Effect of Diaporthe RNA virus 1 (DRV1) on growth and pathogenicity of different *Diaporthe* species

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Abstract A 4.1 kbp positive-strand RNA virus known as Diaporthe RNA virus 1 (DRV1) occurs in hypovirulent, non-sporulating isolates of the fungal pathogen *Diaporthe perjuncta*. A full-length cDNA clone of DRV1 was developed and RNA transcribed from the cDNA clone used to transfect different *Diaporthe* spp. The transfected species included three *D. ambigua* isolates and an unidentified *Phomopsis* asexual state of a *Diaporthe* sp. Successful transfections were confirmed using RT-PCR. Although the in vitro-transcribed positive sense single-stranded RNA used for transfection included vector sequences at both ends, the genomes of progeny virus from DRV1-transfected isolates were free of the vector sequences. Transfection resulted in morphological changes in these fungal pathogens. However, the presence of DRV1 did not reduce growth rate in two of the three *D. ambigua* or the *Phomopsis* sp.

significantly. Pathogenicity studies showed that the transfected isolates have reduced aggresiveness.

Keywords *Diaporthe* · *Phomopsis* · Diaporthe RNA virus 1 · Mycovirus · Transfection · Spheroplast

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Introduction

Mycoviruses persistently infect many species of fungi. Based on the genome organization, capsid structure and nucleotide sequence, most of the characterized double-stranded (ds) RNA mycoviruses belong to families *Totiviridae*, *Partitiviridae*, *Chrysoviridae* and *Reoviridae* (Ghabrial and Suzuki 2009). Interest in mycoviruses has been stimulated by their association with hypovirulence in some pathogens (Nuss 2000; Dawe and Nuss 2001; Nuss 2005). In attempts to identify biological control agents, many classes of plant pathogenic fungi have been screened for the presence of mycoviruses. In many instances, mycoviruses have been found and some have been associated with hypovirulence. These fungi include important plant pathogens such as *Diplodia pinea* (Preisig et al. 1998), *Helminthosporium victoriae* (Sanderlin and Ghabrial 1978), *Ophiostoma novoulmi* (Hong et al. 1999), *Rhizoctonia solani* (Castanho et al. 1978) and various species of *Fusarium* (Nogawa et al. 1993; Compel et al. 1999; Chu et al. 2002).

Mycoviruses move between isolates of a fungus during hyphal anastomosis (Anagnostakis 1977). This

mode of transfer restricts the mycoviral movement between isolates (Choi and Nuss 1992). Transfection and transformation methods have been developed to introduce the *Cryphonectria parasitica* hypovirus into new hosts (Choi and Nuss 1992; Chen et al. 1994; 1996; Chen and Nuss 1999). Transfection involves electroporation of fungal spheroplasts with synthetic single-stranded RNA, in vitro-transcribed from the coding strand of the hypovirus (Chen et al. 1994). Transformation involves delivery of the hypovirus as a cloned cDNA insert into the new host. This involves the integration of hypovirus cDNA in the fungal chromosome (Choi and Nuss 1992).

Knowledge regarding the mode of action of mycoviruses has been largely derived from the interaction of the hypoviruses and their transfected or transformed hosts (Dawe and Nuss 2001). This is despite the fact that several mycoviruses have been characterized at nucleotide sequence level. The development of full-length cDNA clones of other mycoviruses is likely to provide additional knowledge not available from the *Cryphonectria*-hypovirus system. In an effort to develop transfection protocols for mycoviruses other than the Cryphonectria hypovirus, we have developed a full-length cDNA clone of Diaporthe RNA virus 1 (DRV1) and used the in vitro-produced RNA from it to transfect a virus-free *D. perjuncta* isolate (Moleleki et al. 2003). In a related study, Esteban and Fujimura (2003) transformed yeast cells negative for 23S RNA virus and showed transmission of the virus in daughter cells.

DRV1 is a positive-strand RNA mycovirus with dsRNA as a replicative form (Preisig et al. 2000). Since the discovery of DRV1, several other positive-strand RNA mycoviruses such as *Fusarium graminearum virus* DK-21, *Botrytis virus* X, *Oyster mushroom spherical virus*, and two mycoviruses from *Sclerotinia sclerotiorum* have been discovered (Xie et al. 2006; Liu et al. 2009; Howitt et al. 2006; Kwon et al. 2007; Yu et al. 2003). Although DRV1 has not been placed into a virus genus, its C-terminal half shows the highest similarity to *Turnip crinkle virus* (TCV) and *Carnation mottle virus* (CMV) in the genus *Carmovirus*, family *Tombusviridae* (Preisig et al. 2000). The aim of this study was firstly to extend the host range of DRV1 beyond its natural host, *D. perjuncta*. A second aim was to characterize the effects of DRV1 in the new hosts. To achieve these goals, different isolates of *D. ambigua* and an unidentified *Phomopsis* asexual state

of a *Diaporthe* sp. were transfected with DRV1 and subsequently characterized.

