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The Movement of Viral-Like RNA Between Colonies of *Cryphonectria parasitica*

R. M. Martin and N. K. Van Alfen

Department of Biology, Utah State University, Logan 84322-5305 U.S.A.
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The viruslike dsRNA associated with hypovirulence of *Cryphonectria (Endothia) parasitica* moves between strains of the same vegetative compatibility types. To quantitate and determine the location of movement of the viral-like RNA between colonies in culture we have used colony *in situ* hybridization techniques. Virulent and hypovirulent (dsRNA-containing) strains of the fungus *C. parasitica* were inoculated in pairs on agar plates and covered with hybridization membranes at 24-hr intervals. After processing, the membranes were probed with a cDNA clone of part of the sequence of the plus-sense strand of the dsRNA. Presence of dsRNA in virulent colonies was detected between 1 and 2 days after the colonies touched. By 2 days after touching, dsRNA was detected throughout the periphery of the virulent colonies. DsRNA was always found in the highest concentration in the young peripheral mycelium. To confirm the *in situ* experiments, samples of hyphae were periodically removed along the length of paired colonies. The presence of dsRNA in these samples was assessed by dsRNA isolation and by colony morphology. The average rate of movement of the RNA over a 24-hr period was approximately 16 mm. This was three to four times faster than the colony growth rate during the same time period.

Additional keywords: chestnut blight, *Endothia parasitica*.

The filamentous fungus *Cryphonectria (Endothia) parasitica* (Murr.) Barr, the causal agent of chestnut blight, is responsible for the continuing decimation of the American chestnut tree. Hypovirulence of the fungus is associated with presence of dsRNA (Day *et al.* 1977; Fulbright 1984). Movement of the dsRNA between fungal strains occurs as a result of anastomosis of the hyphae of the two strains (Anagnostakis 1984a). This movement of the dsRNA is correlated with a change in the phenotype of the strains acquiring the dsRNA from virulent to hypovirulent. The acquisition of the dsRNA by virulent strains from hypovirulent ones is accompanied by a variety of symptoms in addition to reduced virulence, which may include changes in colony morphology and reduced numbers of pycnidia and conidia (Anagnostakis 1984a; Elliston 1985).

This transmissible hypovirulence has proven to be an effective control of chestnut blight in Europe (Anagnostakis 1984b; Van Alfen 1985; Van Alfen 1988). There has been limited success in the use of transmissible hypovirulence to control chestnut blight in North America, probably as a result of heterogenic and allelic vegetative incompatibility. With increasing numbers of differing alleles of vegetative compatibility (vc) genes, there is a decreasing transmission of hypovirulence between strains (Anagnostakis 1982; Anagnostakis 1984b).

Present address of N. K. Van Alfen: Department of Plant Pathology and Microbiology, Texas A&M University, College Station, Texas 77843-2132 U.S.A.
Present address of R. M. Martin: Department of Plant Pathology, University of California, Davis, 95616 U.S.A.

Address correspondence to N. K. Van Alfen.

The movement of dsRNA between strains is not likely to be the result of passive diffusion. As an initial step in the demonstration of the mechanisms associated with dsRNA movement between and within fungal colonies, we have determined the rate of movement of the dsRNA and identified the tissues of fungal colonies containing the greatest amounts of dsRNA. These studies were facilitated by the development of a method for the *in situ* hybridization of dsRNA in whole colonies of the fungus. Hybridization *in situ* has proven to be a useful tool in understanding developmental and tissue-specific events in a variety of plant tissues (Harris and Croy 1986; Martineau and Taylor 1986). Similar methods have recently been used to determine the location of strain-specific mtDNAs (Gobbi *et al.* 1990) and the locations of expression of mRNAs in fungal tissue (Ruiters and Wessels 1989).

MATERIALS AND METHODS

Fungal strains. The virulent, *met* strain, EP2001, was obtained from S. Anagnostakis of the Connecticut Agriculture Experiment Station, New Haven. The hypovirulent, dsRNA-containing strain, EP113, was obtained from the ATCC (38771a), Rockville, MD. Both strains are of vegetative compatibility (vc) group 10. The strains were stored on PDAmys (Hansen *et al.* 1985) slants at 4° C.

