Characterization of Double-Stranded RNA in Isolates of *Phytophthora infestans* from Mexico, the Netherlands, and Peru

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ABSTRACT

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Seventy-nine isolates of *Phytophthora infestans,* from Mexico, the Netherlands, Peru, Israel, and the United States, were screened for the incidence of double-stranded (ds) RNA. The dsRNA was most abundant in Mexican isolates (83.3%), followed by isolates from the Netherlands (20%) and Peru (2.9%). *P. infestans* isolates from Israel and the United States were dsRNA-free. Double-stranded RNA was found in both A1 and A2 mating-type Mexican isolates, while only A2 mating-type isolates from the Netherlands contained dsRNA. Dutch and Peruvian isolates contained dsRNA segments that were identical in size with dsRNA segments recovered from Mexican isolates. In addition to the dsRNA segments and patterns previously reported in Mexican isolates, three new segments and six new patterns were discovered. The dsRNAs ranged in size from 1.35 to 11.10 kbp, and transmission electron microscopy of the dsRNA revealed linear molecules. Northern blot hybridization analysis showed that sequence homology exists between comigrating dsRNA segments of Mexican, Dutch, and Peruvian isolates, and that at least four nonhomologous groups of dsRNA occur in *P. infestans.*

Additional keywords: epidemiology, late blight, molecular marker.

Phytophthora infestans (Mont.) de Bary is the causal fungus of the most important disease of potato crops worldwide. Recently, Tooley et al (25) reported the existence of dsRNA in 14 isolates of the fungus from Mexico. Three banding patterns, designated A, B, and C, and four dsRNA segments were identified. Double-stranded RNA occurred in both A1 and A2 mating-type isolates. Other isolates examined, from the United States and Europe, were dsRNA-free.

The presence of dsRNA in *P. infestans* suggests the possibility that a mycovirus is present in some isolates, although this is not yet known. Virulence assays performed with the 14 Mexican isolates originally found to contain dsRNA have shown high overall levels of virulence in all but one isolate (25). Thus, there is no apparent connection between presence of dsRNA and lowered virulence, as in *Cryphonectria parasitica* (Murrill) Barr (3,10). Tooley et al (25) proposed that the dsRNA could be valuable as a genetic marker to analyze segregation of cytoplasmic traits in *P. infestans* and as an epidemiological marker to monitor worldwide migration of Mexican isolates.

The objectives of this work were to survey additional isolates of *P. infestans* for the presence of dsRNA, size the dsRNAs with known molecular markers, investigate potential sequence homology between dsRNA segments extracted from different isolates of the fungus, and observe the dsRNA using transmission electron microscopy.

MATERIALS AND METHODS

Isolates. A total of 79 *P. infestans* isolates, from the United States, Mexico, the Netherlands, Israel, and Peru, were screened for the presence of dsRNA (Table 1). All U.S. and Peruvian isolates were of A1 mating type, while the isolates from the Netherlands and Mexico consisted of both A1 and A2 mating types. The Israeli isolate was of A2 mating type. All isolates were grown at 18 C, without being shaken, in Fernbach flasks containing 400 ml of V8 juice medium. After 3 to 4 wk, the flasks were placed on shakers (18 C, 25 rpm) for a few days to allow the colonies to increase in thickness. Mycelial mats were collected on Miracloth (Calbiochem, Inc., La Jolla, CA) via vacuum filtration, and excess moisture was removed by pressing the mats between paper towels. The mats then were frozen at −80 C, lyophilized, and stored desiccated at −20 C.

Extraction of dsRNA. A modification of the method outlined by Tooley et al (25) was used to isolate dsRNA. One or two grams of lyophilized fungal mycelium (~4–8 g fresh weight) was finely ground with a pestle in a mortar containing liquid nitrogen. The resulting powder was transferred to a centrifuge tube, and the following were added: extraction buffer (10 × STE [1.0 M NaCl, 0.5 M Tris, 0.01 M (Na_2) EDTA, pH 6.8] buffer, 100 ml; 10% SDS, 150 ml; 1% PVP-10, 60 ml; and 2-mercaptoethanol, 2.5 ml), 2 or 4 ml; chloroform/pentanol (24:1, v/v), 4 or 8 ml; phenol saturated with 2 × STE, 4 or 8 ml; and 2-mercaptoethanol, 50 or 100 μl. After grinding the mixture with a glass rod for 15 min, 5 or 10 ml of 2 × STE was added to the tube, and the contents were stirred thoroughly. The tube then was centrifuged at 6,723 *g* (4 C) for 10 min, and the aqueous phase was recovered and adjusted to 16.5% ethanol in 1 × STE buffer. The adjusted aqueous phase was added to 1 g of CF-11 cellulose (Whatman Labsales, Inc., Hillsboro, OR) and stirred magnetically for 30 min. The cellulose then was allowed to settle, and the supernatant was decanted. Fresh 16.5% ethanol-1 × STE wash solution was added to the cellulose, and the mixture was poured into a small column. Approximately 150–200 ml of 16.5% ethanol-1 × STE wash was passed through the cellulose in the column, after which the dsRNA was eluted into a polycarbonate centrifuge tube using 10 ml of 1 × STE. The dsRNA was ethanol-precipitated

at −20 C overnight, and pelleted by centrifugation at 7,650 *g* for 30 min at 4 C. The dsRNA pellet was resuspended in 250 μl of 1 × STE and transferred to a sterile 1.5-ml Eppendorf tube for further processing.

