* inhibited fungal growth, deformed mycelia, and reduced development of infection structures and conidiation (Gu et al., 2019; Mosa & Youssef, 2021; Nerva et al., 2020). Our finding that dsRNAs targeting highly transcribed genes significantly reduce urediniospore germination and disease symptoms may suggest that the RNAi mechanism is amplified in rust fungi, in contrast to previous reports for other fungi (Song et al., 2018). The hypothesis that RdRPs detected in our phylogenetic analysis enhance RNAi in rust fungi requires further testing.

RNAi has an advantage over fungicide application and breeding for resistance, for which point mutations in the fungus and outcrossing of plants may overcome disease resistance (McDonald et al., 2019). To develop resistance to sprays containing long dsRNA as the active ingredient, entire targeted genes or RNAi genes must be lost or accumulate mutations at multiple sites in a targeted region to avoid recognition of the corresponding mRNA by siRNAs. New pathogen target sequences can be identified more rapidly than breeding layers of resistance into host plants, and RNAi has fewer environmental impacts than chemical fungicides. Delivery, uptake, and stability of exogenously applied dsRNA are improved when complexed with RNA carriers (Islam et al., 2021; Leber et al., 2017; Mitter et al., 2017; Numata et al., 2014), and with further developments in longevity of dsRNA on the leaf surface, an outcome of our study is a potential disease management strategy to apply in fragile ecosystems or on culturally sacred trees threatened by myrtle rust.

Rust fungi have the largest genomes of all known fungi (Tavares et al., 2014). The six new genomes assembled in the present study were over 500 Mb, larger than the average genome size of rust fungi (300 Mb), with the exception of *C. plumeriae* (c.187 Mb) and *Sphaerophragmium acaciae* (c.244 Mb). These genomes are the first sequenced and assembled for taxa in the Araucariomyceaceae, Phragmidiaceae, and Skierkaceae. The gene tree relationships among conserved RNAi machinery were mostly congruent with the familial relationships recovered by Aime and McTaggart (2021).

Exogenous dsRNA treatment brings a new era for disease management of rust fungi. It probably has order-wide potential based on conservation of genes in the studied taxa. It can be timed with disease outbreaks similar to fungicides, but without negative environmental effects. It has advantages over breeding resistance and fungicide application in that resistance to dsRNA requires loss of essential genes, or loss of the silencing pathway, and our study shows its potential for the management of invasive rust fungi, such as *A. psidii*, in natural environments. We have shown that exogenous application of dsRNA prevents infection by rust fungi, and a future challenge is whether this approach can be used curatively to manage established infections.

4 | EXPERIMENTAL PROCEDURES

4.1 | Preparation of inocula

Urediniospores of *A. psidii* were shaken from infected *S. jambos* plants grown under shade-house conditions. Urediniospores of *C. plumeriae* were vacuumed from infected leaves. For all experiments except dsRNA uptake, spores were dried for 48 h in a desiccator then aliquoted and stored at −80°C. Urediniospores were rehydrated in sterile distilled water and 0.05% Tween 20 for 30 min before use. Inocula were concentrated to 10^6 spores/ml. Urediniospores of *A. psidii* and *C. plumeriae* were germinated at 16 and 26°C, respectively, with 80% relative humidity. These conditions were used for whole-plant inoculations and spore germination experiments.

4.2 | RNA extraction and dsRNA synthesis

Dried urediniospores of *A. psidii* and *C. plumeriae* were ground in liquid nitrogen and total RNA extracted using TRIzol (ThermoFisher

