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Transfection of *Diaporthe perjuncta* with *Diaporthe RNA Virus*

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***Diaporthe perjuncta* is a pathogen of grapevines worldwide. A positive-strand RNA virus, *Diaporthe RNA virus* (DaRV), occurs in hypovirulent isolates of this fungus. A virus-free isolate from a South African grapevine was transfected with in vitro-transcribed positive strands of DaRV. Based on reverse transcription-PCR and partial sequence analysis, the transfected virus was identified as DaRV. The in vitro-transcribed RNA transcripts used to transfect fungal spheroplasts contained parts of the vector at their distal ends. These vector sequences were separated from the DaRV genome during replication in the new host. The transfected isolate had morphological features that differed from those of the isogenic virus-free strain, including production of a yellow pigment, a decreased growth rate, and lack of sporulation. An apple-based pathogenicity test did not reveal any differences in virulence between the virus-free and DaRV-transfected isolates. This study showed that virus-free fungal hosts can be successfully transfected with viruses other than the *Cryphonectria parasitica* hypovirus.**

Mycoviruses from many species of fungi have been observed and characterized at the genomic level (6, 22, 30). The fungal hosts include several well-known plant pathogens, including *Cryphonectria parasitica* (17, 19, 20, 35), *Ophiostoma novo-ulmi* (4, 5, 14, 23, 24, 25), *Helminthosporium victoriae* (34), *Sphaeropsis sapinea* (32, 38), and *Diaporthe perjuncta* (28, 31, 37). In *C. parasitica*, the viruses CHV1-EP713 and CHV1-Euro7 mediate hypovirulence to various degrees (7, 11, 13). Vegetative compatibility groups restrict the spread of such viruses and have reduced the success of biological control through hypovirulence in the United States (1, 2, 3, 26).

The interest in viruses that infect fungi is strongly linked to the potential use of these viruses as biological control agents of plant-pathogenic fungi. An emerging area of interest is the use of mycoviruses to study basic genetic processes that lead to fungal pathogenesis (29). The fungal reaction, commonly manifested as hypovirulence-associated traits, is not a general response of the fungus to hypovirus infection but rather is a response triggered by molecular cues encoded by specific viral sequences (10, 12). Reverse genetics developed for the *C. parasitica* hypovirus has shown that specific mutations and deletions of specific regions of the hypovirus are associated with specific responses in the pathogen. For example, deletion of the papain-like protease, p29, from CHV1-EP713 encoded by open reading frame A restores orange pigmentation and moderately increases sporulation in transfected isolates (15).

Although several mycoviral genomes have been sequenced and characterized, only the *C. parasitica* hypoviruses have been used in transfection and transformation studies (16). Thus, the progress in developing mycoviruses as possible biological control agents has been slow. In order to advance the field further, the reverse genetics developed for *C. parasitica* hypoviruses must be extended to mycoviruses with properties different from those of *C. parasitica* hypoviruses. Furthermore, development of efficient transfection protocols would be useful for studying the life cycles and mechanisms of replication of mycoviruses.

Diaporthe spp. include important pathogens of stone fruits, pome fruits, and grapevines worldwide (27, 36, 37). One of these species, *D. perjuncta*, is an important pathogen of grapevines (27). Isolates of this pathogen have various morphologies and virulence levels. A double-stranded RNA (dsRNA) virus has been recovered from slowly growing, nonsporulating, and hypovirulent strains of this fungus (37). These strains also have hypovirulence-associated traits, such as reduced laccase activity (37). The dsRNA from an infected isolate has been sequenced and characterized and is known as *Diaporthe RNA virus* (DaRV) (28, 31).

The taxonomy of *Diaporthe* spp. is poorly developed, and identification of species is difficult. The hypovirulent *Diaporthe* sp. that is now known as *D. perjuncta* and was the subject of the present study was initially identified incorrectly as *Diaporthe-ambigua*. Recently, however, DNA sequences of the internally transcribed spacer regions of the ribosomal DNA repeat of isolates of this fungus were used to determine that the isolates are *D. perjuncta* and not *D. ambigua* (28).