Fungal colonies. Paired inocula consisting of 3- × 3-mm agar pieces of EP113 and either 3- × 3-mm or 3- × 40-mm agar pieces of EP2001 (taken from the colony margins) were placed end to end, 1 cm apart, on 30-ml PDAmys agar plates and covered with sterile hybridization transfer membranes (NEF-978, NEN Research Products, Boston MA). The membranes were placed on the agar plates with the convex (sensitive) side up, and the plates were incubated inverted under continuous fluorescent light at 26° C until

the desired growth had occurred. As a control, EP113 and EP2001 were also inoculated at opposite ends of a plate and grown under identical conditions.

Colony growth measurements. The paired fungal colonies were grown for 24 hr before measurements began. This allowed the inocula to begin growth onto the agar. At 24 hr, a line was drawn around the periphery of the colonies with a marking pen. This was repeated at 12-hr intervals. The growth rate of the colony was determined by measuring the distance between the lines at 1-cm intervals around the colony (a total of 10 measurements). The measurement intervals used in the calculations were the distance the colonies grew each 24-hr period. Three such 24-hr intervals were measured from each plate at the 10 measurement locations, and these were averaged together. This experiment was repeated five times.

Preparation of membranes for hybridization. When fungal colonies had reached the desired growth, the membranes were peeled from the agar plates with tweezers and the excess agar was removed by scraping with a razor blade. The filters were then washed with 50 mM EDTA (pH 8.0), 2.5% (w/v) β-mercaptoethanol for 30 min, followed by a 3-hr incubation at 45° C in 50 mM sodium citrate buffer (pH 5.8), 1.0 M D-sorbitol, 2% (w/v) Novozyme 234 (NOVO Biolabs, Wilton, CT). The nucleic acid bound on the membranes was then denatured by washing in 1.5 M NaCl, 0.5 M NaOH for 5 min and rinsing in 0.5 M Tris-Cl (pH 7.5), 1.5 M NaCl for 5 min. The membranes were blotted between paper towels between each wash. After the final wash the membranes were air-dried, baked in an oven for 2 hr at 80° C, and stored in a desiccator.

Hybridization with biotin-labeled probe. Dessicated membranes were rewetted in 1% Triton X-100, blotted between Whatman 3MM paper (Whatman 3030917, Maidstone, England), and placed in hybridization bags (BRL-8278BA, BRL, Gaithersburg, MD). The membranes were then incubated overnight at 42° C in a prehybridization solution consisting of deionized formamide (45% v/v), 20× SSC (25% v/v [3.0 M NaCl, 3.0 M sodium citrate, pH 7.0]), 50× Denhart's solution (10% v/v of 0.05% Ficoll, 0.05% polyvinylpyrrolidone, 0.05% BSA pentax fraction V), sodium phosphate buffer 1.0 M, pH 6.8 (5% v/v), 20% sodium dodecyl sulfate (2.5% v/v), dextran sulfate (10% w/v), and sheared salmon sperm DNA (0.1 mg/ml) (Maniatis *et al.* 1982).

A single-stranded cDNA clone of the plus strand of a 194-bp sequence from the homopolymer end of the dsRNA of EP713 was obtained from R. Rhoads, University of Kentucky (Hiremath *et al.* 1986). The clone was labeled with biotin 7-ATP using a 3′ M13 hybridization probe-primer (1202 New England BioLabs, Beverly, MA). After prehybridization, the biotin-labeled M13 probe was added to the hybridization bags (0.015 μg/ml hybridization solution) and the membranes were incubated an additional 5 hr at 42° C. The hybridization solution was then removed from the hybridization bags and stored at 4° C for reuse. The membranes were washed and the detection and visualization reagents were applied as directed in the BluGene nonradioactive nucleic acid detection kit (BRL 8279SA).

DsRNA purification. Inoculum was taken from fungal colonies, grown in liquid culture, and dsRNA was purified from the resultant fungal mass. DsRNA was isolated as described by Hansen *et al.* (1985).

RESULTS

***In situ* hybridization.** Using cDNA probes complementary to the negative strand of the dsRNA, it was possible to detect the dsRNA in the hypovirulent strains of EP113. The blue of the chromophore on hybridized membranes indicated the presence of the dsRNA. No dark blue was found on membranes containing nucleic acid from the non-dsRNA containing strain EP2001. Some areas of the colonies contained fungal pigment, which stained portions of the membranes brown. This, however, was easily distinguished from the dark blue produced by the reaction of the BluGene detection system.