DNase and S_1 nuclease treatments and electrophoresis. Based on the results of preliminary experiments, all extracted nucleic acid from isolates to be screened for dsRNA was subjected to the following treatment to be sure that only dsRNA was ultimately observed. Double-stranded RNA was ethanol-precipitated, and the pellet was dissolved in 100 μl of TNM (0.1 M Tris, 0.1 M NaCl, and 0.1 M MgCl, pH 7.5) buffer, followed by the addition of 5 μl (15 μg) of macaloid-treated (21) DNase I. After incubation at 37 C for 30 min, the dsRNA was ethanol-precipitated, and the pellet was resuspended in 13 μl of 1 × S_1 nuclease reaction buffer (0.05 M sodium acetate, pH 4.6, 0.15 M NaCl, and 0.001 M zinc acetate). Five microliters of a 10 U/μl dilution (in 1 × S_1 reaction buffer) of S_1 nuclease was added to the mixture, and the dsRNA was incubated at 37 C for 30 min. After incubation, 2 μl of 10 × loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll [type 400] in double-distilled H_2O) was added, and the dsRNA was electrophoresed on a neutral agarose (1%) gel (7 × 10 × 0.3 cm) (100 V at 4 C for 1 h), or an acrylamide (5%) gel (10 × 8 × 0.075 cm) (110 V at room temperature for 2 h). The same electrophoresis buffer (1 × = 0.04 M Tris, 0.02 M sodium acetate, and 0.001 M (Na_2) EDTA, pH 7.8) was used for both types of gels. Double-stranded RNA was visualized by staining with ethidium bromide (1 μg/ml).

TABLE 1. Isolates of *Phytophthora infestans* from Mexico, the Netherlands, and Peru that contained double-stranded RNA

Isolate number[a]	Source[b]	Mating type	dsRNA pattern[c]
512	Toluca, Mexico	A1	E[d]
573	Toluca, Mexico	A2	B
575	Toluca, Mexico	A2	B
576	Toluca, Mexico	A2	F
577	Toluca, Mexico	A2	D
578	Toluca, Mexico	A2	D
579	Toluca, Mexico	A2	A
580	Toluca, Mexico	A1	F
616	Toluca, Mexico	A1	G
617	Toluca, Mexico	A1	G
618	Toluca, Mexico	A2	G
619	Toluca, Mexico	A1	A
620	Toluca, Mexico	A1	G
621	Toluca, Mexico	A1	G
622	Toluca, Mexico	A1	G
623	Toluca, Mexico	A1	A
1104	Nagele, Netherlands	A2	H
1106	Kooienburg, Netherlands	A2	I
1108	Kooienburg, Netherlands	A2	I
1109	Kooienburg, Netherlands	A2	I
819	Huanuco, Peru	A1	G

[a] The following additional isolates were tested and did not contain dsRNA: 572, 574, and 581 (Mexico); 1070, 1074–1076, 1078, 1098–1100, 1110–1113, 1115, 1116, 1118, and 1119 (Netherlands); 800–818 and 820–833 (Peru); 105, 120, 123, 131, 134, 136 (United States); and 1069 (Israel).

[b] All Mexican isolates originated from the Toluca region. Isolates 576–580 were obtained from wild *Solanum* species, while all other isolates came from potato plants growing in late blight field plots. All isolates from the Netherlands were collected by L. C. Davidse, Department of Phytopathology, Agricultural University, Wageningen, Netherlands. The Peruvian isolates were obtained from V. Otazú and E. R. French, Pathology Department, International Potato Center, Lima, Peru. They were collected from the foliage of blighted potato plants growing in the central highlands of Peru by Hans Pinedo of the International Potato Center.

[c] Double-stranded RNA patterns are illustrated in Figure 1.

[d] Isolate 512 was originally thought to possess dsRNA with pattern C (25). In this study, it was found to have the high molecular weight segment as well, which made it the only isolate showing dsRNA pattern E (Fig. 1).

Sizing of dsRNA bands. A 0.25 to 9.50 kb single-stranded RNA (ssRNA) ladder (BRL Life Technologies, Inc., Gaithersburg, MD), brome mosaic virus (BMV) replicative form dsRNA (1,2) (isolated from *Hordeum vulgare* L. 'Henry'), and high molecular weight dsRNA isolated from *Phaseolus vulgaris* L. 'Black Turtle Soup' (BTS) (30) were used as references to size the dsRNA segments obtained from *P. infestans* isolates.

P. infestans dsRNAs, and all reference nucleic acids, were denatured in a formamide/formaldehyde buffer and electrophoresed as outlined by Fourney et al (9). Gels were photographed with Polaroid type 55 film, and an LKB Ultroscan laser densitometer (Pharmacia, LKB Biotechnology, Inc., Piscataway, NJ) was used to scan the resulting negatives to analyze the positions of denatured dsRNAs of *P. infestans* relative to the size standards. Plots subsequently were made, similar to the method of Bozarth and Harley (4), to estimate the sizes of denatured *P. infestans* dsRNA.

Northern blotting. Three micrograms of dsRNA and known molecular weight standards λ *Hind*III digest DNA and 0.25–9.50 kb ladder ssRNA (both BRL Life Technologies, Inc.) were denatured, electrophoresed, and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) membranes as described by Fourney et al (9). After blotting, the membranes were air-dried and baked under vacuum at 80 C for 2 h. The baked blots were stored desiccated before hybridization.