Our objective in this study was to determine the effects of DaRV on a host following transfection of a virus-free isolate of *D. perjuncta*. We think that DaRV might potentially be developed as a biological control agent for *Diaporthe* canker. This study is the first transfection study of an RNA virus of a filamentous fungus other than the *C. parasitica* hypoviruses.

MATERIALS AND METHODS

Fungal isolates and culture conditions. The fungal isolates used in this study are maintained as fresh cultures in the culture collection of the Forestry and Agricultural Biotechnology Institute. *D. perjuncta* isolates CMW3407, CMW8597-WT, and CMW8597-DaRV were maintained on 2% potato dextrose agar (PDA) (Biolab, Midrand, South Africa) (28).

In vitro transcription of DaRV. A full-length cDNA copy of the genome of DaRV was cloned as pDV3 in the pGEM-T Easy vector (Promega, Madison, Wis.) as described by Preisig et al. (31). Positive-strand RNA was synthesized from *Sal*I-linearized pDV3 by using T7 RNA polymerase (Roche Molecular

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Biochemicals, Mannheim, Germany) in the presence of a ribonucleoside triphosphate (Roche Molecular Biochemicals) mixture containing ATP, CTP, GTP, and UTP. The reaction mixture contained ca. 150 ng of linearized plasmid DNA, each nucleoside triphosphate at a concentration of 1 mM, 1× transcription buffer, 10 U of T7 RNA polymerase, and 12 U of RNase inhibitor. The reaction was performed for 2 h at 30°C. In vitro transcription products were analyzed on a 1% agarose gel.

Preparation of fungal spheroplasts. Spheroplasts were prepared by the method of Royer and Yamashiro (34), with minor modifications. Ten-day-old mycelium was suspended in a mixture of chitinase (Fluka, Buchs, Switzerland) and cellulase (Sigma, St. Louis, Mo.) dissolved in 1 M $MgSO_4 \cdot 7H_2O$ overnight at room temperature. The mycelium was gravity filtered through a 120-μm-pore-size nylon mesh (Swiss Silk Bolting Cloth Manufacturing Co. Ltd., Zurich, Switzerland). An equal volume of 1 M sorbitol (Sigma) was added to the spheroplasts and centrifuged at 2,700 × *g* for 5 min at 4°C. The resulting pellet was washed with ice-cold sorbitol, resuspended in 500 μl of STC (1 M sorbitol, 50 mM Tris HCl [pH 8], 50 mM $CaCl_2$), and centrifuged as described above. The spheroplasts were resuspended in a solution containing STC, PTC (40% polyethylene glycol 6000, 50 mM Tris HCl [pH 8], 50 mM $CaCl_2$), and dimethyl sulfoxide (80:20:1) and used immediately or stored at −80°C.

Transfection of fungal spheroplasts with in vitro-produced viral RNA. Freshly prepared fungal spheroplasts were transfected by electroporation with a multiporator (Eppendorf, Hamburg, Germany) as described by Chen et al. (8), with minor modifications. Spheroplasts were resuspended in 85 μl of ice-cold 1 M sorbitol and placed on ice. RNase inhibitor (12 U) was added to a 15-μl transcription reaction mixture containing the in vitro-produced positive-strand viral RNA transcripts. The viral transcripts were added to the spheroplast suspension, and the suspension was placed on ice for 5 min. The spheroplast-RNA suspension was transferred to a prechilled, 1-mm-gap-width, 100-μl electroporation cuvette (Eppendorf) and then pulsed three times at 1.8 kV. A 750-μl portion of ice-cold 1 M sorbitol was added to the cuvette, which was placed on ice for 10 min. Portions (200 μl) of the transfected spheroplasts were pipetted into 90-mm-diameter petri dishes. Regeneration medium (1 g of casein hydrolysate per liter, 1 g of yeast extract per liter, 342 g of sucrose per liter, 16 g of agar per liter) at 48°C was added to each petri dish around the spheroplasts from the edge to the center. The petri dishes were placed on a laminar flow bench until the agar solidified. The petri dishes were sealed with Parafilm (American National Can, Chicago, Ill.), transferred to a bench top, and left there overnight. Beginning the next morning, the dishes were incubated at 25°C for 48 h, and this was followed by incubation for 5 to 10 days on a bench top at room temperature (20 to 25°C). Blocks of agar were cut arbitrarily from the edges of each culture and placed close together on PDA plates.

