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Detection of dsRNA from Cleistothecia and Conidia of the Grape Powdery Mildew Pathogen, *Uncinula necator*

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ABSTRACT

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Double-stranded RNA species ranging in molecular weight from 0.95 to 6.3×10^6 were detected in grapevines in New York. We recently showed that two of the species ($M_r = 5.3$ and 4.4×10^6) are associated with rupestris stem pitting disease. In this report, we show that the other eight detectable dsRNA species are associated with the powdery mildew fungus, *Uncinula necator*. These dsRNAs associated with the powdery mildew fungus were previously detected in leaves and epidermal stem tissue of grapevines infected with powdery mildew. The same dsRNA species were also detected from extracts of isolated cleistothecia and conidia of *U. necator* devoid of plant tissue. Isometric and rigid rodlike particles were observed in single cleistothecia preparations when examined under transmission electron microscopy.

Rupestris stem pitting (rSP) is a graft-transmissible grapevine disease of undetermined causal agent. It was first recognized in California by the pitting symptoms induced on the woody indicator *Vitis rupestris* Scheele 'St. George.' Because the disease has not been transmitted mechanically, biological indexing on St. George remains the only diagnostic test available. A recent work (1) showed the presence of two distinct dsRNA species (B and C) ($M_r = 5.3$ and 4.4×10^6, respectively) from rSP-diseased vines in California and Canada. In New York, however, the dsRNA analysis of several grapevine cultivars indicated the presence of different dsRNA species ranging in molecular weight between 0.95 and 6.3×10^6. Among these dsRNAs, two species were similar, if not identical, to the dsRNA species B and C detected in stem phloem tissue extracts of rSP-diseased vines from California and Canada. The other dsRNAs, eight detectable species, were identified in leaf extracts of both rSP-negative and rSP-positive samples. The estimated M_r of these species (designated A and D–F) was $0.95–6.3 \times 10^6$. Because species A and D–F were detected only in New York, we investigated their origin further.

Based on the observation that dsRNA detection in the greenhouse samples was closely associated with leaves infected with the powdery mildew fungus, we tested the hypothesis that dsRNAs detected in the leaves were associated with *Uncinula necator* (Schwein.) Burrill, the causal agent of grape powdery mildew. In this paper, we report that dsRNA species observed in leaf extracts were indeed associated with *U. necator*.

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MATERIALS AND METHODS

Greenhouse experiment. Grape leaves from the cultivars Colobel (rSP-positive vines 256 and 257), Villard blanc (rSP-negative), and Pinot noir (a leafroll-diseased grapevine collected from a vineyard in Hammondsport, NY) were used for this experiment. Seven to 10 cuttings from each sample were grafted onto St. George, and dsRNA was extracted from leaves that emerged from the grafted bud. Grafts were made in October 1988, and testing began in February 1989 and continued monthly until June 1989. Fifteen grams of young leaves was collected from each of the four samples and processed for dsRNA extraction. Healthy St. George leaves were also processed for dsRNA.

DsRNA extraction from grape and fungal tissue. Extraction of dsRNA was by the method of Hu et al (7). Fifteen grams of leaf and 10 g of stem tissue per field sample was processed during early September 1989. Cleistothecia were also harvested at that time from leaves of *Vitis* interspecific hybrid Colobel (grown in the field) and cv. Rosette (grown in the field, growth chamber, or tissue cultured tubes). Cleistothecia (from Rosette grown in the field and in the growth chamber) and conidia (originally from *V. champini* Planch. in the field but grown on Rosette in tissue cultured tubes) were provided by David Gadoury, New York State Agricultural Experiment Station, Geneva. Thirty grams of leaves bearing cleistothecia was shaken vigorously in water to release ascocarps, and the resultant suspension was poured through a 170-mesh Cobb sieve. Cleistothecia retained by the sieve were suspended in 45 ml of distilled water and stored at −20 C until used. Conidia were collected by vortexing infected Rosette plants grown in tissue culture tubes (grape seedling plants grown under sterile conditions to ensure the presence of only the conidia of powdery mildew) in a 30-ml tube containing 25 ml of distilled water and Tween 20 (0.01%) for a few minutes. Leaf and stem debris were picked out of the tube by hand or by forceps, and the conidial suspension was processed for dsRNA extraction, as such, without grinding in liquid nitrogen.

