Trends in
Plant Pathology
Vol. 1 (1997)

Mini Review

Biological control of *Diaporthe* canker: relatedness of dsRNA isolated from hypovirulent fungal strains

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ABSTRACT

Diaporthe canker of pome and stone fruit trees, caused by *Diaporthe ambigua*, is a newly recognized disease in the deciduous fruit growing areas of South Africa. A Potential biological control strategy for *Diaporthe* canker would make use of viruses or virus-like agents capable of debilitating *D. ambigua*. Hypovirulence-associated double-stranded RNA (dsRNA) was isolated from a number of different *D. ambigua* strains. In all cases a single dsRNA segment was observed. This is in contrast to the related *Cryphonectria parasitica* where it is more common to find many different sizes of dsRNA. The *D. ambigua* dsRNA segments showed similar electrophoretic mobility in agarose gels, and the size was estimated to be approximately 4.3 kb. Hybridization between the different dsRNAs showed a high degree of homology. However, no hybridization was evident between the dsRNA in *D. ambigua* and *C. parasitica* even though the hybridization stringency was decreased in an attempt to reveal possible homology. The presence of *D. ambigua* strains in a localized area, carrying a single dsRNA segment of similar size and homology suggests that natural spread of dsRNA within the *D. ambigua* population has occurred. The lack of homology between dsRNA from South African hypovirulent strains of *D. ambigua* and dsRNA from North American and European hypovirulent strains of *C. parasitica* suggests different origins.

INTRODUCTION

The presence of endogenous virus-like double-stranded RNA (dsRNA) genetic elements has been correlated with altered virulence in several plant pathogenic fungi [17]. DNA-mediated transformation studies employing full length and partial cDNA clones of the large viral dsRNA (L-dsRNA) isolated from the hypovirulent *Cryphonectria parasitica* (Murr.) Barr strain EP713, provided the first direct evidence that viral RNA is responsible for the hypovirulence phenotype [3,4].

Slow-growing strains of *Diaporthe ambigua* Nits. were recently recovered in culture that were hypovirulent on apple rootstocks in comparison with typical, virulent strains [25,26]. All hypovirulent strains of *D. ambigua* were found to contain a single dsRNA segment, and converted virulent strains of the same vegetative compatibility (VC) group [28] to hypovirulence after hyphal anastomosis. In addition to hypovirulence, the converted strains also exhibited various hypovirulence-associated traits [26]. This is in contrast to *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. where no association was detected between the presence of dsRNA and virulence, toxin production, growth rate or the activity of phenol oxidase [15].

D. ambigua is a newly reported stem canker pathogen of pome and stone fruit trees in South Africa [27] and therefore represents a system in which virus-induced hypovirulence could potentially be a useful means of biological control. Since this fungus is reasonably closely related to *C. parasitica* [13], numerous comparisons can be made between the two hypovirulence systems. Findings from one system may contribute to our understanding of hypovirulence in the other system as well.

The number and size of dsRNA segments in *C.*

parasitica varies among strains [11,18]. The dsRNA segments within a strain have been found to share sequence homology and termini [22]. However, strains may carry dsRNA segments that do not share homology [29]. A study of the interrelatedness of dsRNAs from *C. parasitica* has, for example, indicated that the virus of the North American strain NB58 is related to those of European origin (EP713; EP747), but not related to seven other dsRNAs of North American origin [11].

Knowledge of the similarities and differences among dsRNA found in *D. ambigua* and *C. parasitica* may also provide clues to the origin of the dsRNAs and their ability to spread geographically. In this review we report that cDNA from South African strains of *D. ambigua* hybridizes to dsRNA from identical as well as non-identical South African strains of *D. ambigua*. We also provide evidence that these dsRNAs have little or no similarity to *C. parasitica* dsRNAs of North American, French, and Italian origin.

SOURCE OF STRAINS

The ability of cDNA made from dsRNA from South African strains (GR20; ORR7; GR216[GR7]; ORR27[ORR17]) of *D. ambigua* to hybridize to dsRNA from South African strains (GR7; GR20; ORR7; ORR17; GR216[GR7]; GR216[GR20]; ORR27[ORR7]; ORR27[ORR17]) of *D. ambigua* and *C. parasitica* dsRNA from North American (NB58; 155[58]), French (EP713; EP802), and Italian (EP748(B); EP748(W); EP747) origin, was examined (Table 1) [24,26].