Detection of DaRV by RT-PCR. dsRNA was extracted and purified by CF11 cellulose (Whatman, Maidstone, England) column chromatography as described by Valverde et al. (39), with minor modifications. The isolated dsRNA or total nucleic acids from the naturally infected and DaRV-transfected fungal isolates were used for reverse transcription (RT)-PCR performed with DaRV-specific primers Oli64 (5′-GTCGCATCTCACAGCCGAGCG-3′) and Oli80 (5′-CTCA CCAGCCTCCAACCG-3′) as described by Preisig et al. (31). These primers amplify a ca. 600-bp product. Sequencing of RT-PCR products was performed by using an ABI PRISM BigDye terminator v3.0 cycle sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.). The elongation products from the sequencing reactions were separated and analyzed with an ABI PRISM 3100 DNA sequencer (Applied Biosystems).

Determination of the sequences of the distal ends of the viral genome. The distal ends of DaRV RNA isolated from transfected fungi were amplified by the 5′-3′ rapid amplification of cDNA ends (RACE) approach by using a 5′/3′ RACE kit (Roche Molecular Biochemicals) (21). The 5′ and 3′ ends of DaRV were reverse transcribed by using avian myeloblastosis virus reverse transcriptase with primers Oli73 (5′-GTGCCCTGCACAAACAACTC-3′) and Oli75 (5′-TC CATCTCACCGGAGCGGCAG-3′), respectively. A poly(A) tail was added to the cDNA product by using terminal transferase in the presence of 0.2 mM dATP. A nested PCR was performed with an oligo(dT) primer and either Oli78 (5′-CCTGGGTGACGGTTGTTACAC-3′) for the 5′ end or Oli81 (5′-TTGAA CGATGGGTGTAGGTGG-3′) for the 3′ end. The PCR products were cloned in the pGEM T-Easy vector (Promega), and the plasmid inserts were sequenced and analyzed as described above.

Growth rates of fungal isolates. Mycelial plugs of *D. perjuncta* CMW3407, CMW8597-WT, and CMW8597-DaRV (diameter, 5 mm) from the edges of actively growing 1-week-old fungal isolates were transferred to 10 2% PDA plates. The plates were incubated in the dark at 25°C. Colony diameters were measured 2, 3, 4, and 5 days after inoculation. The colony diameters were used to calculate the radii of the colonies. The radii of the colonies were used to estimate the areas covered by the fungal colonies (area = πr^2, where r is the radius). The mean growth rates of the fungal isolates were determined and analyzed by using a one-way analysis of variance (ANOVA) (SYSTAT, version 7.0.1; SPSS Inc., Chicago, Ill.). The entire trial was repeated once, and the correlation between the results of the two trials was determined by a Pearson correlation test.

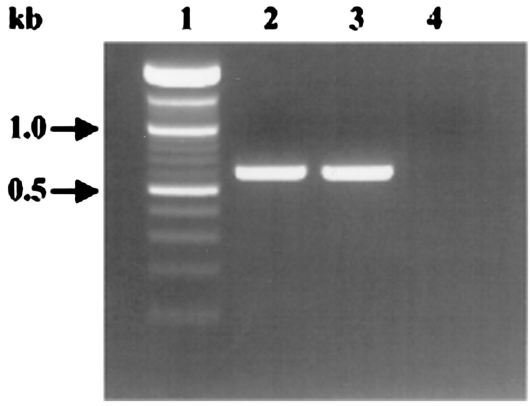

FIG. 1. Confirmation of the presence of DaRV. Lane 1, molecular weight marker (100-bp ladder); lane 2, CMW3407; lane 3, CMW8597-DaRV; lane 4, CMW8597-WT. The bands were separated on a 2% agarose gel stained with ethidium bromide.