End-labeling of dsRNA. Except where noted, standard procedures and solutions were used as indicated by Sambrook et al (19). Double-stranded RNA to be 5′ end-labeled was treated with DNase I and S_1 nuclease, extracted twice with phenol/chloroform/isoamyl alcohol, and gel-purified from low melting point agarose (BRL Life Technologies, Inc.). Two to four micrograms of dsRNA were alkaline-hydrolyzed and ethanol-precipitated overnight at −20 C. The dsRNA was resuspended in double-distilled H_2O (ddH_2O), denatured at 100 C for 3 min, and quenched in liquid nitrogen for 30 s. Denatured dsRNA was end-labeled using 100 μCi of 5′-(γ-^{32}P) ATP (3,000 Ci/mmol, Amersham Corp., Arlington Heights, IL). The kinase reaction was allowed to proceed overnight at 37 C, after which the dsRNA was precipitated twice at −20 C for 2 h. Specific activity of the labeled dsRNA was determined by TCA precipitation and liquid scintillation counting. Specific activity approaching 1 × 10^6 cpm/μg was obtained for each probe.

Hybridization. Northern blots were prehybridized, hybridized, and washed (low and high stringency) using standard procedures and solutions listed by Sambrook et al (19). The end-labeled ^{32}P probes in ddH_2O were denatured by heating at 100 C for 5 min and quenching on ice for 3 min before their use in hybridization. From 300,000 to 500,000 cpm/ml of denatured dsRNA probe was added to each blot. Autoradiographs were made by exposing Kodak XAR-5 film to the membranes for the appropriate interval at room temperature.

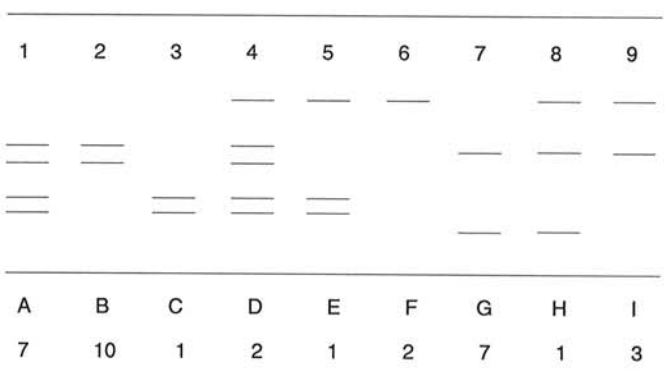

Fig. 1. Graphic representation comparing all of the double-stranded RNA banding patterns found in *Phytophthora infestans* isolates from Mexico, the Netherlands, and Peru. Letters designate dsRNA patterns and numbers represent the number of isolates exhibiting each pattern. Patterns designated A, B, and C were described previously (25).

Transmission electron microscopy of dsRNA. Double-stranded RNA from Mexican *P. infestans* isolate 511 (25) was isolated as described previously and purified by treatment with DNase I and S_1 nuclease. The dsRNA then was extracted twice with equal parts phenol (saturated with ddH_2O) and chloroform/isoamyl alcohol (24:1, v/v), then ethanol-precipitated overnight at −20 C. The dsRNA was pelleted as before, resuspended in sterile ddH_2O, and checked for purity by running an aliquot on a neutral agarose (1.0%) gel.

The balance of the DNase I and S_1 nuclease-treated dsRNA was prepared for transmission electron microscopy using the modified Kleinschmidt method according to Fishel and Warner (8). The grids were examined and dsRNA photographed in a Zeiss 10A transmission electron microscope at 60 kV.

RESULTS

Double-stranded RNA in *P. infestans*. Treatment of extracted dsRNA with S_1 nuclease removed ssRNA contamination and did not affect migration of the dsRNAs relative to untreated controls (data not shown). Typical yields of dsRNA amounted to 1 μg/10 g mycelial fresh weight. Some isolates lost some or all of their dsRNA segments after repeated transfer and storage in the laboratory and had to be regenerated from stock cultures stored in liquid nitrogen to obtain the original dsRNA patterns from them.

Isolates of *P. infestans* from Mexico, the Netherlands, and Peru contained dsRNA (Table 1). The dsRNA occurred most frequently in Mexican (83.3%) isolates, followed by isolates from the Netherlands (20.0%) and Peru (2.9%). Double-stranded RNA was not detected in isolates from the United States or Israel. A total of six new banding patterns (Figs. 1–3) and three new segments (Figs. 1–3) of dsRNA were identified. When added to the segments and patterns described by Tooley et al (25) (Figs. 1 and 2), the totals are nine banding patterns and seven segments (Fig. 1) among all *P. infestans* isolates found to contain dsRNA. For discussion purposes, the dsRNA segments will be referred to as high, top doublet, intermediate, bottom doublet, and low.

A graphical representation of the sizes of *P. infestans* dsRNA segments, relative to the known sizes of dsRNA from BMV and BTS, are shown in Figure 4. The results of scanning laser densitometry of denaturing gel negatives suggested that all high, intermediate, and low bands, whether they occurred in isolates from Mexico (Figs. 2 and 3; patterns D–G), the Netherlands (Figs. 1 and 3; patterns H and I), or Peru (Fig. 3; pattern G), were 11.10, 3.15, and 1.35 kbp, respectively. Likewise, the dsRNA segments that formed the top and bottom doublets (Fig. 2; patterns A–E) in Mexican isolates were 3.25, 2.80, 1.67, and 1.54 kbp in all isolates in which they occurred.

The dsRNA-containing Mexican isolates exhibited seven of the nine banding patterns (Figs. 2 and 3). Four of the seven patterns and three of the dsRNA segments differ from those first found by Tooley et al (25) (Fig. 1). The new segments included an 11.10-kbp segment that occurred alone (Fig. 2), or in conjunction with the top and bottom doublets (Fig. 2), or just in the bottom doublet (Fig. 2). Four of the five Mexican isolates that possessed the 11.10-kbp segment were originally isolated from wild *Solanum demissum* L. plants (Table 1). Minor dsRNA segments occasionally were seen along with the 11.10-kbp segment (Fig. 2, lane 5), but not consistently. The other new segments were the intermediate (3.15 kbp) and low (1.35 kbp) segments (Fig. 3). Double-stranded RNA was found in relatively equal proportions in both A1 and A2 mating-type Mexican isolates (Table 1).