Virulent (GR216; ORR27), and hypovirulent (GR7; GR20; ORR7; ORR17) strains of *D. ambigua* were isolated [16,30,31] from M793 rootstocks in a pome and stone fruit nursery in Simondium, Cape Province, South Africa [26,27]. DsRNA-free strains of *D. ambigua* were converted to hypovirulence (GR216[GR7]; GR216[GR20]; ORR27[ORR7]; ORR27[ORR17]) [26]. Two dsRNA-containing strains (GR7; GR20), representing one VC group, were used to convert dsRNA-free strain GR216. Two dsRNA-containing strains from a different VC group (ORR7; ORR17), were concurrently used to convert dsRNA-free strain ORR27 [1,28].

Seven hypovirulent strains of *C. parasitica* were used for comparative purposes [11]. They included strain NB58, a hypovirulent dsRNA-containing field strain from New Jersey (ATCC 76220). Hypovirulent *C. parasitica* strain 155[58] was produced through transfer of dsRNA from the North American hypovirulent strain NB58 to the North American virulent strain EP155 (ATCC 38755) by anastomosis. Hypovirulent *C. parasitica* strain EP713 (ATCC 52571) was originally produced by transfer of dsRNA from the French hypovirulent strain EP113 (not shown) to the North American virulent strain EP155. Hypovirulent *C. parasitica* strain EP802 was constructed by transfer of French dsRNA from the North American hypovirulent strain EP713 to the Italian virulent strain EP67 (ATCC 38753). The virulent counterpart of *C. parasitica* strain EP747 (ATCC 52575) was also EP155, but the dsRNA was of Italian origin. Both *C. parasitica* stains EP748(B) (CMW 2362) and EP748(W) (ATCC 52572) represent Italian dsRNA in Italian *C. parasitica* background.

RELATEDNESS OF dsRNA

Banding patterns of *D. ambigua* dsRNA differed from those of *C. parasitica* dsRNA in 1% agarose electrophoresis [24,26]. A single dsRNA segment, approximately 4.3 kb in size, was isolated from all hypovirulent strains (GR7; GR20; ORR7; ORR17), as well as converted strains (GR216[GR7]; GR216[GR20]; ORR27[ORR7]; ORR27[ORR17]) of *D. ambigua* (Table 1, Fig. 1) [24,26]. The *D. ambigua* dsRNA comigrated with the 4.3 kb position on the λ DNA (*Eco* RI and *Hind* III digest) ladder. The exact size of the *D. ambigua* dsRNA has not yet been determined with RNA standards [26]. The nature of the dsRNA was confirmed by its resistance to DNase I, resistance to RNase A at high (0.3 M) sodium chloride concentration and susceptibility to RNase A at low (0.03 M) sodium chloride concentration. The number and size of dsRNA segment(s) isolated from North American and European strains of *C. parasitica* were as described by Hillman et al. [11].

Complementary DNA from dsRNA of *D. ambigua* strain GR20, when used as a probe, hybridized to itself and to all other dsRNAs isolated from *D. ambigua* strains of South African origin (GR7; ORR7; ORR17; GR216[GR7]; GR216[GR20]; ORR27[ORR7]; ORR27[ORR17]) (Tables 1 & 2) [24,26]. It did,

Table I. Strains of *Diaporthe ambigua*[1] and *Cryphonectria parasitica*[2] used in hybridization tests

Strain	Description	Origin
GR216[1]	Virulent	Simondium, Cape SA
ORR27[1]	Virulent	Simondium, Cape SA
GR7[1]	Hypovirulent	Simondium, Cape SA
GR20[1]	Hypovirulent	Simondium, Cape SA
ORR7[1]	Hypovirulent	Simondium, Cape SA
ORR17[1]	Hypovirulent	Simondium, Cape SA
GR216[GR7][1]	Converted hypovirulent	Simondium, Cape SA[a]
GR216[GR20][1]	Converted hypovirulent	Simondium, Cape SA
ORR27[ORR7][1]	Converted hypovirulent	Simondium, Cape SA
ORR27[ORR17][1]	Converted hypovirulent	Simondium, Cape SA
EP155[2]	Virulent, ATCC[g] 38755	America
NB58[2]	Hypovirulent, ATCC 76220	American dsRNA
155[58][2]	Hypovirulent	American dsRNA[c]
EP713[2]	Hypovirulent, ATCC 52571	French dsRNA[b]
EP802[2]	Hypovirulent	French dsRNA[e]
EP67[2]	Virulent, ATCC 38753	Italy
EP748(B)[2]	Hypovirulent, CMW[f] 2362	Italian dsRNA[d]
EP748(W)[2]	Hypovirulent, ATCC 52572	Italian dsRNA[d]
EP747[2]	Hypovirulent, ATCC 52575	Italian dsRNA[b]