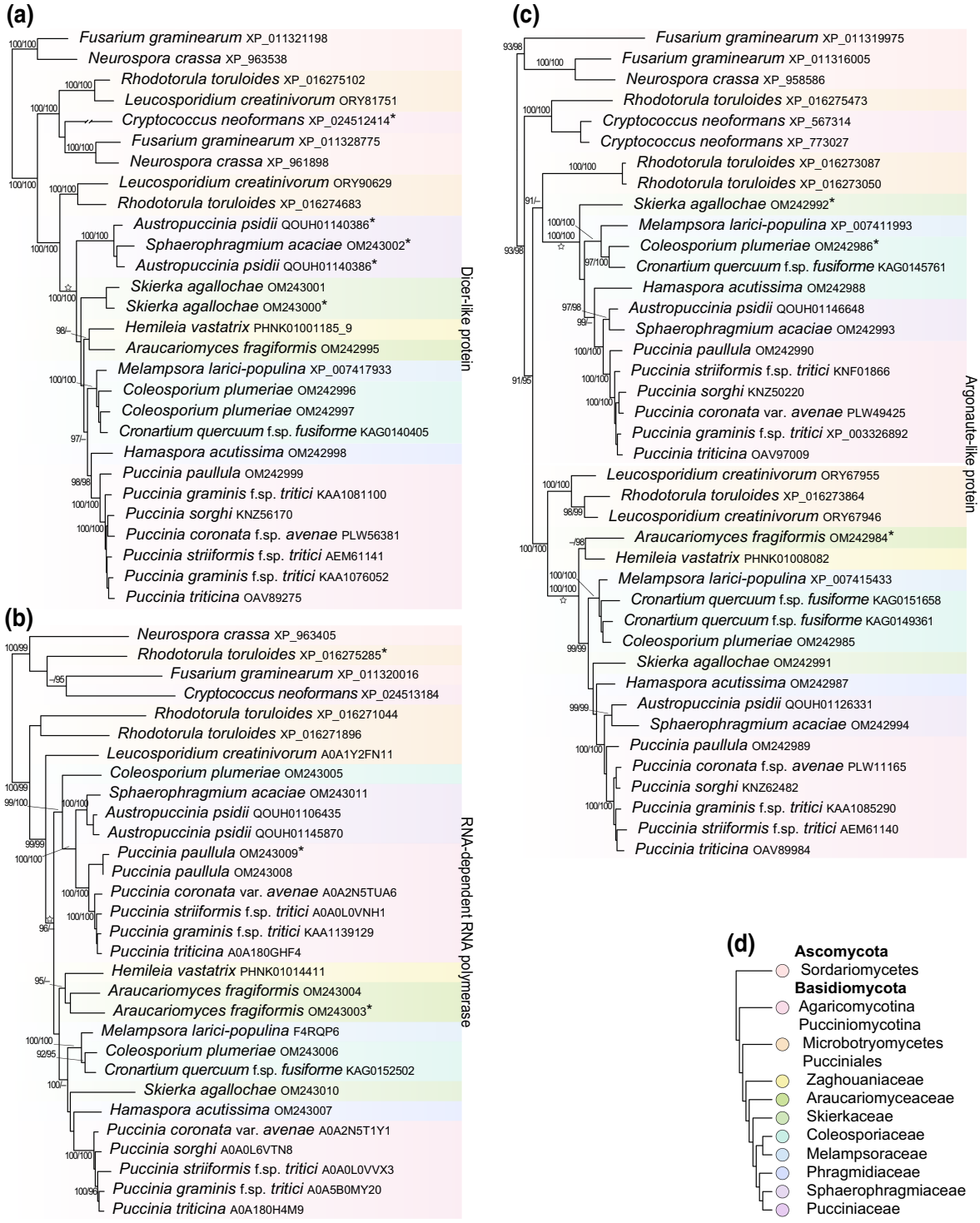


FIGURE 7 Phylograms of RNAi machinery genes annotated from model fungi and Pucciniomycotina. Support above nodes from 10,000 approximate likelihood ratio tests (≥90) and ultrafast bootstraps (≥95) in IQTree v. 2.0.6. Homologues of (a) *Dicer-like*, (b) *RNA-dependent RNA polymerase*, and (c) *Argonaute-like* genes. *Denotes truncated proteins in an alignment. Taxa are coloured by their phylum, subphylum, class or familial rank classification shown in (d) and based on Aime and McTaggart (2021). Monophyletic groups of rust fungi are marked with a star at their most recent common ancestor.

Scientific) as per the manufacturer's instructions. cDNA was synthesized from extracted RNA using the ProtoScript II First Strand cDNA Synthesis Kit, following the standard protocol (New England BioLabs). Target sequences (300–400 bp long) were amplified from cDNA using sequence-specific primers containing T7 promoter sequences at the 5′ end (Table S1). Contigs that contained target genes in assemblies of *A. psidii* and *C. plumeriae* were identified by tBLASTn, and exons were predicted in UniProt.

dsRNA was synthesized in vitro with a HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs) using the T7 PCR products as template. Sense and antisense sequences were synthesized in the same reaction. Following dsRNA synthesis, RNA was DNase-treated and purified using TRIzol and annealed at 95°C for 5 min. For Cy3 dsRNA synthesis, 4.5 μl of 5 mM Cy3 CTP (GE Healthcare) and 1.125 μl of 100 mM CTP were used instead of 1.5 μl of 100 mM CTP nucleotides. RNA was purified using the Monarch RNA Cleanup Kit (New England BioLabs).