The isolates from the Netherlands possessed dsRNAs in common with the Mexican isolates, namely, the high, intermediate, and low segments (Fig. 3). However, the arrangement of the segments accounted for the two dsRNA patterns not seen in Mexican isolates (Figs. 1 and 3; patterns H and I). All of the dsRNA-containing isolates from the Netherlands exhibited the A2 mating type (Table 1).

The Peruvian isolate positive for dsRNA possessed the same banding pattern (intermediate and low segment; pattern G) found in six of the Mexican isolates (Table 1) (Fig. 3). The intermediate and/or low segments also occurred in isolates from the Netherlands, but only in combination with the high segment (Figs. 1 and 3).

Hybridization analysis. Preliminary hybridization results (data not shown) demonstrated that a probe made to 511 dsRNA (four segments; pattern A) (25) showed sequence homology with comigrating dsRNA segments (top and bottom doublets) in Mexican isolate 578 (pattern D). However, no homology was detected between 511 dsRNA and the high molecular weight dsRNA segment in isolate 578. In this study, end-labeled probes were made using enzyme-treated, gel-purified dsRNAs from Mexican isolates 511 and 622.

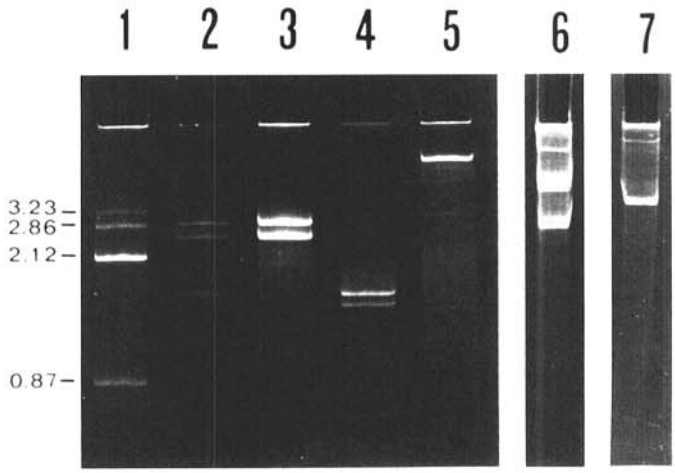

Fig. 2. Banding patterns of double-stranded RNAs from brome mosaic virus (BMV) and Mexican isolates of *Phytophthora infestans* on a 5% polyacrylamide gel after treatment with DNase I and S_1 nuclease. Lane 1, BMV; lane 2, *P. infestans* isolate 511 (pattern A); lane 3, isolate 547 (pattern B); lane 4, isolate 560 (pattern C); lane 5, isolate 580 (pattern F); lane 6, isolate 578 (pattern D); lane 7, isolate 512 (pattern E). The high molecular weight segments and bottom doublets in lanes 6 and 7 are not aligned because the gels were run for different lengths of time when compared with each other and the dsRNA segments in lanes 1–5. Sizes are expressed in kilobase pairs.

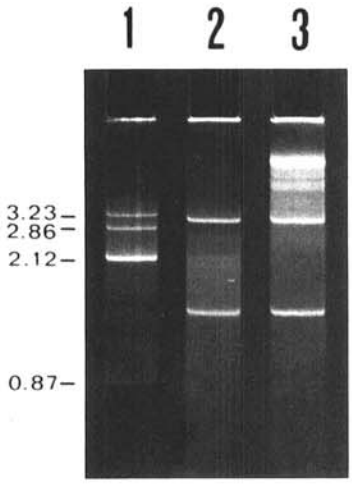

Fig. 3. Banding patterns of double-stranded RNA from brome mosaic virus (BMV) and *Phytophthora infestans* isolates from Mexico, the Netherlands, and Peru on a 5% polyacrylamide gel after DNase I and S_1 nuclease treatment. Lane 1, BMV; lane 2, *P. infestans* isolate 622 (Mexico) (pattern G); lane 3, isolate 1104 (the Netherlands) (pattern H). Peruvian isolate 819 had the same pattern as Mexican isolate 622 in lane 2. Sizes are expressed in kilobase pairs.

The first hybridization experiment involved two individual probes made using all segments of dsRNA from Mexican isolates 511 (four segments; pattern A) and 622 (two segments; pattern G). These isolates do not contain comigrating bands. Duplicate northern blots containing dsRNA segments from isolates 511, 622, 1104 (three segments; pattern H), and 819 (two segments; pattern G) were made. A dsRNA-free Mexican isolate, 506, was used as a negative control on each blot. The dsRNA patterns of the isolates on the blots are shown in Figure 1. One blot was hybridized with the 511 probe and the duplicate blot was hybridized with the 622 probe.

Sequence homology was detected between the 622 probe and the 3.15- and 1.35-kbp segments in Dutch isolate 1104 and Peruvian isolate 819 (Fig. 5). However, the 622 probe did not show homology with the top and bottom doublets of 511, or the high molecular weight segment of 1104 (Fig. 5). The 511 probe only showed homology with itself (Fig. 5). Neither probe hybridized to dsRNA-free isolate 506 (Fig. 5).