Purified dsRNA preparations from cleistothecia or conidia were suspended in 20 μl of sterile water and loaded on a 6% polyacrylamide gel (10 × 10 cm). This 20-μl volume represented cleistothecia from the 30 g of leaf tissue and conidia from the two tissue cultured plants. The dsRNA concentration was not measured. Electrophoresis was performed for 11 hr at 20 mA. Gels were stained in ethidium bromide (50 ng/ml) for 10 min, destained in distilled water for 5 min, and photographed on a transilluminator with Polaroid 667 film.

Detection of virus particles. Attempts were made to purify virus particles from Colobel leaves bearing cleistothecia with the procedure described by Hu et al (7). Additionally, groups or single cleistothecia that were mixed with water and squeezed under a glass slide were placed on 400-mesh electron microscope grids, stained with 1% uranyl acetate for 1–2 min, and examined under the electron microscope.

Table 1. Detection of dsRNA from leaf samples of greenhouse-grown *Vitis* interspecific hyb Colobel-256 and -257, Villard blanc, and Pinot noir after grafting onto *V. rupestris* 'St. George

Sampling date	dsRNA detected from cultivar[a]				
	Colobel 256	Colobel 257	Villard blanc	Pinot noir	Healthy St. George
2/14/89	+[b]	+	−	+	−
3/10/89	+	+	−	+	−
4/08/89	−	+	−	−	−
5/05/89	+	+	−	+	−
6/03/89	+	+	−	−	−

[a] Colobel-256 and -257 are infected with rupestris stem pitting and Pinot noir with grapevine leafroll. Colobel-256, -257, and Pinot noir were infected with powdery mildew, whereas Villard blanc and St. George were not. Ungrafted healthy St. George was used as a negative control.
[b] + = dsRNA species A and D–F were detected, − = dsRNA species were not detected.

RESULTS

Greenhouse experiment. Because we detected different dsRNA species from both Colobel (rSP-positive) and Villard blanc (rSP-negative) vines, we grafted these different vines onto St. George in the greenhouse to monitor the detection of dsRNAs in the young shoots and to check whether there was any association between rSP disease and the detected dsRNA species. However, 3 mo after grafting, young shoots of Colobel-256 and -257 and Pinot noir were found covered with powdery mildew mycelia. The mycelia were not observed on other cultivars.

The results of dsRNA analysis are shown in Table 1 and Figure 1. The dsRNA pattern (species A and D–F) was identical to that observed earlier in 1987 from the leaves of Colobel (1). The M_r of species A and D were estimated as 6.3×10^6 and 3.4×10^6, respectively. The molecular weights of the smaller dsRNA species were estimated as 2.1, 1.9, and 1.75×10^6 for species E, which enclosed a group of closely migrating dsRNA species; and 1.1, 1.05, and 0.95 $\times 10^6$ for the group species F. DsRNA species A and D–F were detected in Colobel-257, but they were not detected in Villard blanc during the entire testing period. DsRNA species were not detected in Colobel-256 during April. Similarly, dsRNA species were not detected in Pinot noir in April and June. The intensity of the dsRNA species was not consistent for each testing period. For example, dsRNA species A was very faint in the February test (Fig. 1).