Materials and methods

Fungal cultures

All isolates in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. The *Phomopsis* sp. cultures (CMW5588-WT and CMW5588-DRV1) and three different isolates of *D. ambigua* (CMW5587-WT and CMW5587-DRV1; CMW5288-WT and CMW5288-DRV1; CMW5287-WT and CMW5287-DRV1), and the naturally-infected *D. perjuncta* (CMW3407) were grown on 2% potato dextrose agar (PDA) (Biolab, Midrand, South Africa) as previously described (Moleleki et al. 2002).

In vitro transcription and transfection

Synthetic DRV1 RNA transcripts were in vitro-transcribed using T7 RNA polymerase (Roche Diagnostics, Mannheim, Germany) from *Sal*I-linearized pDV3 as described by Moleleki et al. (2003). Fungal spheroplasts were prepared by digesting ten-day-old fungal mycelium with a mixture of chitinase (Fluka, Buchs, Switerland) and cellulase (Sigma, St. Louis, MO) as described previously (Moleleki et al. 2003). The spheroplasts were pulsed three times at 1.8 kV with the viral transcripts using multiporator (Eppendorf, Hamburg, Germany). The transfected spheroplasts were regenerated on regeneration medium (1 g/l casein hydrolysate; 1 g/l yeast extract; 342 g/l sucrose; 16 g/l agar) for 10 days and transferred to PDA.

Analysis of DRV1 dsRNA isolated from transfected fungi

Fungal mycelium was freeze-dried, after which approximately 0.1 g mycelium was ground to a fine powder in liquid nitrogen using a pestle and mortar. Double-stranded RNA was extracted and purified from the mycelium of transfected fungal isolates using the method described by Valverde et al. (1990). A rapid test for successful transfection was done by isolating total nucleic acids from the transfected fungi using 2 X STE (0.5 M Tris–HCl, 1 M

NaCl, 10 mM EDTA, pH 6.8). The nucleic acids were purified sequentially using phenol, phenol/chloroform and chloroform. RT-PCR was done using the DRV1-specific primer pair, Oli64 (5′-GTCGCATCTCA CAGCCGAGCG-3′) and Oli80 (5′-CTCAC CAGCCTCCAACCG-3′) using the Titan One Tube RT-PCR System (Roche Diagnostics, Mannheim, Germany) (Moleleki et al. 2003). Double stranded RNA was denatured for 3 min at 98°C. The conditions for RT-PCR were 50°C for 30 min, 96°C for 2 min, 10 cycles at 94°C for 30 s, 64°C for 30 s and 68°C for 1 min. This was followed by further 30 cycles at 94°C for 30 s, 64°C for 30 s and 68°C for 1 min with a cycle elongation time increase of 5 s for each cycle. A final elongation step at 68°C for 10 min was added. The RT-PCR products were separated on a 1% agarose gel and sequenced using ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA).

The distal ends of the genome of DRV1 from different transfected isolates were amplified by 5′/3′ RACE approach using a 5′/3′ RACE kit (Roche Diagnostics) (Frohman 1994). The primers Oli73 (5′-GTGCCCTGCACAAACAACTC-3′) and Oli75 (5′-TCCATCTCACCGGAGCGGCAG-3′) were used to reverse-transcribe the 5′ and 3′, respectively (Moleleki et al. 2003). After polyadenylating these ends, a nested PCR was used to amplify the 5′ end using Oli78 (5′-CCTGGGTGACGGTTGTTACAC-3′) and the 3′ end using Oli81 (5′-TTGAACGATGGGTGTAGGTGG-3′) as described previously (Moleleki et al. 2003). These products were cloned in pGEM T-Easy vector (Promega, Madison, WI) and sequenced using ABI PRISM 3100 DNA sequencer.

Evaluation of phenotypic characteristics

Ten mycelial plugs of each isolate were cut from actively growing one-week-old cultures, plated on PDA medium and incubated in the dark at 25°C. Differences in culture morphology were examined for the transfected and wild-type isolates. Growth of cultures was compared by measuring colony diameters of all isolates after 2, 3 and 4 days. SAS (SAS Institute Inc., Cary, NC) was used to analyze mean growth rates of cultures. The entire experiment was repeated once.