The presence of dsRNA is known to affect the protein composition of infected cells (Powell and Van Alfen 1987). To assure that there was no differential, nonspecific binding of the biotin reagents to fungal proteins or other fungal components, a control membrane was hybridized with biotin-labeled lambda DNA. There was no evidence of nonspecific binding to lambda DNA by either EP113 or EP2001 (data not shown).

To assure that there was no differential enzyme digestion occurring between the thin, actively growing peripheral tissue and the older, denser tissue at the center of the fungal colonies, enzyme-lysed colonies were hybridized with ^{32}P-labeled DNA plasmid specific for mitochondrial DNA (pUV10 [Gobbi *et al.* 1990]). This resulted in a uniform hybridization reaction across the entire colony (see Gobbi *et al.* 1990).

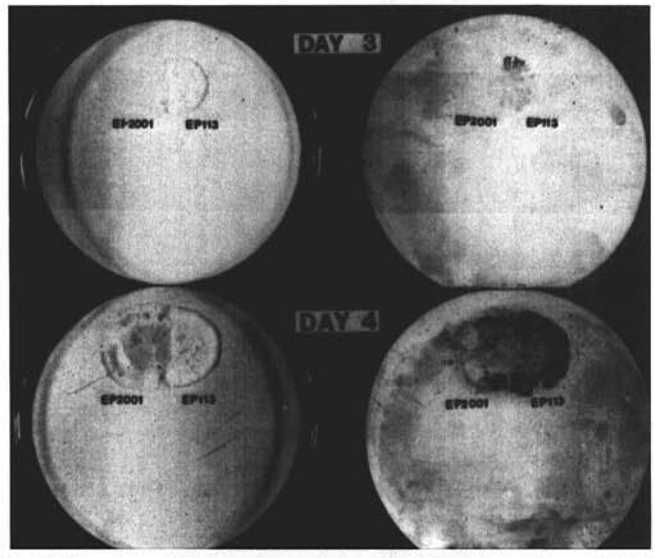

Fig. 1. *Cryphonectria parasitica*, strains EP2001 (virulent) and EP113 (hypovirulent), were inoculated together on agar plates and allowed to grow through hybridization membranes. The membranes were probed with a cDNA clone of the viral dsRNA. The lefthand plates show the colonies growing through the membranes before processing and the righthand column shows the results of probing with the cDNA clone. After 3 days, both strains had grown through the membranes, but dsRNA was only detected in EP113. After 4 days, dsRNA could be detected throughout both colonies.

Movement of dsRNA. PDAmys plates with paired inocula of EP113 and EP2001 were used to monitor the movement of dsRNA between strains over 24-hr intervals (Fig. 1). The hybridization membranes were removed from all plates at the same time and processed simultaneously. At 2 days after inoculation, growth of the fungus was visible through the membranes. At 3 days, the colonies had reached a diameter of about 20 mm, with contact between the colonies being along a 10 mm length, but no transfer of dsRNA to EP2001 was detected. At 4 days, the colonies had each grown to about 25 mm in diameter. *In situ* hybridization revealed that dsRNA was present throughout the colonies of both strains. From 4 days after inoculation, the dsRNA became more densely concentrated in the peripheral regions than in the center of the colonies. This experiment was repeated four times with nearly identical results. Figure 1 depicts a representative experiment.

Samples of mycelium were taken from the periphery of the plate 6 days after inoculation and assayed for dsRNA by gel electrophoresis. The presence of dsRNA was verified in the periphery of both EP113 and EP2001 (Fig. 2).

To confirm and extend these observations concerning viral movement, a long, thin piece of inoculum of EP2001 was placed on an agar plate with a small piece of inoculum of EP113 placed near one end. Small samples were removed at 24-hr intervals along the periphery of the growing colony (Fig. 3). The samples were plated on PDAmys and grown for 2–3 wk until a distinctive, hypovirulent, or converted hypovirulent colony morphology could be detected (Fig. 4). After a distinctive colony morphology was observed, the colonies were assayed for presence of dsRNA using agarose gel electrophoresis. DsRNA was found to be present in all samples showing either hypovirulent or converted hypovirulent colony morphology. No dsRNA was found in samples showing virulent colony morphology. This experiment was repeated three times with the presence of dsRNA always correlating to colony morphology.