[a] Designates a converted strain: Strain GR216 is converted by strain GR7, etc.
[b] American background (EP155, ATCC 38755)
[c] American background (EP155, ATCC 38755), with dsRNA from NB58
[d] Italian background (EP67, ATCC 38753)
[e] Italian background (EP67, ATCC 38753), with dsRNA from EP713
[f] Culture collection of M. J. Wingfield, Department of Microbiology and Biochemistry, UFS, Bloemfontein, South Africa
[g] American Type Culture Collection, Rockville, Maryland

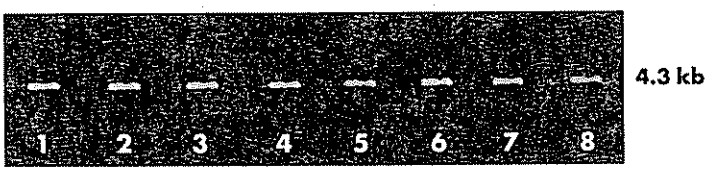

Fig. 1. Agarose gel (1%) electrophoresis of dsRNA [26] extracted from original (lanes 1-4) and converted (lanes 5-8) hypovirulent strains of *Diaporthe ambigua*. Lanes 1, GR7; 2, GR20; 3, ORR7; 4, ORR17; 5, GR216[GR7]; 6, GR216[GR20]; 7, ORR27[ORR7]; 8, ORR27[ORR17].

however, not hybridize to dsRNA from *C. parasitica* strains of North American, (NB58; 155[58]), French (EP713; EP802), or Italian (EP748(B); EP748(B); EP747) origin (Tables 1 & 2) [24,26]. Similarly, individual cDNA probes from dsRNA of *D. ambigua* strains ORR7, GR216[GR7], and ORR27[ORR17] hybridized to themselves and to all dsRNAs isolated from *D. ambigua* strains of South African origin, but did not hybridize to dsRNAs from *C. parasitica* originating from North America, France or Italy

Table II. Summary of hybridization results[a] between dsRNA from *Diaporthe ambigua* strains, and dsRNA from North American and European strains of *Cryphonectria parasitica* [24,26]

Source of dsRNA[c] used in hybridizations	Source of probe cDNA[b]				
	GR20	ORR7	GR216 [GR7]	ORR27 [ORR17]	NB58
Cape					
GR7	+	+	+	+	-
GR20	+	+	+	+	-
ORR7	+	+	+	+	-
ORR17	+	+	+	+	-
GR216[GR7]	+	+	+	+	-
GR216[GR20]	+	+	+	+	-
ORR27[ORR7]	+	+	+	+	-
ORR27[ORR17]	+	+	+	+	-
North America					
NB58	-	-	-	-	+
155[58]	-	-	-	-	+
France					
EP713	-	-	-	-	+
EP802	-	-	-	-	nd
Italy					
EP748(B)	-	-	-	-	nd
EP748(W)	-	-	-	-	nd
EP747	-	-	-	-	+

[a] Scores: + = clear hybridization, - = no hybridization, nd = not done

[b] dsRNA was denatured in 50 mM methyl mercury hydroxide for 15 min at room temperature before adding to the reaction mixture. Reaction mixtures for first strand cDNA synthesis using random oligonucleotide primers (Hexanucleotide mixture, Boehringer Mannheim), were prepared according to the method of Dickinson et al. [5]. ^{32}P-labelled cDNA was heat denatured [21]