The second hybridization experiment was conducted using just the top doublet segments of dsRNA from Mexican isolate 511. The blot to which the probe was hybridized contained all dsRNA segments from Mexican isolates 511 (four segments; pattern A), 533 (two segments; pattern B), 560 (two segments; pattern C), 578 (five segments; pattern D), and 512 (three segments; pattern E). Again, dsRNA-free Mexican isolate 506 was used as a negative control on the blot. The dsRNA patterns of the isolates on the blot are shown in Figure 1.

Sequence homology was detected between the 511 probe and the same size dsRNA segments in Mexican isolates 533 and 578 (Fig. 6). No homology existed between the 511 probe and the bottom doublet segments of 511, 560, 578, and 512, or the high molecular weight segment of 578 and 512 (Fig. 6). The 511 probe did not hybridize to dsRNA-free isolate 506 (Fig. 6).

Electron microscopy of *P. infestans* dsRNA. Transmission electron microscopy of the top and bottom doublet segments from Mexican isolate 511 revealed that the dsRNA is linear (Fig. 7). In addition, four main groups of dsRNA molecules, based on length, were identified on the grids. A background that consisted of many very small pieces of nucleic acid was present on the grids interspersed among dsRNA molecules of the four major groups.

DISCUSSION

Tooley et al (25) first reported dsRNA in a member of the class Oomycetes, *P. infestans*. Three distinct patterns of dsRNA were described in 36% of the Mexican isolates screened. Over 80% of the Mexican isolates surveyed in the current study contained dsRNA, and three new segments and four new patterns were identified. Also, dsRNA was found to be relatively equally distributed among A1 and A2 mating-type isolates. This indicates that the prevalence and diversity of dsRNA among Mexican isolates is much greater than first realized, and suggests that dsRNA-containing isolates of *P. infestans* first appeared in Mexico.

The sizes obtained for the denatured dsRNA segments of the top doublet (3.25 and 2.80 kb) were larger than those reported by Tooley et al (25) for the same two segments (2.75 and 2.50 kb) in a neutral polyacrylamide gel. This was not surprising, as it has been known for some time that mobility of dsRNA in polyacrylamide gels, as a function of molecular weight, is not linear (7). We consider the denatured sizes to be the most accurate to date.

Hybridization experiments showed that sequence homology exists between comigrating dsRNA segments from Mexican, Dutch, and Peruvian isolates of *P. infestans*. In addition, the results indicate that at least four nonhomologous groups of dsRNA are present in *P. infestans*. Based on the current results, the high molecular weight segment represents one group, the top and bottom doublets compose a second and third group, respectively, and the intermediate and low segments are members of a fourth group. Thus, some isolates of *P. infestans*, including 512, 578, and 1104, harbor mixed populations of dsRNA.

An important feature of dsRNA presence in *P. infestans* is the consistency with which the major bands appear. This fact, along with the hybridization results showing a number of isolates harboring mixed infections, raises the possibility that mycoviruses are present in *P. infestans* (5). Except for the high band, the sizes of the dsRNA segments from *P. infestans* are within the range of typical isometric mycovirus dsRNAs (5). Bacilliform particles were seen in nuclei of *P. infestans* by Styer and Corbett (22), but no attempt was made to extract dsRNA from mycelia of the isolates examined. A recent attempt to extract dsRNA from *P. infestans* isolates in which intranuclear particles have been seen was unsuccessful (25). Mycoviruses or viruslike particles never have been found in the cytoplasm of oomycetous fungi (5).

Only seven major segments of dsRNA were associated with *P. infestans* isolates from Mexico, the Netherlands, and Peru. When the top and bottom doublets are considered as single entities (since the two dsRNA segments of each doublet never appeared alone), the number of different segments or combinations of segments drops to five. This is remarkable considering that the isolates

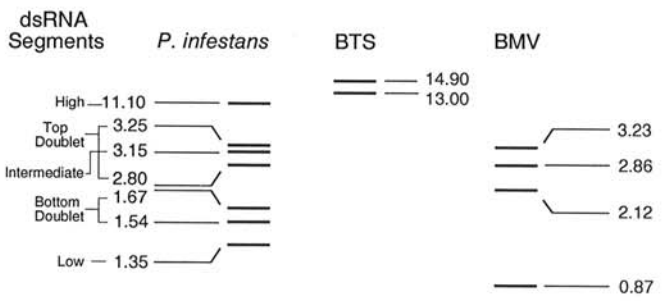

Fig. 4. Graphic representation comparing the sizes of dsRNA segments extracted from *Phytophthora infestans* isolates as determined using agarose (1%) formaldehyde denaturing gels. Brome mosaic virus (BMV) dsRNAs and high molecular weight dsRNAs from the Black Turtle Soup (BTS) cultivar of *Phaseolus vulgaris* were used as size markers.