The presence of dsRNA in EP2001 was first detected, by dsRNA isolation and colony morphology changes, between 48 and 72 hr after inoculation. By 96 hr, 24 hr after first being detected in EP2001, the dsRNA had moved an average of 21 mm. The rate of colony growth averaged 4.4 mm over the same period of time (Table 1). All of the colonies measured had similar growth rates.

To determine if hyphae from EP113 are able to penetrate into the EP2001 colony, inoculum was taken from various locations of 5-day-old paired colonies and grown on mini-

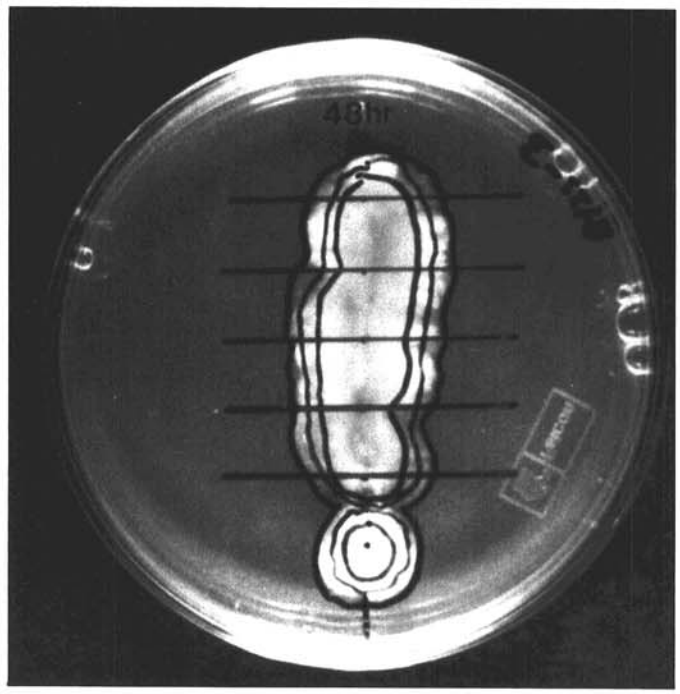

Fig. 3. A 3- × 3-mm piece of a culture of EP113 and a 3- × 40-mm piece of EP2001 were placed 1 cm apart on an agar plate. Lines perpendicular to the length of the EP2001 inoculum were drawn every centimeter starting at the center of the EP2001 inoculum site. The perimeter of each colony was traced, using a marking pen, at 12-hr intervals beginning at 24 hr (this figure shows a plate at 48 hr after inoculation). Growth of the colony over a 12-hr period was determined by measuring the distance between the marked growth rings. Separate plates with similar growth rates were used for isolations for each time period. Samples were removed along the entire width of the colony at the perpendicular lines. The samples were transferred to PDAmys and grown until colony morphologies could be distinguished.

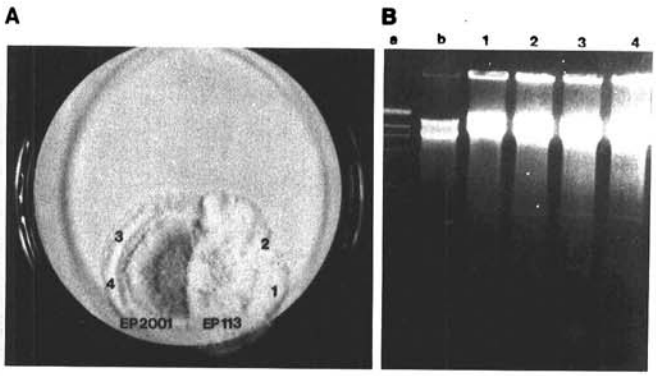

Fig. 2. A, Paired colonies of EP113 and EP2001 were grown together through a hybridization membrane for 5 days. **B,** Samples of mycelium were taken from under the membrane and assayed for dsRNA by gel electrophoresis. Lane a is lambda DNA digested with *Hin*dIII. Lane b is a dsRNA standard isolated from strain EP113. Lanes 1–4 show dsRNA from samples taken from plate A where indicated by the numbers.