Pathogenicity tests with apples. The pathogenicities of *D. perjuncta* isolates CMW3407, CMW8597-WT, and CMW8597-DaRV were compared by using an apple-based test (18). 'Golden Delicious' apples at room temperature were surface disinfected with 70% ethanol prior to inoculation. Holes that were approximately 15 mm deep were punched in the apples with a 5-mm-diameter cork borer. For each strain 10 apples were inoculated with similar-size disks of mycelia, and the inoculation sites were covered with masking tape to reduce desiccation. Sterile uninoculated PDA disks were placed in 10 apples and served as negative controls. A dead culture was not used as a negative control as it would not have been possible to identify toxicity due to small molecules, including toxins and proteins excreted into the medium that are not associated with continued growth of the fungus. After 10 days, the masking tape was removed from the inoculated apples, and the diameters of the necrotic lesions were measured. The areas covered by the circular lesions on apple surfaces were estimated as described above for estimating the areas covered by fungal colonies. The data were analyzed by using a one-way ANOVA (SYSTAT, version 7.0.1; SPSS Inc.). The entire trial was repeated once. A Pearson correlation test was performed for the results of the two experiments.

Virus transfer to ascospores. Isolates were induced to produce ascospores as described by Smit et al. (37, 38). After sporulation, single-ascospore cultures were prepared. The presence of DaRV was tested by using both CF11 cellulose column chromatography and RT-PCR with the DaRV sequence-derived primers Oli64 and Oli80.

RESULTS

Transfection of spheroplasts with in vitro-produced viral RNA. *D. perjuncta* isolate CMW8597-WT was successfully transfected with DaRV. dsRNA was isolated from the transfected isolate (CMW8597-DaRV) but not from the isogenic virus-free isolate (CMW8597-WT). RT-PCR performed with DaRV-specific primers Oli64 and Oli80 amplified 600-bp products from the dsRNA samples and total nucleic acids isolated from the transfected and naturally infected isolates (Fig. 1).

Sequences of the distal ends of the viral genome from the transfected fungus. Linearization of pDV3 with *Sal*I for in vitro production of positive-strand RNA added 35 vector-derived nucleotides to the 3′ end of DaRV. The 5′ end of the virus had 61 vector-derived nucleotides since T7 RNA poly-

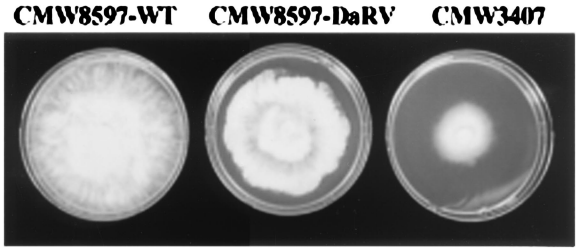

FIG. 2. Colonies of the isogenic virus-free (CMW8597-WT), DaRV-transfected (CMW8597-DaRV), and naturally infected (CMW3407) isolates of *D. perjuncta* grown on PDA plates. The transfected isolate grew more slowly than the isogenic virus-free isolate and had fluffy yellow aerial mycelia, while the isogenic virus-free isolate had compressed white mycelia.

merase initiates transcription 61 nucleotides from the DaRV cDNA insert. RT-PCR products amplified from both ends of DaRV by using the 5′/3′ RACE approach showed that there were no vector-derived nucleotides at either end of the DaRV genome in the transfected isolate (CMW8597-DaRV).