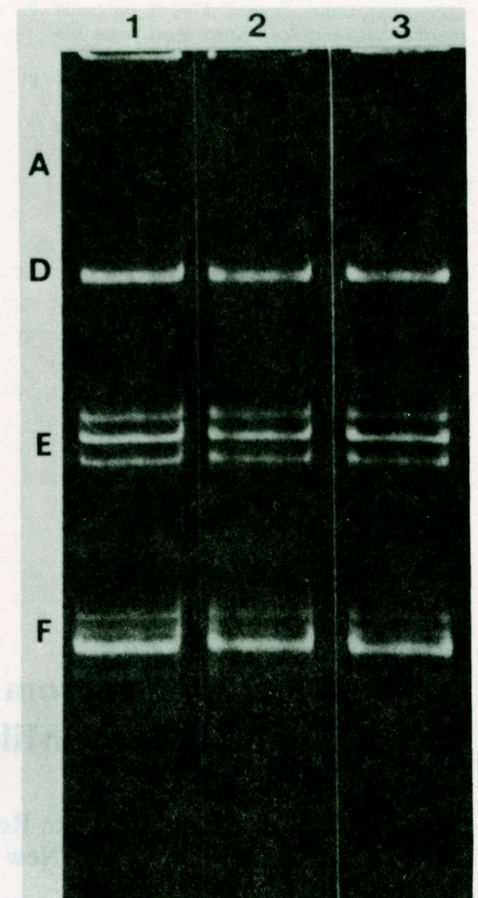

Fig. 1. Electrophoretic analysis in a 6% polyacrylamide gel of dsRNA species detected in **(lane 1)** Colobel-256, **(lane 2)** Colobel-257, and **(lane 3)** Pinot noir. Colobel-256 and -257 indexed positive and Pinot noir indexed negative for rupestris stem pitting. The gel was electrophoresed at 20 mA for 11 hr and stained in ethidium bromide. The molecular weight (M_r) for dsRNA species A and D was estimated as 6.3 and 3.4×10^6. For the smaller group species E and F, the estimated M_r was 2.1, 1.9, and 1.75×10^6, and 1.1, 1.05, and 0.95×10^6 respectively.

Fungal origin of dsRNA. The presence of powdery mildew on grape cultivars that tested positive for these dsRNAs in the greenhouse, and the fact that there was no observed association between rSP disease and these dsRNA species, led us to investigate the possibility that these dsRNAs originated from the grape powdery mildew fungus. Leaves and stems exhibiting powdery mildew symptoms were collected from a Colobel-256 vine in the field in early September 1989. Each sample of leaves and stems was divided into two subsamples. Leaf subsamples included 15 g of leaves bearing cleistothecia, and a second subsample was made up of 15 g of leaves from which cleis-

...ecia had been removed from the surface by tissue paper. One stem subsample consisted of 10 g of epidermal tissue from stems covered with cleistothecia and the other consisted of 10 g of phloem tissue that did not have detectable fungal growth. DsRNA was detected from leaves bearing cleistothecia but not from the leaves that had been washed (Fig. 2, lanes 3 and 4). The group of dsRNA species (F) that migrated faster was also detected from epidermal and phloem tissue of stems (Fig. 2, lanes 1 and 2). DsRNA species (B and C) that were associated with rSP (1) were detected only in the stem phloem tissue (Fig. 2, lane 1).

Evidence that dsRNA is associated with cleistothecia and conidia of *Uncinula necator*. Further experiments were done in an effort to obtain unequivocal evidence that the dsRNA species were associated with *U. necator* and not grape tissue. Cleistothecia were harvested from leaves of Colobel and Rosette, the latter being highly susceptible to powdery mildew. Leaves from which cleistothecia had been removed by vigorous washing and wiping by tissue paper were included as controls in the dsRNA test. DsRNA species were observed only from extracts of cleistothecia ($>10^5$) and not from the washed leaves (Fig. 3). The relative concentration of these dsRNA species was higher in extracts from cleistothecia harvested from Rosette than from Colobel. When 100, 1,000, and 10,000 cleistothecia were extracted, dsRNA species F was observed only in extracts from 10^4 cleistothecia. On the other hand, dsRNA species A and D–F were observed in extracts containing $>10^4$ conidia.