Pathogenicity tests were carried out using Golden Delicious apple tree twigs. Twigs of roughly uniform thickness and 40 cm in length were cut from Golden Delicious apple tree branches and sealed with molten wax at both ends. Bark disks were removed with a 5-mm diameter cork borer to expose cambium on twigs. Mycelial discs of equal size were placed into the wounds. Ten twigs were inoculated with each fungal isolate. The inoculated wounds were covered with masking tape to reduce desiccation and incubated in moist chambers at 25°C for 1 month. The lesion lengths were measured and differences in these lengths were analyzed using SAS.

Small blocks of wood were cut from along the length of the discoloured wood under the bark of inoculated twigs and transferred to fresh PDA plates. The plates were incubated at 25°C. The cultures were purified by successive sub-culturing on PDA plates. Total nucleic acids were isolated from these cultures and RT-PCR was performed using the primer pair Oli64 and Oli80. The products were separated on a 1% agarose gel.

Fungal isolates were induced to sporulate as described by Smit et al. (1996). Conidial masses from pycnidia were suspended in distilled water, serially diluted and spread onto PDA plates. More than a hundred single germinating conidia were lifted from the agar with a sterile needle and transferred to new PDA plates. Additionally, masses of conidia from five pycnidia were streaked directly onto the surface of PDA plates and allowed to grow. RT-PCR was performed on total nucleic acids isolated from mycelium of these single conidial cultures to confirm the presence or absence of DRV1.

Results

Transfection of fungal isolates with DRV1

DRV1 RNA transcripts used to transfect spheroplasts were obtained from pDV3, a full-length cDNA clone of the RNA genome of DRV1. In vitro transcription of linearized pDV3 yielded a single band of RNA. Spheroplasts of three isolates of *D. ambigua* and one isolate of *Phomopsis* sp. were transfected with the in vitro-produced viral RNA or with water as a negative control. Transfections were confirmed by isolating dsRNA from the regenerated mycelium. Isolation resulted in a single 4 kbp band similar in size to DRV1, in cases where transfection was successful. RT-PCR using DRV1 sequence-derived primers Oli64

and Oli80 amplified a band of about 600 bp, thus confirming that the dsRNA band from transfected mycelium is DRV1 (Fig. 1). The sequences of these RT-PCR products matched those of DRV1 exactly. The sequences of the 5′ and 3′ termini were consistently identical to those described previously for the genome of DRV1 by Preisig et al. (2000). DRV1 RNA could not be recovered from the transfected mycelium of C. cubensis.

Assessment of phenotypic characteristics

There were no discernible morphological differences between the DRV1-transfected Phomopsis sp. (CMW5588-DRV1) and its isogenic virus-free (CMW5588-WT) analogue, except that the former produced smaller pycnidia, which did not readily exude conidia. CMW5588-WT produced larger pycnidia that readily exuded conidia as the colony aged. There were distinct morphological differences between the transfected and isogenic wild-type isolates of D. ambigua (CMW5587-WT). The DRV1-transfected D. ambigua (CMW5587-DRV1) grew sparsely so that the agar under the mycelium could be seen. The mycelium was closely appressed to the agar surface. The isogenic wild-type (CMW5587-WT) isolate of this fungus produces dense aerial mycelium on the agar plates.

The principal difference between the DRV1-transfected D. ambigua (CMW5288-DRV1) and its isogenic virus-free (CMW5288-WT) isolate was that the aging mycelium of the former isolate aggregated in tufts and exposed the agar underneath the mycelium. In contrast, the isogenic virus-free isolate grew evenly over the agar surface without aggregation of the mycelium. The DRV1-transfected D. ambigua
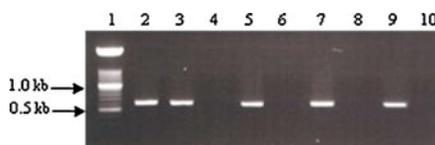
Fig. 1 RT-PCR analysis of transfected and virus-free Phomopsis sp. and D. ambigua isolates. Lane 1: 100 bp ladder; Lane 2: D. perjuncta CMW3407; Lane 3: Phomopsis sp. CMW5588-DRV1; Lane 4: Phomopsis sp. CMW5588-WT; Lane 5 D. ambigua CMW5587-DRV1; Lane 6: D. ambigua CMW5587-WT; Lane 7: D. ambigua CMW5288-DRV1; Lane 8: D. ambigua CMW5288-WT: Lane 9: D. ambigua CMW5287-DRV1: Lane 10: D. ambigua CMW5287-WT. The RT-PCR products were separated on a 1% agarose gel

(CMW5287-DRV1) produced mycelium that resembled that of the naturally-infected D. perjuncta isolate (CMW3407). The mycelium was fluffy and did not produce the pinkish-yellow colour that develops as cultures of this fungus become old. The isogenic virus-free (CMW5287-WT) isolate produced fluffy mycelium that aggregated in tufts and produced the pinkish-yellow pigment. This isolate produced black perithecia after prolonged growth on PDA plates.