Phenotypic changes caused by DaRV. Transfection resulted in slight morphological changes to isolate CMW8597-DaRV. The transfected isolate grew in sectors that formed rings of raised mycelium on PDA plates, while the isogenic virus-free fungus grew evenly on the plates (Fig. 2). The mycelium of the transfected isolate had more aerial growth and a fluffier appearance than the mycelium of the isogenic virus-free fungus. Furthermore, the mycelium of the DaRV-transfected isolate produced a yellow pigment on PDA plates.

Growth rates in culture. There were significant differences among the growth rates of the DaRV-transfected, isogenic virus-free, and naturally infected isolates in both replicates of the experiment ($F = 390$, df = 2, and $P < 0.001$; $F = 810$, df = 2, and $P < 0.001$). The DaRV-transfected isolate grew 600 ± 12 mm^2/day (replicate 1) and 570 ± 9 mm^2/day (replicate 2). These rates were significantly lower than those of the isogenic virus-free isolate, which grew 830 ± 13 mm^2/day (replicate 1) and 900 ± 14 mm^2/day (replicate 2). The naturally infected *D. perjuncta* isolate had the lowest growth rate; its growth rate was 310 ± 14 mm^2/day in the first replicate and 270 ± 10 mm^2/day in the second replicate. There was a good correlation (98%) between the two replicates of the experiment. The Bonferroni pairwise comparison indicated that the growth rates of the three isolates used in this study differed significantly from each other.

Pathogenicity tests with 'Golden Delicious' apples. Necrotic lesions were observed on all the apples inoculated with the naturally infected (CMW3407), DaRV-transfected (CMW8597-DaRV), and isogenic virus-free (CMW8597-WT) isolates of *D. perjuncta*. There were no necrotic lesions on apples inoculated with sterile PDA blocks, which served as negative controls. The data did not meet the assumptions of ANOVA as they were too skewed, but log-transformed data were normally distributed. There were significant differences in the lesion sizes produced on the apples by the naturally infected, DaRV-transfected, and isogenic virus-free isolates used for inoculation in both the first replicate ($F = 45$, df = 3, $P < 0.001$) and the second replicate ($F = 33$, df = 3, $P < 0.001$) of the experiment (Table 1). However, a Bonferoni pairwise comparison indicated that there were no significant differences between mean lesion sizes (± standard errors of the means) for either replicate of the experiment. The Pearson correlation test showed that there was a good correlation (96%) between the two replicates of the experiment.

Virus transfer to ascospores. After 3 months, perithecia were observed on the surfaces of the apple stem pieces inoculated with the virus-free *D. perjuncta* isolate (CMW8597-WT). When examined microscopically, the perithecia were found to contain ascospores. The DaRV-transfected *D. perjuncta* isolate (CMW8597-DaRV) and the naturally infected isolate (CMW3407) produced neither ascospores nor perithecia.

DISCUSSION

We transfected an isolate of *D. perjuncta* with DaRV. Transfection of filamentous fungi with mycoviruses has been reported previously only for the mild (7) and aggressive (8, 11, 40) variants of the *C. parasitica* hypovirus. To the best of our knowledge, this is the first time that a virus of a filamentous fungus other than the *C. parasitica* hypoviruses has been used to transfect spheroplasts derived from a filamentous fungus.

Using RT-PCR, we showed that the dsRNA isolated from the transfected isolate was derived from DaRV. The ends of the dsRNA elements did not contain nucleotides from the vector, which were introduced during the in vitro production of viral RNA. A similar observation was made by Chen et al. (9), who suggested that the vector-derived nucleotides are spliced from the pre-mRNA during trafficking from the nucleus to the cytoplasm. Even though hypovirus replication was initiated from a plasmid construct integrated into the fungal genome, it is possible that the same mechanism operates on DaRV RNA in the cytoplasm of *D. perjuncta* after transfection. Alternatively, the viral RNA-dependent RNA polymerase might specifically recognize the ends of DaRV during the initiation of replication.