4.3 | dsRNA uptake assays

An aliquot of 4 μl of 250 ng/μl Cy3-labelled *GFP* dsRNA was mixed with 4 μl of fresh rust spores (10^4–10^6 spores/ml) and plated on 1% water agar drops on microscope slides or directly on the microscope slides, according to the method described by Odell and Smith (1982). Slides were placed on moist filter paper in a Petri dish sealed with Parafilm. Spores were incubated for 6 or 8 h in the dark at room temperature, treated with 75 U of micrococcal nuclease (New England BioLabs) for 30 min at 37°C, and examined under a Zeiss LSM700 confocal microscope for Cy3 fluorescence (excitation at 555 nm and detection at 559–640 nm).

4.4 | Fluorescence lifetime imaging microscopy

Spores were set up directly on glass slides as detailed above but with 0.5 μg of dsRNA/8 μl drop instead of 1 μg. Spores were incubated in the dark at room temperature for 4 h before treating with micrococcal nuclease. Imaging was performed on a Leica Microsystems SP8 system, with Cy3 excited using a 40 MHz pulsed, tunable white light laser at 555 nm and detected with HyD detectors at 560–620 nm. FLIM was performed to distinguish the autofluorescence from the spore and the Cy3 fluorescence signal. Data were collected to obtain a minimum of 100 photons per pixel to ensure reliable modelling of the fluorescence decay. Leica Application Suite X (LAS X) software v. 5.0.2 (https://imb.uq.edu.au/facilities/microscopy/microscopy-hardware-software) was used to produce FLIM images and phasor plots. ImageJ v. 1.53q was used for processing images and *Z*-stack projections.

4.5 | Detached leaf assays

Young, emerging *S. jambos* leaves were excised at the base of the petiole using a scalpel. In a laminar flow, leaves were surface sterilized in 1% sodium hypochlorite for 1 min and washed for 3 × 1 min in Milli-Q water. Then, 1% agar was pipetted along one side of sterile Petri dishes, approximately 3 ml, covering a quarter of the Petri dish. Leaves were transferred onto the Petri dishes using sterile forceps, with the petiole penetrating the medium. Inoculum (10^6 spores/ml in 0.05% Tween 20) was pipetted onto leaves in 5-μl droplets, with two to 10 droplets per leaf, depending on leaf size. Inoculum was resuspended by inverting every 30 s to prevent settling of spores. Plates were sealed using Parafilm and left in the dark for 24 h at 18°C, 80% humidity. After 24 h, plates were moved to an incubator (25°C, 16 h day, 8 h night cycle) for 14 days.

For assays including dsRNA, aliquots of *A. psidii* inoculum were prepared in PCR tubes from a 10^6 spores/ml stock solution and 1 μg of dsRNA was added per 10 μl of *A. psidii* inoculum. Each plate contained one control (−dsRNA) and one treatment leaf (+dsRNA), with the same number of drops on each leaf. Control and treatment leaves in each plate were from the same *S. jambos* plant and were of the same age and growth stage to ensure minimal variation in cuticle thickness and other infection-impacting features.

4.6 | Urediniospore germination assays on PDMS discs

Ten microlitres of *A. psidii* or *C. plumeriae* inocula were mixed with 1 μg of *GFP*, *EF1-a*, *28S rRNA*-1 or *β-TUB* dsRNA, with 0.05% Tween 20 as the negative control, and pipetted in 2 μl aliquots onto discs. Urediniospores were incubated for 24 h at 18°C, 80% humidity, then photographed using a Leica microscope with Nomarski interference and a DFC550 camera. Germinated urediniospores were counted using Adobe Photoshop v. 22.1.0.

4.7 | dsRNA dose-dependent assays

Urediniospores were germinated on discs made of polyvinylpyrrolidone (PVP, Kollidon 25; Sigma-Aldrich)-treated PDMS (Sylgard 184, 10:1 wt/wt base to curing agent ratio) (Soffe et al., 2019). A standard PDMS microfabrication processes produced discs 2 mm high, and subsequent PVP treatment made surfaces hydrophilic to facilitate wetting with spore germination buffer. PDMS discs were placed in Petri dishes on damp filter paper.