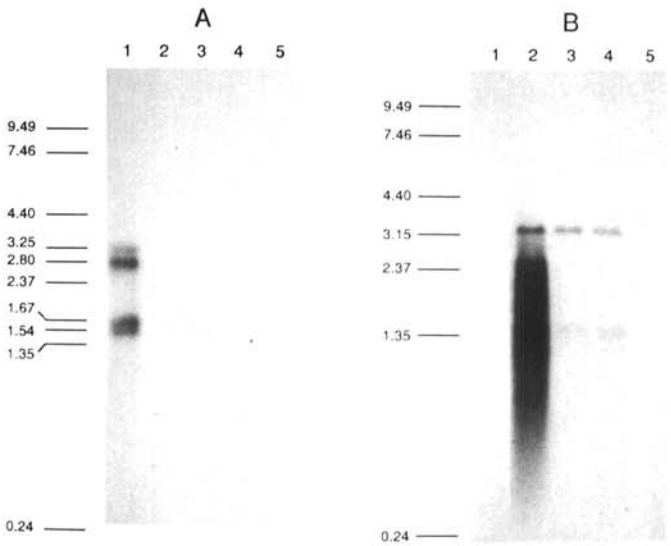

Fig. 5. Results of hybridization studies involving dsRNAs from Mexican, Dutch, and Peruvian isolates of *Phytophthora infestans*. **A,** Northern blot probed with 5′ end-labeled dsRNA from Mexican isolate 511; **B,** northern blot probed with 5′ end-labeled dsRNA from Mexican isolate 622. **A** and **B,** lane 1, *P. infestans* isolate 511 (Mexico) (pattern A); lane 2, isolate 622 (Mexico) (pattern G); lane 3, isolate 1104 (the Netherlands) (pattern H); lane 4, isolate 819 (Peru) (pattern G); lane 5, isolate 506 (Mexico) (dsRNA-free). Migration of the ssRNA segments of the 0.25–9.50 kb RNA ladder on each denaturing gel relative to the *P. infestans* dsRNA segments is shown for reference. Sizes of the *P. infestans* dsRNA segments are expressed in kilobases.

of *P. infestans* found to contain dsRNA represent three different populations of the fungus on two continents. In comparison, dsRNA-containing isolates of *C. parasitica,* even those obtained from the same geographic location, show great variation in banding patterns, usually with no bands in common. Indeed, Van Alfen (29) stated that it has not been possible to classify the multiple segments of dsRNA extracted from hypovirulent isolates of *C. parasitica* into electrophoretic patterns that could represent specific mycoviral types. Clearly, the dsRNA patterns and hybridization results obtained in this study show *P. infestans* to be a good candidate for the development of a dsRNA-type classification scheme. However, the construction of such a system should not be attempted until the presence or absence of mycoviruses associated with the dsRNA is determined.

Transmission electron microscopy studies showed the *P. infestans* dsRNA to be linear. The possibility that the dsRNA was originally a circular form that was nicked and linearized by S_1 nuclease is remote, because the electrophoretic migration of enzyme-treated dsRNA was identical with that of nontreated dsRNA. The small pieces of nucleic acid interspersed among molecules of dsRNA in Figure 7 probably represented degradation products of ribosomal ssRNA that resulted from S_1 nuclease digestion. *C. parasitica* also has been shown to possess linear dsRNA (31) and a unique cytoplasmic viruslike particle (15,16). Although preliminary TEM examination of freeze-substituted dsRNA-containing *P. infestans* hyphae did reveal the presence of suspicious isometric structures in the cytoplasm (J. R. Newhouse, *unpublished*), more work needs to be done before this can be confirmed.

This is the first report of dsRNA in European isolates of *P. infestans.* Since isolates of the A2 mating type first appeared in Europe in the early 1980s (12,13,20,23), speculation has persisted concerning their origin. In this study, dsRNA was found only in A2 mating-type isolates from the Netherlands, and all three segments were identical in size with segments first found in Mexican isolates. In addition, sequence homology was detected between dsRNA from a Mexican isolate and comigrating dsRNA segments from a Dutch isolate. This finding supports the hypothesis that the dsRNA-containing A2 isolates in the Netherlands originally came from Mexico. Other pieces of evidence exist that support this theory. First, A2 mating-type isolates, and the sexual stage of the fungus, initially were discovered in central Mexico decades ago (11) and were not found anywhere else in the world until recently. Also, the A2 mating type in Mexico occurs in approximately equal frequency with A1 mating-type isolates. Indeed, central Mexico is believed to be the center of origin of the *Solanum-P. infestans* pathosystem (17,18,26,28).

Second, Mexican isolates show the greatest incidence and diversity of dsRNA when compared with other isolates of *P. infestans* from around the world, including the Netherlands, strongly suggesting that dsRNA-containing isolates originated in central Mexico. Third, recent work by Tooley and Therrien (27) showed that polyploidy is usually found in *P. infestans* isolates from temperate climates, while diploidy is predominant among sexually reproducing Mexican isolates of the fungus. The dsRNA-containing A2 mating-type isolates from the Netherlands were either diploid or diploid-polyploid heterokaryons (24), making