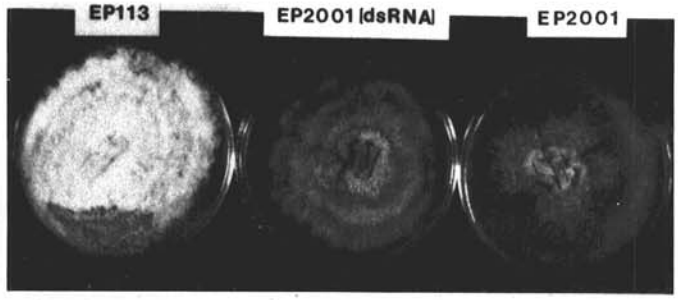

Fig. 4. Samples removed along the length of paired colonies as shown in Figure 3 were transferred to potato-dextrose agar. Three colony morphology types were subsequently observed. All colonies were tested for the presence or absence of dsRNA by gel electrophoresis. Colony morphology was found to correlate completely with the presence of dsRNA. The dsRNA-containing colonies EP113 and converted EP2001 (EP2001[dsRNA]), had growth rings and fluffy aerial hyphae. EP2001 colonies were darkly pigmented and showed little aerial hyphae (contained no dsRNA).

mal media. EP2001 is *met* auxotroph and will not grow on minimal media, but the prototrophic strain EP113 will. Inocula taken from the EP2001 colonies, including areas close to the zone of contact between EP2001 and EP113, were not able to grow on minimal media. Hyphae from EP113 did not penetrate into the EP2001 colony beyond the zone of contact (Fig. 5).

DISCUSSION

In studying the movement of the viruslike RNA responsible for hypovirulence of *C. parasitica*, we have limited our studies to the dsRNA form of the viruslike genetic element. Our detection of the dsRNA has been based on two different methods. One is the direct isolation of the dsRNA from fungal tissue. The other is *in situ* hybridization with a cDNA clone of the plus-sense strand of the dsRNA. Thus each of these methods detect the double-stranded RNA form of the virus. Movement of the viruslike genetic element may be either as dsRNA within fungal vesicles or as ssRNA, such as described for tobacco mosaic virus (Citovsky *et al.* 1990). The methods used in this study are not suitable for addressing the question of what is actually moving.

Using *in situ* hybridization with a biotin-labeled probe specific to the minus strand of dsRNA, areas within fungal colonies containing dsRNA were visualized. Because *in situ* hybridization to dsRNA in paired hypovirulent and virulent colonies revealed a rapid movement of the dsRNA (Fig. 1), further tests were done to determine the rate of movement of the viruslike genetic element through fungal colonies. Long pieces of EP2001 inoculum were paired with small pieces of inoculum of EP113 so presence of the dsRNA along the new growth of EP2001 could be detected. The presence or absence of dsRNA at a given distance along the length of the new growth from the inoculum of EP2001 was determined by purification procedures. The dsRNA was isolated from colonies that grew from small pieces of agar transferred from EP2001 after interaction with EP113. The colonies that grew after transfer had distinctive colony morphologies if dsRNA was present and they could easily be distinguished from colonies that did not contain dsRNA.

We were able to detect the transfer of the dsRNA from EP113 to EP2001 within 3 days after inoculation (Table 1). Using the *in situ* hybridization method the transfer of the virus from EP113 to EP2001 was detectable 4 days after inoculation (Fig. 1). This difference might be due to the inability of the *in situ* hybridization technique to distinguish low concentrations of dsRNA from background. On day 3, using isolation procedures, dsRNA was detected in the EP2001 only 5 mm from the point of contact of the two colonies. By day 4, dsRNA could be detected as far as 35 mm from the point of contact (Table 1), which is similar to the results from *in situ* hybridization. These results indicated that the dsRNA moved up to 30 mm through the recipient colony in one day. Colony growth was measured perpendicular to the length of the EP2001 inoculum. The measured movement of the dsRNA was perpendicular to the hyphal growth. Although movement also occurs in the direction of hyphal growth, it is too rapid in this direction to distinguish from hyphal growth. It is hypothesized that the viruslike genetic element is moving laterally through the recipient colony via a network of interconnected lateral hyphae.