Electron microscope detection of isometric viruslike particles from cleistothecia. The identification of dsRNA in *U. necator* cleistothecia suggested the presence of a virus. Three single cleistothecia isolated from the surface of Colobel leaves, groups of cleistothecia, and partially purified preparations (following the method of Hu et al [7]) of Colobel leaves bearing or not bearing cleistothecia were examined by electron microscopy. Isometric viruslike particles were observed in both single and groups of cleistothecia squashes and in leaves containing these bodies. In addition, a few rigid rodlike particles were examined in one of three single cleistothecia squashes and two of five leaf preparations bearing cleistothecia (Fig. 4). Rigid rodlike particles were not seen in group extracts of cleistothecia or in washed leaves. (The experiment was repeated twice.) The size of the rigid particles ranged from 200 to 600 nm in length, and they were too few to make an accurate estimate. The size of isometric particles ranged from 25 to 35 nm in diameter (100 particles counted).

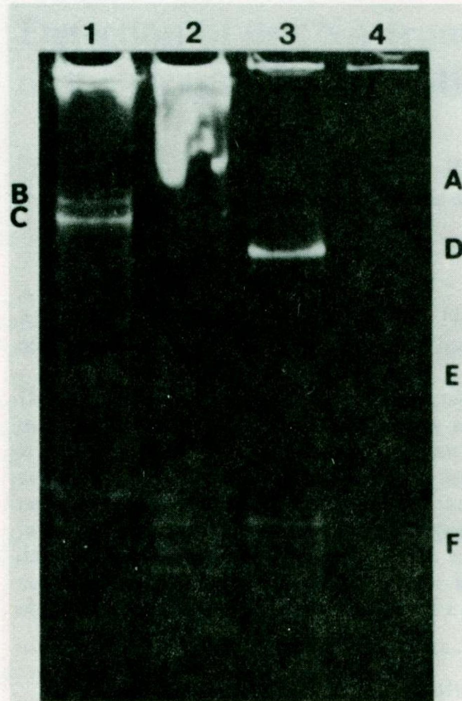

Fig. 2. DsRNA from **1)** stem phloem tissue, **2)** stem epidermal tissue, **3)** leaves bearing cleistothecia, and **4)** leaves without cleistothecia. Purified dsRNA preparations from 15 g of leaves and 10 g of stem tissue of Colobel-256 infected with powdery mildew were suspended in 30 μl and were electrophoresed through 6% polyacrylamide gel for 11 hr at 20 mA, and the gel was stained in ethidium bromide.

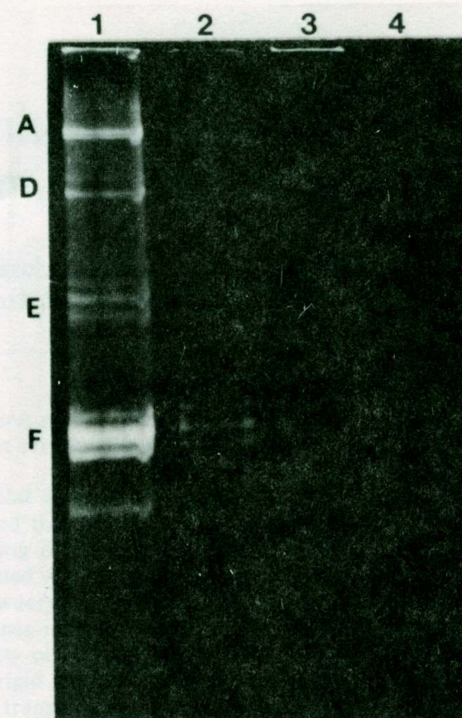

Fig. 3. Electrophoretic analysis in a 6% polyacrylamide gel of dsRNA extracted from **1)** pure cleistothecia from Rosette, **2)** pure cleistothecia from Colobel-256, **3)** Colobel-256 leaves cleaned of cleistothecia, and **4)** Rosette leaves cleaned of cleistothecia. Each cleistothecia sample was collected from 30 g of leaf tissue and each leaf sample consisted of 15 g. Both leaf samples were included as controls. Samples were analyzed by electrophoresis in 6% polyacrylamide gel for 11 hr at 20 mA, and the gel was stained in ethidium bromide.