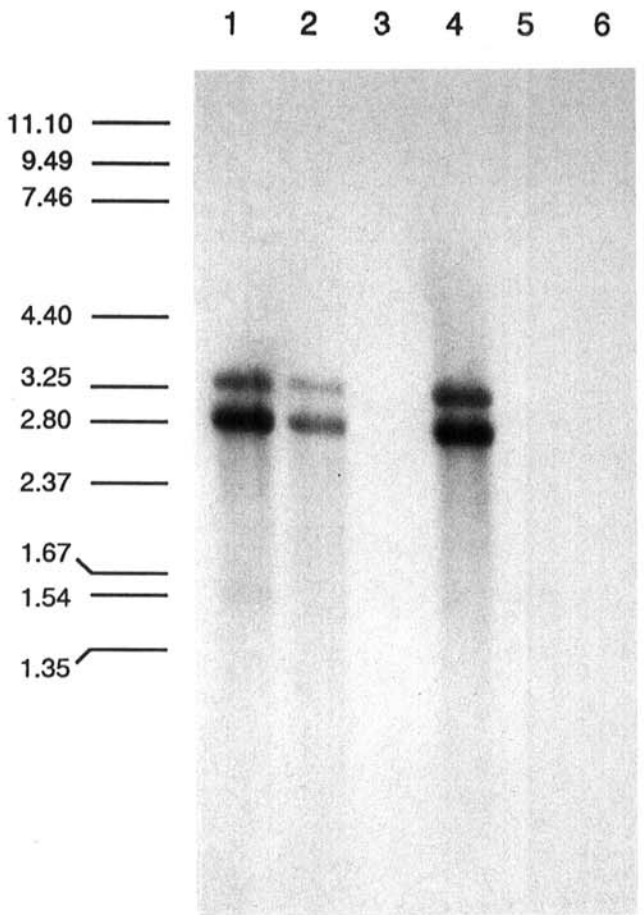

Fig. 6. Results of hybridization study in which dsRNAs from Mexican isolates of *Phytophthora infestans* were probed with 5′ end-labeled top doublet dsRNA from Mexican isolate 511. Lane 1, *P. infestans* isolate 511 (pattern A); lane 2, isolate 533 (pattern B); lane 3, isolate 560 (pattern C); lane 4, isolate 578 (pattern D); lane 5, isolate 512 (pattern E); and lane 6, isolate 506 (dsRNA-free). Migration of the ssRNA segments of the 0.25–9.50 kb RNA ladder on the denaturing gel relative to the *P. infestans* dsRNA segments is shown for reference. Sizes of the *P. infestans* dsRNA segments are expressed in kilobases.

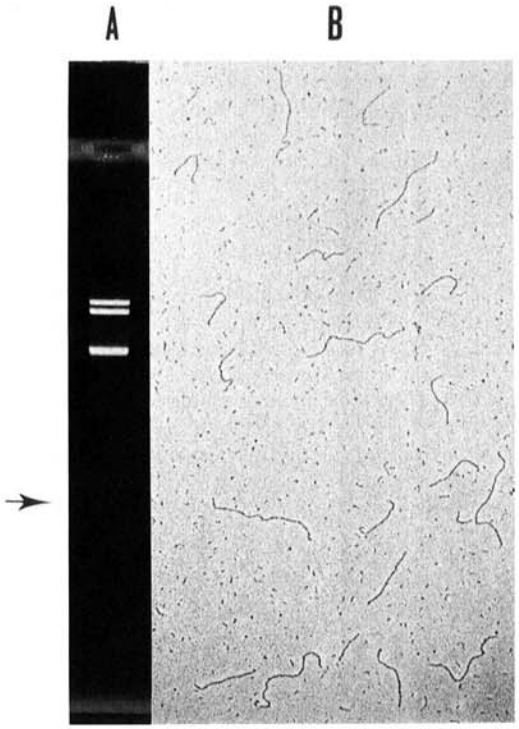

Fig. 7. Transmission electron microscopy of double-stranded RNA from Mexican *Phytophthora infestans* isolate 511. **A,** Aliquot of 511 dsRNA treated with DNase I and S_1 nuclease checked for purity on a neutral agarose (1%) gel. The bottom doublet segments did not resolve on this gel and appear as a single band. The balance of the DNase I- and S_1 nuclease-treated dsRNA was observed in the electron microscope. The arrow marks degradation products observed to be scattered among the dsRNA strands in **B. B,** Ultrastructure of dsRNA segments from isolate 511. The dsRNA is linear and four major lengths of molecules were identified from the micrographs, which was consistent with the number of segments present in isolate 511.

them similar to Mexican isolates in that regard. The dsRNA-free isolates from the Netherlands mostly were A1 mating type (81.3%), and 60% were diploid-polyploid, triploid, or tetraploid heterokaryons, the type of ploidy that would be expected in native Dutch isolates.

Therrien et al (24) recently examined A1 and A2 mating-type *P. infestans* isolates from the Netherlands that had been isolated before 1987 for metalaxyl resistance. Included in their work were all but one of the isolates from the Netherlands examined for dsRNA content in this study. All of the dsRNA-containing A2 mating-type isolates from the Netherlands were metalaxyl-sensitive, whereas 80% of the dsRNA-free isolates from the Netherlands were metalaxyl-resistant. The remaining 20% of dsRNA-free isolates that were metalaxyl-sensitive also were of the A2 mating type. Since our results suggest that dsRNA-containing A2 mating-type isolates from the Netherlands originally came from Mexico, this would support the hypothesis that A2 mating types appeared in the Netherlands after the emergence of metalaxyl resistance, which occurred following extensive metalaxyl use after a late blight outbreak in 1980 (6).

This also is the first report of dsRNA in a Peruvian isolate of *P. infestans*. Again, both dsRNA segments found in the Peruvian isolate were identical in size with two segments initially discovered in a Mexican isolate, and sequence homology was detected between comigrating Mexican and Peruvian dsRNA segments. These results suggest a Mexican origin for the Peruvian isolate.

In this study, we have demonstrated the potential of dsRNA as an epidemiological marker for determining the international movement of an important plant pathogen. Certainly, A2 mating-type isolates of *P. infestans* from other parts of the world, such as Egypt (20), England (23), and Japan (14), should be screened for dsRNA in an attempt to determine their origins. Considering the widespread occurrence of dsRNA in fungi, usually with no apparent deleterious effects, this type of molecular tracking system may be possible for other pathosystems.