Purified *GFP* (nonspecific control), *EF1-a*, *β-TUB*, and *28S rRNA*-1 dsRNA were diluted to three concentrations: 100 ng/μl, 10 ng/μl, and 1 ng/μl. dsRNAs were mixed with either *A. psidii* or *C. plumeriae* urediniospores at 10^6 spores/ml and 10 μl of this solution was aliquoted in 2 μl droplets onto PVP-coated PDMS discs. Inoculum only (−dsRNA) was also included as a negative control. Urediniospores were incubated for 24 h at 18°C, 80% humidity and germinated spores were quantified using a Leica microscope.

4.8 | Shade-house assays

One-year-old cuttings of *S. jambos* plants were grown in a shade-house, watered twice daily for 5 min with an overhead sprinkler system and fertilized with Nitrosol 2 weeks prior to infection to encourage growth of new leaves. *A. psidii* inoculum was aliquoted into 1 ml fine-spray bottles with 100 ng/μl of dsRNA for each treatment.

Young flush was sprayed until saturated (c.1 ml). Plants were covered and incubated in the dark for 24 h at 18°C, 80% humidity. Coverings were removed and plants were returned to the shade-house (ambient temperature, natural light, and overhead watering for 2 × 5 min daily). Infections were monitored and final disease incidence was assessed 2 weeks postinoculation. Disease was scored based on manual measurements of lesion size and percentage coverage of diseased tissue using the Leaf Doctor application (Pethybridge & Nelson, 2015).

4.9 | Northern blots

Commercially produced *GFP* dsRNA (Genolution Inc.) was pipetted onto the upper surface of *S. jambos* leaves. 20–40 µl of 250 ng/µl dsRNA in 0.01% Pulse penetrant (Nufarm Australia) was applied per leaf depending on the size. dsRNA was applied slowly and spread across the leaves via pipetting to promote even spread of dsRNA. Leaves were harvested 1 week later and RNA was extracted using the Norgen Plant/Fungi Total RNA Purification Kit following the manufacturer's protocol (Norgen Biotek Corp.). Extracted RNA was run on a 1% agarose formaldehyde gel (10 µl/lane) and then transferred onto a Hybond-N^+ nylon membrane (GE Healthcare). The blot was probed with a digoxigenin (DIG)-labelled *GFP* DNA probe (Roche), which was detected using an anti-digoxigenin AP-conjugate (Roche) and visualized using the CDP Star chemiluminescent substrate (Sigma Aldrich) on an iBright imaging system (Thermo Fisher).

4.10 | Genome sequencing and assembly

We sequenced and assembled draft genomes of *A. fragiformis* (Araucariomyceaceae), *C. plumeriae* (Coleosporiaceae), *Hamaspora acutissima* (Phragmidiaceae), *Puccinia paullula* (Pucciniaceae), *S. agallochae* (Skierkaceae), and *S. acaciae* (Sphaerophragmiaceae). Genomic DNA was extracted from environmental samples of rust fungi (Table 1). Urediniospores were vacuumed directly from leaf material, ground in liquid nitrogen, and DNA was extracted following the protocol of a Machery Nagel NucleoSpin Plant II DNA extraction kit. The Australian Genome Research Facility (AGRF) prepared a Chromium 10× library and sequenced all libraries on an Illumina NovaSeq 6000. Chromium sequencing data were assembled using Supernova v. 2.1.1 (Weisenfeld et al., 2017).

4.11 | RNAi gene discovery

Published genomes of *A. psidii* (McTaggart et al., 2018), *Cronartium quercuum* f. sp. *fusiforme* (Coleosporiaceae) (Pendleton et al., 2014), *H. vastatrix* (Zaghouaniaceae) (Cristancho et al., 2014), *Melampsora larici-populina* (Melampsoraceae) (Duplessis et al., 2011), *Puccinia coronata* f. sp. *avenae* (Pucciniaceae) (Miller et al., 2018), *Puccinia graminis*