The naturally-infected D. perjuncta (CMW3407) isolate had the slowest growth rate of all the isolates at 25°C (Fig. 2). The virus did not result in significant differences in growth rate of the transfected (CMW5588-DRV1) and corresponding isogenic virus-free (CMW5588) isolate of the Phomopsis sp. The same situation was observed for the transfected (CMW5587-DRV1 and CMW5287-DRV1) and the isogenic virus-free isolates (CMW5587-WT and CMW5287-WT) of D. ambigua. However, DRV1 reduced the growth rate of one isolate of D. ambigua (CMW5288-DRV1).

No necrosis of the bark was observed on any of the inoculated twigs after one month. When the bark was removed to expose the cambium, brownish discoloration associated with the inoculations was observed. There were significant differences in pathogenicity of the isolates ($p < 0.05$) (Fig. 3). Diaporthe perjuncta (CMW3407) was the least pathogenic isolate. The DRV1-transfected isolates were associated with significantly smaller lesions than the corresponding isogenic virus-free isolates ($p < 0.05$). The DRV1-transfected Phomopsis sp. (CMW5588-DRV1) formed lesions that were 32% smaller than those formed by the corresponding isogenic virus-free isolate. The DRV1-transfected D. ambigua (CMW5288-DRV1) isolate gave rise to lesions that were 12% smaller that those associated with the isogenic virus-free isolate. The D. ambigua isolates CMW5287-DRV1 and CMW5587-DRV1 were associated with lesions that were 51% and 59% smaller than those associated with the isogenic wild-type isolates CMW5287-WT and CMW5587-WT, respectively.

An RT-PCR product of approximately 600 bp was amplified from the total nucleic acids of the DRV1-transfected isolates that were re-isolated from the inoculated twigs. In contrast, there was no amplification from the total nucleic acids of the wild-type isolates (data not shown).
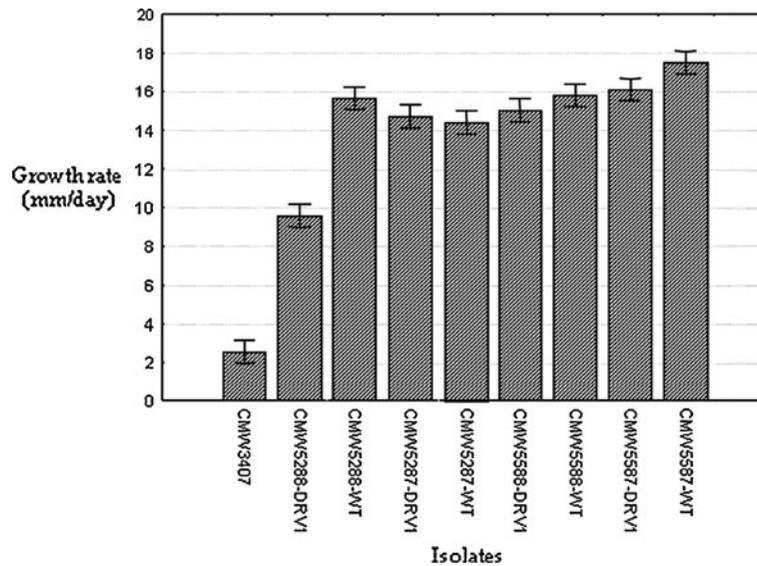
Fig. 2 Comparison of the effect of DRV1 on the growth rate (mm/day) of the transfected and isogenic virus-free isolates. The growth rate of the naturally-infected *D. perjuncta* (CMW3407) isolate is also included. The fungi are: the DRV1-transfected *D. ambigua* isolates (CMW5288-DRV1, CMW5287-DRV1), the DRV1-transfected *Phomopsis* sp. (CMW5588-DRV1), the DRV1-transfected *D. ambigua* isolate (CMW5587-DRV1) and the corresponding isogenic virus-free isolates (CMW5288-WT, CMW5287-WT, CMW5588-WT and CMW5587-WT)

All the DRV1-transfected *D. ambigua* isolates failed to produce ascopores while the isogenic virus-free isolates produced fertile ascospores. In contrast, both DRV1-transfected and isogenic virus-free *Phomopsis* sp. isolates gave rise to pycnidia producing conidia. RT-PCR with Oli64 and Oli80 performed on total nucleic acid preparations from fifty single-conidia cultures and five mass spore cultures of the transfected *Phomopsis* sp. (CMW5588-DRV1) isolate did not result in the amplification of the characteristic 600 bp product.