The average growth rate of the fungal colonies over each 24-hr period was 4.4 mm. This can be compared with an average movement of the dsRNA of 16 mm between days 3 and 4 (Table 1). The rapid movement of the dsRNA within recipient colonies compared with fungal growth rate suggests an active transport mechanism. Transport of the virus may be linked to membrane vesicle transport because of the association of dsRNA with fungal vesicles (Hansen *et al.* 1985). Perhaps these dsRNA-containing vesicles are transported toward growing hyphal tips as are other vesicles

Table 1. Growth rate of paired EP113 and EP2001 colonies compared with movement of dsRNA

Sampling Time (hr)	Growth previous 24 hr[a] (mm)	Presence of dsRNA Distance from EP113-EP2001 interface (mm)			
		5	15	25	35
48	4.1[b]	−	−	−	−
	4.6	−	−	−	−
	4.5	−	−	−	−
	4.2	−	−	−	−
	4.4	−	−	−	−
	Avg. 4.4				
72	4.3	+	−	−	−
	4.5	+	−	−	−
	4.6	+	−	−	−
	4.3	+	−	−	−
	4.2	+	−	−	−
	Avg. 4.4				
96	4.1	+	+	−	−
	4.7	+	+	+	−
	4.5	+	+	−	−
	4.3	+	+	+	+
	4.4	+	+	−	−
	Avg. 4.4				

[a] Growth measurements were made from five separate plates.
[b] Average of 10 measurements around the periphery of a single colony. Data from five colonies are used to determine average growth at each sampling time. See Figure 3.

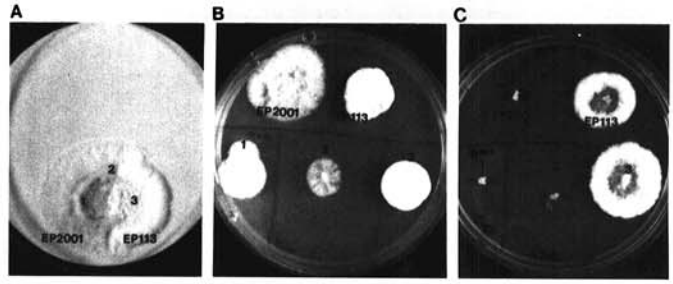

Fig. 5. A, Paired pieces of inoculum of EP113 and EP2001 were covered with hybridization membrane and grown for 5 days. **B,** Mycelial samples were taken at locations indicated by numbers 1–3 and transferred to potato-dextrose agar along with inocula from stock EP113 and EP2001 cultures. **C,** Mycelia from these same samples were also transferred to minimal media plates. Only the EP113 samples grew on minimal media. EP2001 is a methionine auxotroph and will not grow on minimal media.

in fungal hyphae. The movement of nuclei and other organelles within fungal hyphae is common, and often occurs very rapidly (Ross 1976; McKerracher and Heath 1987). Active transport of dsRNA in *Ceratocytis ulmi* has also been postulated, because of the observed rapid movement of a dsRNA-associated phenotype into recipient colonies (Brasier 1986).

Movement of TMV, an ssRNA plus-sense virus, within plants has been shown to be facilitated by a movement protein that binds to the ssRNA. It is postulated that this protein makes possible the observed transmembrane movement of TMV (Citovsky *et al.* 1990; Wolf *et al.* 1989). Movement proteins have been described for a number of different viruses, but it is not known if they each have the same mode-of-action suggested for the movement protein of TMV (Hull 1989). There is currently no evidence to suggest that the viruslike RNA of *C. parasitica* is able to move across the plasma membrane of the fungus nor has a movement protein been described for this genetic element. The mechanism of movement of the viruslike RNA of *C. parasitica* within the fungal hyphae and between vegetatively compatible strains is yet to be resolved.

A concentration of dsRNA in the peripheral regions of the colonies was detected by *in situ* hybridization. It is known that dsRNA is in the sporulating tissue, even though we did not detect it by *in situ* hybridization (Elliston 1987). The detection of greater amounts of dsRNA in the periphery of the colony is probably not an artifact of the method, since mtDNA was equally detectable throughout the colony (Gobbi *et al.* 1990).

This study has shown that the viruslike RNA moves tangentially through the fungal colony at a rate that cannot be explained by the growth of hyphae. This rapid movement appears to occur in nature because a small amount of inoculum of a hypovirulent strain can be placed on the growing edge of a large virulent canker, and the virulent canker will become hypovirulent (Grente 1965). For this to occur the viruslike RNA must move completely around or through the virulent canker.

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