[c] dsRNA was denatured by heating at 65°C for 5 min in 50% formamide (v/v), 6% formaldehyde (v/v), and 1 x MOPS buffer (20 mM 3-(N-morpholino) propanesulfonic acid), 5 mM sodium acetate, pH 7.0, and 1 mM EDTA). The samples were then adjusted to 5% Ficoll (MW ~ 400 000) (w/v), 5 mM EDTA, 0.004% bromophenol blue, and loaded onto denaturing gels. The gels (1% agarose; 6% formaldehyde), were pre-run for 30 min at 40 V in MOPS buffer. Lanes containing size standards were cut off the gel and stained with ethidium bromide (0.17 µg/ml). The dsRNA was transferred from the gel to Hybond-N nylon membranes (Amersham) by capillary blotting in 20 x SSC (1 x SSC= 0.15 M NaCl, 0.015 M sodium citrate). Membranes were air dried and baked at 80°C for 2 h, and prehybridized and hybridized in 50% deionized formamide, 1% blocking reagent, 5 x SSC, 0.02% SDS (sodium dodecyl sulphate), and 0.1% SLS (N-Lauroylsarcosine, Na-salt) [21]

(Tables 1 & 2) [24,26]. Complementary DNA from dsRNA of *C. parasitica* strain NB58, however, hybridized to dsRNA isolated from *C. parasitica* strains of North America (NB58; 155[58]), France (EP713), and Italy (EP747), but did not hybridize to dsRNA isolated from *D. ambigua* strains of South African origin (GR7; GR20; ORR7; ORR17; GR216[GR7]; GR216[GR20]; ORR27[ORR7]; ORR27[ORR17]) (Tables 1 & 2) [24,26].

No hybridization was evident between the dsRNA in *D. ambigua* and *C. parasitica* even though the hybridization stringency was decreased in an attempt to reveal possible homology [24,26]. No hybridization was ever observed in lanes containing extracts from *D. ambigua* strains GR216 or ORR27, and *C. parasitica*

strains EP155 or EP67, all virulent strains lacking detectable dsRNA [24,26].

CONCLUSIONS

A number of different *D. ambigua* strains from South Africa were found to contain hypovirulence-associated dsRNA. In all cases a single dsRNA segment, approximately 4.3 kb was observed in these strains [24,26]. Single dsRNA segments associated with hypovirulence have also been found in fungi such as *C. parasitica* [11,19] and *Sclerotinia sclerotiorum* (Lib.) de Bary [2]. This is in contrast to most fungal strains, including well-studied strains of *C. parasitica*, where a complexity of dsRNA banding patterns have been observed [1,6,8,10,12,14,20]. In *C. parasitica* strain EP713, combined results suggest that all genetic information of the hypovirulence-associated virus resides within the single largest dsRNA [23]. Furthermore, Shapira et al. [23] demonstrated that most of the complexity observed for dsRNAs present in hypovirulent *C. parasitica* strains is a result of the generation and maintenance of internally deleted defective forms of the single largest dsRNA.

Hybridization amongst the different single dsRNA segments from *D. ambigua* strains showed a high degree of homology [24,26]. The presence of *D. ambigua* in a localized area carrying a single dsRNA segment of similar size and homology suggests that natural spread of dsRNA within the *D. ambigua* population has occurred. These results also indicate establishment of a single size dsRNA population in the region, excluding cross-hybridization with other potential dsRNA populations [26]. An ideal opportunity appears to exist for biological control of *D. ambigua* using hypovirulence. Moreover, results of these studies could provide insight into our understanding of engineering of hypovirulence in the future.

No homology was evident between the dsRNA in *D. ambigua* strains originating from South Africa, and the dsRNA(s) in *C. parasitica* strains originating from North America, France, and Italy [24,26]. According to Paul and Fulbright [18], homology relationships among dsRNAs from different geographical areas are more complex than previously reported. Paul and Fulbright [18] as well as Enebak et al. [9] reported dsRNAs of American sampled *C. parasitica* strains that do not share homology. Despite the inability to cross-hybridize, nucleotide sequence analysis of well-studied American dsRNAs indicated phylogenetic relatedness [7]. Although dsRNAs may have ancestors in common, they could have diverged to an extent where they no longer cross-hybridize with their more distant relatives. The lack of homology between dsRNA from South African hypovirulent strains of *D. ambigua* and dsRNA from North American, French, and Italian hypovirulent strains of *C. parasitica* suggests different origins. However, the possibility of phylogenetic relatedness to some of those distant dsRNAs can not be excluded.

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