Discussion

In this study, three different isolates of *D. ambigua* and an unidentified *Phomopsis* asexual state of a

Fig. 3 Comparison of lesion sizes on apple twigs caused by the naturally-infected *D. perjuncta* isolate (CMW3407), the DRV1-transfected *D. ambigua* isolates (CMW5288-DRV1, CMW5287-DRV1), the DRV1-transfected *Phomopsis* sp. (CMW5588-DRV1), the DRV1-transfected *D. ambigua* isolate (CMW5587-DRV1) and the corresponding isogenic virus-free isolates (CMW5288-WT, CMW5287-WT, CMW5588-WT and CMW5587-WT)
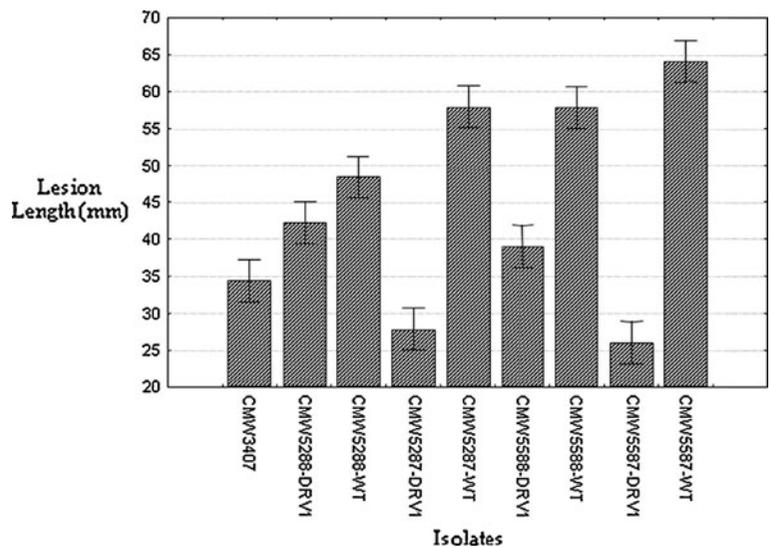
Diaporthe sp. were transfected with DRV1. The virus-infected isolates thought to be *D. ambigua* are in fact *D. perjuncta*. Therefore, the designation of the naturally-occurring virus as DaRV is no longer valid, and the virus infecting *D. perjuncta* is designated DRV1. DRV1 is not known to occur naturally in both *D. ambigua* and *Phomopsis*. DRV1, a positive-strand RNA virus isolated from *D. perjuncta* was used to transfect a virus-free *D. perjuncta* isolate (Moleleki et al. 2003). In that study, the virus did not significantly reduce the pathogenicity of the transfected isolate.

Phomopsis sp. and *Diaporthe* spp. are important pathogens in Japanese orchards where they cause canker and rot diseases of peach and grapevine (Kanematsu et al. 1999; Kajitani and Kanematsu 2000). Phomopsis canker is controlled by surgical removal of cankers followed by local application of fungicides (Sasaki et al. 2002). Thus, the development of a transfection protocol for these fungi may promote opportunities for biological control of these diseases.

Previously, we have shown that the vector sequences at both ends of DRV1 introduced by the cDNA construct are trimmed off the replicating viral RNA in the transfected isolate (Moleleki et al. 2003). The same phenomenon was observed in this study. This confirms the suggestion that during initiation of replication, the RNA-dependent RNA polymerase of DRV1 specifically recognizes the ends of DRV1 (Moleleki et al. 2003). In an earlier study, it was also shown that vector sequences are trimmed from the hypovirus pre-mRNA during its trafficking from the nucleus to the cytoplasm (Chen et al. 1994). However, in the study of Chen et al. (1994), the hypovirus was transformed into the new host as a plasmid construct.

DRV1-transfected *D. ambigua* isolates in this study had different morphology to those of the isogenic virus-free isolates (data not shown). In *C. parasitica*, it is known that the different phenotypic changes associated with the hypovirus infection are a result of perturbation of gene expression (Choi and Nuss 1992). By constructing different chimeric viruses from CHV1-EP713 and CHV1-Euro7, Chen et al. (2000) showed that differences in fungal colony morphology were encoded by specific regions on the hypovirus genome. Thus, it would be interesting to consider whether the differences in morphology between the DRV1-transfected and DRV1-free isolates

of *D. ambigua* are a result of specific regions on the genome of DRV1.