DISCUSSION

We have shown that dsRNA species detected from some New York grapevines are associated with *U. necator*. DsRNA was detected from cleistothecia that are usually produced on the surface of infected leaves and stems beginning in mid-summer (5,10). Moreover, dsRNA was detected in conidia of *U. necator* grown on tissue cultured plants.

Detection of dsRNA from plant pathogenic fungi is common (2,3,8), however, we believe this is the first report on the detection of dsRNA from *U. necator*. The fact that dsRNA was recovered from cleistothecia and conidia devoid of any plant tissue shows that these different dsRNA components are associated with the fungus and not with rSP (1).

The detection of dsRNA species F (Fig. 2) in the stem phloem tissue sample might be attributed to a contamination in the sample itself, because it is sometimes difficult to separate the phloem from the epidermal tissue. The variability of dsRNA patterns seen at different times might be related to different concentrations of fungal tissue present when extractions were made. The fact that the vines in the field were sprayed with fungicides early in the growing seasons of 1988 and 1989 might explain why dsRNA detection in the field was delayed until early fall.

The rigid rodlike particles observed in single cleistothecia and in the leaves containing cleistothecia resemble tobacco mosaic virus (TMV). TMV-like particles have been reported from powdery mildews and rusts (9,11). The authors claimed these particles were TMV strains from conidia of barley powdery mildew caused by *Erysiphe graminis* DC. and oak powdery mildew caused by *Sphaerotheca lanestris* Harkn. But further work on particles isolated from *Uromyces phaseoli* (Pers.) G. Wint. var. *vignae*

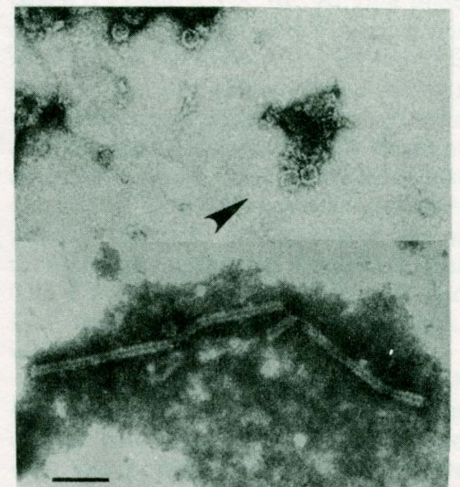

Fig. 4. Isometric and rigid rod viruslike particles as seen under the electron microscope in **(top)** a single cleistothecium extract and **(bottom)** partially purified leaf tissue (50 g) bearing cleistothecia. The grid was stained with 1% uranyl acetate. Bar = 100 nm.

showed that these TMV-like particles might simply be rigid rods with ends butted to spherical vesicles and not related to any strain of TMV found in the plants (2). In this investigation, we did not consistently isolate these rigid rod-shaped particles from leaf tissue bearing cleistothecia or single cleistothecia preparations. Often, they appeared to be aggregates of short particles. More work is needed in this area.

The finding that both isometric and rigid rodlike particles may exist in the same fungus body is common, and there are several reports that have shown morphologically distinct types of viruslike particles present together in the same fungal culture (4,6).

This study raises a number of important questions. Are dsRNAs common in *U. necator* from different vineyards or geographic regions? What is the significance of the viruslike particles in *U. necator*? Are these viruslike particles or dsRNAs associated with hypovirulence? Is the virulence level of the fungus affected by these viruslike particles or dsRNAs? And what is the specific association of these dsRNAs with the virus-like particles?

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