LITERATURE CITED

1. Ahlquist, P., Dasgupta, R., and Kaesberg, P. 1984. Nucleotide sequence of the brome mosaic virus genome and its implications for viral replication. J. Mol. Biol. 172:369-383.
2. Ahlquist, P., Lucknow, V., and Kaesberg, P. 1981. Complete nucleotide sequence of brome mosaic virus RNA3. J. Mol. Biol. 153:23-38.
3. Anagnostakis, S. L., and Day, P. R. 1979. Hypovirulence conversion in *Endothia parasitica*. Phytopathology 69:1226-1229.
4. Bozarth, R. F., and Harley, E. H. 1976. The electrophoretic mobility of double-stranded RNA in polyacrylamide gels as a function of molecular weight. Biochim. Biophys. Acta 432:329-335.
5. Buck, K. W. 1986. Fungal virology–An overview. Pages 1-84 in: Fungal Virology. K. W. Buck, ed. CRC Press, Boca Raton, FL.
6. Davidse, L. C., Looijen, D., Turkensteen, L. J., and Van Der Wal, D. 1981. Occurrence of metalaxyl-resistant strains of *Phytophthora infestans* in Dutch potato fields. Neth. J. Plant Pathol. 87:65-68.
7. Dodds, J. A., Morris, T. J., and Jordan, R. L. 1984. Plant viral double-stranded RNA. Annu. Rev. Phytopathol. 22:151-168.
8. Fishel, R. A., and Warner, R. C. 1986. Novel dimeric configurations from bacteriophage G4 replicative form DNA. Virology 148:198-209.
9. Fourney, R. M., Miyakoshi, J., Day, R. S., and Paterson, M. C. 1988. Northern blotting: Efficient RNA staining and transfer. Focus 10:5-7.
10. Fulbright, D. W. 1984. Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. Phytopathology 74:722-724.
11. Gallegly, M. E., and Galindo, J. 1958. Mating types and oospores of *Phytophthora infestans* in nature in Mexico. Phytopathology 48:274-277.
12. Hohl, H. R., and Iselin, K. 1984. Strains of *Phytophthora infestans* with A_2 mating type behavior. Trans. Br. Mycol. Soc. 83:529-531.
13. Malcolmson, J. F. 1985. *Phytophthora infestans:* A_2 compatibility type recorded in Great Britain. Trans. Br. Mycol. Soc. 85:531.
14. Mosa, A., Kato, M., Sato, N., Kobayashi, K., and Ogoshi, A. 1989. Occurrence of the A_2 mating type of *Phytophthora infestans* on potato in Japan. Ann. Phytopathol. Soc. Jpn. 55:615-620.
15. Newhouse, J. R., Hoch, H. C., and MacDonald, W. L. 1983. The ultrastructure of *Endothia parasitica:* Comparison of a virulent with a hypovirulent isolate. Can. J. Bot. 61:389-399.
16. Newhouse, J. R., MacDonald, W. L., and Hoch, H. C. 1990. Virus-like particles in hyphae and conidia of European hypovirulent (dsRNA-containing) strains of *Cryphonectria parasitica*. Can. J. Bot. 68:90-101.
17. Niederhauser, J. S., and Cobb, W. C. 1959. The late blight of potatoes. Sci. Am. 200:100-112.
18. Reddick, D. 1939. Whence came *Phytophthora infestans?* Chron. Bot. 5:410-412.
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
20. Shaw, D. S., Fyfe, A. M., Hibberd, P. G., and Abdel-Sattar, M. A. 1985. Occurrence of the rare A_2 mating type of *Phytophthora infestans* on imported Egyptian potatoes and the production of sexual progeny with A_1 types from the U.K. Plant Pathol. 34:552-556.
21. Smith, O. P. 1989. Small-scale preparation of RNase-free DNase. Biotechniques 6:846-847.
22. Styer, E. L., and Corbett, M. K. 1978. Intranuclear virus-like particles of *Phytophthora infestans* and *P. parasitica* var. *parasitica*. Mycovirus Newsl. (6):16-18.
23. Tantius, P. M., Fyfe, A. M., Shaw, D. S., and Shattock, R. C. 1986. Occurrence of the A_2 mating type and self fertile isolates of *Phytophthora infestans* in England and Wales. Plant Pathol. 35:578-581.
24. Therrien, C. D., Ritch, D. L., Davidse, L. C., Jespers, B. K., and Spielman, L. J. 1989. Nuclear DNA content, mating type and metalaxyl sensitivity of eighty-three isolates of *Phytophthora infestans* from the Netherlands. Mycol. Res. 92:140-146.
25. Tooley, P. W., Hewings, A. D., and Falkenstein, K. F. 1989. Detection of double-stranded RNA in *Phytophthora infestans*. Phytopathology 79:470-474.
26. Tooley, P. W., Sweigard, J. A., and Fry, W. E. 1986. Fitness and virulence of *Phytophthora infestans* isolates from sexual and asexual populations. Phytopathology 76:1209-1212.
27. Tooley, P. W., and Therrien, C. D. 1987. Cytophotometric determination of the nuclear DNA content of 23 Mexican and 18 non-Mexican isolates of *Phytophthora infestans*. Exp. Mycol. 11:19-26.
28. Tooley, P. W., Therrien, C. D., and Ritch, D. L. 1989. Mating type, race composition, nuclear DNA content, and isozyme analysis of Peruvian isolates of *Phytophthora infestans*. Phytopathology 79:478-481.
29. Van Alfen, N. K. 1986. Hypovirulence of *Endothia* (*Cryphonectria*) *parasitica* and *Rhizoctonia solani*. Pages 143-162 in: Fungal Virology. K. W. Buck, ed. CRC Press, Boca Raton, FL.
30. Wakarchuk, D. A., and Hamilton, R. I. 1985. Cellular double-stranded RNA in *Phaseolus vulgaris*. Plant Mol. Biol. 5:55-63.
31. Wheeler, R. A. 1986. The structure and chemical composition of the dsRNA associated with strain 43 of *Endothia parasitica*. Ph.D. dissertation. West Virginia University, Morgantown.