Two of the transfected *D. ambigua* isolates (CMW5587-DRV1 and CMW5287-DRV1) did not produce the pinkish-yellow pigmentation that is produced by aging wild-type isolates. One of the transfected isolates (CMW5288-DRV1) displayed reduced production of this pigment. Changes in pigmentation of virus-transfected isolates of other fungi have been recorded. For example, CHV1-EP713-free isolates of *C. parasitica* are known to produce orange, yellow and red pigments but the CHV1-EP713-infected isolates display reduced pigment production (Chen et al. 1996). Similarly, isolates of the *Eucalyptus* pathogen *C.cubensis* transfected with the hypovirus, but not the isogenic wild-type isolates, are known to produce bright yellow pigments (Chen et al. 1996; van Heerden et al. 2001).

DRV1 repressed the growth of one isolate (CMW5288-DRV1) of *D. ambigua* while the growth rates of two other isolates of this fungus and that of *Phomopsis* sp. were not affected by DRV1. The repression of growth by DRV1 was also shown in a previous study where DRV1 reduced the growth rate of a transfected *D. perjuncta* isolate (Moleleki et al. 2003). A well-known effect of the hypovirus on *C. parasitica* is that it reduces the growth of the fungus (Chen et al. 1996). Pathogenicity studies on apple twigs also showed that the transfected isolates had reduced aggressiveness. However, additional transfection studies and pathogenicity tests are required to understand the potential value of DRV1 as a biological control agent.

Successful use of mycoviruses to control plant pathogenic fungi depends on several factors. One of the most important of these is that the virus must reduce pathogenicity of the fungus, while not reducing its ecological fitness (MacDonald and Fulbright 1991). DRV1 does not adversely affect the growth rate of transfected hosts, thus making it an ideal candidate for use in biological control. However, the virus apparently results in loss of the production of the sexual state in the *Diaporthe* spp. and the virus is lost during the production of conidia. Reduction in sporulation and loss of DRV1 from single conidial cultures of transfected fungal isolates would greatly compromise potential use of a hypovirulence-based biological control strategy (Anagnostakis 1977).

Infectious cDNA clones have been developed for four mycoviruses, CHV1-EP713, CHV1-Euro7, DRV1 and 23S RNA virus of *Saccharomyces cerevisiae* (Choi and Nuss 1992; Chen and Nuss 1999; Moleleki et al. 2003; Esteban and Fujimura 2003; Nuss 2005). All these mycoviruses share a few characters in common. Firstly, there are no true virions associated with these viruses. Secondly, all these are positive sense RNA viruses. Thus in vitro produced ssRNA representing the positive strand of the full-length virus is sufficient to initiate infection of a new host. Therefore, we can speculate that transfection is successful fundamentally because these viruses are positive-sense RNA viruses. Our laboratory has sequenced the genomes of two dsRNA viruses, *Sphaeropsis sapinea RNA virus 1* (SsRV1) and *Sphaeropsis sapinea RNA virus 2* (SsRV2). Full-length cDNA clones of these viruses were developed together with the cDNA clone of DRV1. We have attempted transfection of different *Diplodia pinea* (formerly *Sphaeropsis sapinea*) isolates with both SsRV1 and SsRV2. More than one hundred attempts under different conditions failed (Moleleki, unpublished results). This could explain why there are no other reports of successful fungal transfection with mycoviruses belonging to families Totiviridae and Partitiviridae. Both these mycoviruses are totiviruses (Preisig et al. 1998). However, it has to be noted that successful infections with mycoviruses with rigid virus particles have been achieved using mycoviruses from families *Reoviridae* (Hillman et al. 2004; Sasaki et al. 2007; Kanematsu et al. 2010) and *Partitiviridae* (Sasaki et al. 2006; Kanematsu et al. 2010). In these studies, infection was achieved by inoculating fungal protoplasts with virus particles.

We have attempted to transfect different isolates of *C. cubensis* with DRV1. These attempts did not result in successful transfections. This could be due to the fact that DRV1 is specific to the genus *Diaporthe*. In previous studies, CHV1-EP714 has been successfully transfected into sphaeroplasts of *C. parasitica*, *C. radicalis*, *C. cubensis* and *Endothia gyrosa* (Chen et al. 1996; van Heerden et al. 2001; Nuss 2005). A deletion mutant of the hypovirus could be transfected into the sphaeroplasts of the above-mentioned fungi with the exception of those of *C. cubensis* (Chen et al. 1996).

The development of a cDNA of DRV1 for transfection studies should allow for better understanding of mechanisms underlying fungal pathogenesis and aid efforts to develop biological control of plant pathogenic fungi using mycoviruses. Such studies will include construction of recombinant DRV1 by mutation of targeted regions on its cDNA, in order to optimize its interaction with the host. In addition, the availability of this cDNA will aid investigations considering DRV1 replication, transcription, and translation.

References

Anagnostakis, S. L. (1977). Vegetative incompatibility in *Endothia parasitica*. *Experimental Mycology, 1*, 306–316.