TABLE 1 Assembly statistics of genomes assembled in the present study

Taxon	Specimen number	Family	DNA (ng) for library preparation	No. of reads (millions)	Assembly size (Mb) scaffolds >10 kb	Effective coverage (%)	Repetitive fraction (%)	GC content (%)	N50 (kb)
Araucariomyces fragiformis	BRIP 68996	Araucariomyceaceae	3016	890.11	622.65	20.49	28.73	51.97	33.4
Coleosporium plumeriae	BRIP 69006	Coleosporiaceae	239	822.42	186.92	18.73	92.65	43.19	22.6
Hamaspora acutissima	BRIP 69009	Phragmidiaceae	1462	965.77	708.12	21.87	70.36	53.45	52.0
Puccinia paullula	BRIP 69007	Pucciniaceae	2105	925.67	941.82	23.16	41.89	46.15	40.3
Skierka agallochae	BRIP 69008	Skierkaceae	2400	909.86	1060	30.39	27.39	33.66	26.5
Sphaerophragmium acaciae	BRIP 69010	Sphaerophragmiaceae	3360	881.55	244.19	19.4	76.26	42.16	20.2

f. sp. *tritici* (Duplessis et al., 2011), *Puccinia sorghi* (Rochi et al., 2018), *Puccinia striiformis* f. sp. *tritici* (Cuomo et al., 2017), and *Puccinia triticina* (Kiran et al., 2016) were mined for copies of RNAi genes (*DCL*s, *RdRP*s and *AGO*s) using tBLASTn (Altschul et al., 1990). We searched for homologues of genes in the RNAi pathway in *C. neoformans* (Janbon et al., 2010), *F. graminearum* (Chen et al., 2015), and *Cryphonectria parasitica* (Zhang et al., 2014). Coding sequences of potential homologues were predicted using comparative models of *P. graminis*, *P. sorghi*, and *M. larici-populina* on UniProt (Bateman et al., 2020). Predicted coding sequences were aligned with 100 HMM iterations in Clustal Omega using Ugene (Okonechnikov et al., 2012). The most likely tree was searched for from each alignment using IQTree v. 2.0.6 with the best fit model of evolution (command-m TEST), and 10,000 ultrafast bootstraps and approximate likelihood ratio tests (Hoang et al., 2018; Nguyen et al., 2015).

4.12 | Data analysis and visualization

Student's *t* tests were computed in R v. 4.0.3 (R Core Team, 2020) using the *t*-test function and the *dplyr* package (Wickham et al., 2020). *p* values of <0.05 were considered significant. Data were plotted in R v. 4.0.3 (R Core Team, 2020) using bar (geom_bar) and box (geom_box) plots with the *ggplot2* package (Wickham et al., 2020).

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CONFLICT OF INTEREST

The authors declare no competing financial and nonfinancial interests.

DATA AVAILABILITY STATEMENT

Draft assemblies of all genomes sequenced in the present study are available at Zenodo (10.5281/zenodo.6650763) or at https://drive.google.com/drive/folders/1nDRWYLEO80R6CCxdnMzkaFY3DqUKn6Vo?usp=sharing. Raw sequencing data are available on request from ARM. All sequences used for analyses of genes in the RNAi pathway have been deposited in GenBank. Raw germination rate and disease assessment data that support the findings of this study are made available publicly through Dryad (10.5281/zenodo.7018192).

ACCESSION NUMBERS

Afragiforme_AGO_620928, OM242984; Coleosporium_AGO_674944, OM242985; Coleosporium_AGO_675696, OM242986; Hamaspora_AGO1_977380, OM242987; Hamaspora_AGO2_613271, OM242988; PucciniaP_AGO_894603, OM242989; PucciniaP_AGO_941392, OM242990;Skierka_AGO_935665,OM242991;Skierka_AGO_990216, OM242992; Sphaerophragmium_AGO2_1035541, OM242993; Sphaerophragmium_AGO_6394, OM242994; Afragiforme_DCL_1423528, OM242995; Coleosporium_DCL1_4378, OM242996; Coleosporium_DCL_651311, OM242997; Hamaspora_DCL1_931648, OM242998; PucciniaP_DCL_897422, OM242999; Skierka_DCL_951268, OM243000; Skierka_DCL_984643, OM243001; Sphaerophragmium_DCL1_94389, OM243002; Afragiforme_RDRP_1026684, OM243003; Afragiforme_RDRP_1457563, OM243004; Coleosporium_RDRP_11429, OM243005; Coleosporium_RDRP_686038, OM243006; Hamaspora_RDRP_618127, OM243007; PucciniaP_RDRP_859667, OM243008; PucciniaP_RDRP_906243, OM243009; Skierka_RDRP_950972, OM243010; Sphaerophragmium_RDRP_460606, OM243011.

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SUPPORTING INFORMATION

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

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