The morphological differences between the transfected and isogenic virus-free isolates of *D. perjuncta* were not as striking as those observed in hypovirus-transfected *C. parasitica* and *Cryphonectria cubensis* isolates (8, 9, 12, 13, 40). However, the growth rate of the DaRV-transfected isolate was significantly lower than that of the isogenic virus-free isolate. One of the effects of the hypovirus on *C. parasitica*, as well as on *C. cubensis*, is that transfected isolates grow more slowly than

TABLE 1. Mean lesion sizes on 'Golden Delicious' apples after inoculation with naturally infected (CMW3407), DaRV-transfected (CMW8597-DaRV), and isogenic virus-free (CMW8597-WT) isolates of *D. perjuncta*

Isolate	Lesion size (mm^2)[a]		Significant difference[b]
	Replicate 1	Replicate 2	
CMW3407	355 ± 30	370 ± 10	b
CMW8597-DaRV	630 ± 40	1,020 ± 80	c
CMW8597-WT	600 ± 50	890 ± 120	c
Agar	113 ± 0	113 ± 0	a

[a] Lesion sizes were determined by calculating the areas covered by the necrotic lesions and are expressed as means ± standard errors of the means.

[b] Isolates with different letters differ significantly from each other, as determined by a Bonferroni pairwise comparison ($P = 0.001$).

isogenic, virus-free isolates (8, 11, 13, 40). A distinguishing feature of the DaRV-transfected isolate was that it produced a yellow pigment on PDA. The yellow color was not observed in cultures of the naturally infected isolate. The *C. parasitica* hypovirus also induces production of a bright yellow-orange pigment in transfected *C. cubensis* isolates (8, 40). The DaRV-transfected isolate also produced more abundant aerial mycelium than the wild-type isolate produced. A similar change has been observed with hypovirus-transfected *Cryphonectria havanensis* (8).

We detected no significant differences between the pathogenicity of the isogenic virus-free isolate and the pathogenicity of the DaRV-transfected isolate with an apple-based screening procedure. Since the naturally infected *D. perjuncta* isolate was hypovirulent (37), we expected the transfected isolate also to have reduced pathogenicity. Smit et al. (37) showed that when the virus was transmitted to virus-free *Diaporthe* sp. by anastomosis, recipient isolates in the same vegetative compatibility group also became hypovirulent. The difference could be due to transfer of cytoplasmic organelles and nuclear genetic information along with the virus during viral transfer by anastomosis (7). The mycovirus, in concert with these factors, might be responsible for the hypovirulence observed in the naturally infected *D. perjuncta* isolate. Alternatively, the *D. perjuncta* isolate used in transfection studies may be a weak pathogen, which would make detection of differences in pathogenicity between the DaRV-transfected and isogenic virus-free isolates more difficult.

The transfected *D. perjuncta* isolate and the naturally infected isolate did not produce ascospores, whereas the isogenic virus-free isolate produced ascospores in culture after 3 months. Thus, we could not determine if ascospore progeny carried the virus. Our results are similar to those of Chen et al. (8), who reported that transfected *C. parasitica* and *Endothia gyrosa* also failed to produce ascospores. However, *C. parasitica* sporulated when it was exposed to high-intensity light. In the same study, hypovirus-transfected *Cryphonectria radicalis* and *C. havanensis* produced ascospores, and 5 and 86% of the cultures derived from these ascospores contained the virus. The inability of the transfected *D. perjuncta* isolate to produce ascospores has a negative impact on biological control, as it reduces the number of propagules available to disseminate the virus. More than 50 attempts to cure the naturally infected *D. perjuncta* isolate with a cycloheximide procedure have failed (unpublished data). Therefore, an isogenic virus-free isolate of this isolate could not be assessed for the ability to produce ascospores.

Although some viral effects were observed, DaRV does not appear to have much potential for biological control. Since the transfected and wild-type isolates used in this study had the same genetic background, the effects observed on the transfected isolate could be ascribed to DaRV exclusively. Transfection of other mycoviruses whose complete nucleotide sequences are known should increase the chance of discovering mycoviruses that can be utilized for biological control.

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