Castanho, B., Butler, E. E., & Sheperd, R. J. (1978). The association of double-stranded RNA with Rhizoctonia decline. *Phytopathology, 68*, 1515–1519.

Chen, B., & Nuss, D. L. (1999). Infectious cDNA clone of hypovirus CHV1-Euro7: a comparative virology approach to investigate virus-mediated hypovirulence of the chestnut blight fungus *Cryphonectria parasitica*. *Journal of Virology, 73*, 985–992.

Chen, B., Craven, M. G., Choi, G. H., & Nuss, D. L. (1994). cDNA-derived hypovirus RNA in transformed chestnut blight fungus is spliced and trimmed of vector nucleotides. *Virology, 202*, 441–448.

Chen, B., Chen, C.-H., Bowman, B. H., & Nuss, D. L. (1996). Phenotypic changes associated with wild-type and mutant hypovirus RNA transfection of plant pathogeneic fungi phylogenetically related to *Cryphonectria parasitica*. *Phytopathology, 86*, 301–310.

Chen, B., Geletka, L. M., & Nuss, D. L. (2000). Using chimeric hypoviruses to fine-tune the interaction between a pathogenic fungus and its plant host. *Journal of Virology, 74*, 7562–7567.

Choi, G. H., & Nuss, D. L. (1992). A viral gene confers hypovirulence-associated traits to chestnut blight fungus. *The EMBO Journal, 11*, 473–477.

Chu, Y.-M., Jeon, J.-J., Yea, J.-S., Kim, Y.-H., Yun, S.-H., Lee, Y.-W., et al. (2002). Double-stranded RNA mycovirus from *Fusarium graminearum*. *Applied and Environmental Microbiology, 68*, 2529–2534.

Compel, P., Pappi, I., Bibo, M., Fekete, C., & Hornok, L. (1999). Genetic interrelationships and genome organization of double-stranded RNA elements in *Fusarium poae*. *Virus Genes, 18*, 49–56.

Dawe, A. L., & Nuss, D. L. (2001). Hypoviruses and chestnut blight: exploiting viruses to understand and modulate fungal pathogenesis. *Annual Review of Genetics, 35*, 1–29.

Esteban, R., & Fujimura, T. (2003). Launching the yeast 23S RNA Narnavirus shows 5' and 3' cis-acting signals for replication. *Proceedings of the National Academy of Sciences of the United States of America, 100*, 2568–2573.

Frohman, M. A. (1994). On beyond RACE (rapid amplification of cDNA ends). *PCR Methods and Applications, 4*, S40–S58.

Ghabrial, S. A., & Suzuki, N. (2009). Viruses of plant pathogenic fungi. *Annual Review of Phytopathology, 47*, 353–384.

Hillman, B. I., Supyani, S., Kondo, H., & Suzuki, N. (2004). A reovirus of the fungus *Cryphonectria parasitica* that is infectious as particles and related to the *Coltivirus* genus of animal pathogens. *Journal of Virology, 78*, 892–898.

Hong, Y., Dover, S. L., Cole, T. E., Brasier, C. M., & Buck, K. W. (1999). Multiple mitochondrial viruses in an isolate of the Dutch elm disease fungus *Ophiostoma novo-ulmi*. *Virology, 258*, 118–127.

Howitt, R. L. J., Beever, R. E., Pearson, M. N., & Forster, R. L. S. (2006). Genome characterization of a flexuous rod-shaped mycovirus, Botrytis virus X, reveals high amino acid identity to genes from plant 'potex-like' viruses. *Archives of Virology, 151*, 563–579.

Kajitani, Y., & Kanematsu, S. (2000). *Diaporthe kyushuensis* sp. nov., the teleomorph of the causal fungus of grapevine swelling arm in Japan, and its anamorph *Phomopsis vitimegaspora*. *Mycoscience, 41*, 111–114.

Kanematsu, S., Kobayashi, T., Kudo, A., & Ohtsu, Y. (1999). Conidial morphology, pathogenicity and cultural characteristics of *Phomopsis* isolates from peach, Japanese pear and apple in Japan. *Annals of the Phytopathological Society of Japan, 65*, 264–273.

Kanematsu, S., Sasaki, A., Onoue, M., Oikawa, Y., & Ito, T. (2010). Extending the fungal host range of a partitivirus and a mycoreovirus from *Rosellinia necatrix* by inoculation of protoplasts with virus particles. *Phytopathology, 100*, 922–930.

Kwon, S. J., Lim, W. S., Park, S. H., Park, M. R., & Kim, K. H. (2007). Molecular characterization of a dsRNA mycovirus, Fusarium graminearum virus-DK21, which is phylogentically related to hypoviruses but has a genome organization and gene expression strategy resembling those of plant potex-like viruses. *Molecules and Cells, 23*, 304–315.

Liu, H., Fu, Y., Jiang, D., Li, G., Xie, J., Peng, Y., et al. (2009). A novel mycovirus that is related to the human pathogen *Hepatitis E virus* and rubi-like viruses. *Journal of Virology, 83*, 1981–1991.

MacDonald, W. L., & Fulbright, D. W. (1991). Biological control of chestnut blight: use and limitations of transmissible hypovirulence. *Plant Disease, 75*, 656–661.

Moleleki, N., Preisig, O., Wingfield, M. J., Crous, P. W., & Wingfield, B. D. (2002). PCR-RFLP and sequence data delineate three *Diaporthe* species associated with stone and pome fruit trees in South Africa. *European Journal of Plant Pathology, 108*, 909–912.

Moleleki, N., van Heerden, S. W., Wingfield, M. J., Wingfield, B. D., & Preisig, O. (2003). Transfection of *Diaporthe*

perjuncta with *Diaporthe RNA virus*. *Applied and Environmental Microbiology, 69*, 3952–3956.

Nogawa, M., Kageyama, S. T., & Okazaki, M. (1993). A double-stranded RNA mycovirus from the plant pathogenic fungus *Fusarium solani f.* sp. *robiniae*. *FEMS Microbiology Letters, 110*, 153–158.

Nuss, D. L. (2000). Hypovirulence and chestnut blight: from the field to the laboratory and back. In J. W. Kronstad (Ed.), *Fungal pathology* (pp. 149–170). The Netherlands: Kluwer.

Nuss, D. L. (2005). Hypovirulence: mycoviruses at the Fungal-plant interface. *Nature Reviews. Microbiology, 3*, 632–642.

Preisig, O., Wingfield, B. D., & Wingfield, M. J. (1998). Coinfection of a fungal pathogen by two distinct double-stranded RNA viruses. *Virology, 252*, 399–406.

Preisig, O., Moleleki, N., Smit, W. A., Wingfield, B. D., & Wingfield, M. J. (2000). A novel RNA mycovirus in a hypovirulent isolate of the plant pathogen *Diaporthe ambigua*. *The Journal of General Virology, 81*, 3107–3114.

Sanderlin, R. S., & Ghabrial, S. A. (1978). Physicochemical properties of two distinct types of virus-like particles from *Helminthosporium victoriae*. *Virology, 87*, 142–151.

Sasaki, A., Onoue, M., Kanematsu, S., Suzaki, K., Miyanishi, M., Suzuki, N., et al. (2002). Extending chestnut blight hypovirus host range within Diaporthales by biolistic delivery of viral cDNA. *Molecular Plant-Microbe Interactions, 15*, 780–789.

Sasaki, A., Kanematsu, S., Onoue, M., Oyama, Y., & Yoshida, K. (2006). Infection of *Rosellinia necatrix* with purified viral particles of a member of *Partitiviridae* (RnPV1-W8). *Archives of Virology, 151*, 697–707.

Sasaki, A., Kanematsu, S., Onoue, M., Oikawa, Y., Nakamura, H., & Yoshida, K. (2007). Artificial infection of *Rosellinia necatrix* with purified viral particles of a member of the genus *Mycoreovirus* reveals its uneven distribution in single colonies. *Phytopathology, 97*, 278–286.

Smit, W. A., Wingfield, B. D., & Wingfield, M. J. (1996). Reduction of laccase activity and other hypovirulence-associated traits in dsRNA-containing strains of *Diaporthe ambigua*. *Phytopathology, 86*, 1311–1316.

Valverde, R. A., Nameth, S. T., & Jordan, R. L. (1990). Analysis of double-stranded RNA for plant virus diagnostics. *Plant Disease, 74*, 255–258.

van Heerden, S. W., Geletka, L. M., Preisig, O., Nuss, D. L., Wingfield, B. D., & Wingfield, M. J. (2001). Characterization of South African *Cryphonectria cubensis* isolates infected with a *C. parasitica* hypovirus. *Phytopathology, 91*, 628–632.

Xie, J., Wei, D., Jiang, D., Fu, Y., Li, G., Ghabrial, S., et al. (2006). Characterization of debilitation-associated mycovirus infecting the plant-pathogeneic fungus *Sclerotinia sclerotiorum*. *The Journal of General Virology, 87*, 241–249.

Yu, H. J., Lim, D., & Lee, H. S. (2003). Characterization of a novel single-stranded RNA mycovirus in pleurotus ostreatus. *Virology, 314*, 9–15.