Strawberry Pallidosis Disease: Distinctive dsRNA Species Associated with Latent Infections in Indicators and in Diseased Strawberry Cultivars

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ABSTRACT

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Double-stranded RNA (dsRNA) was extracted from *Fragaria* plants and analyzed by electrophoresis on 5% polyacrylamide gel, followed by silver stain. The dsRNA band patterns were determined for a virus-tested clone of strawberry cultivar Northwest and for nongrafted plants of several common strawberry virus indicators, including seedling line Alpine of *Fragaria vesca* var. *semperflorens* and cultivars UC-10, UC-11, and UC-12 of *F. virginiana*. No dsRNA bands in the M_r range above 4×10^6 were found in these plants. On the other hand, plants of indicator cultivars UC-4, UC-5, and UC-6 of *F. vesca*, maintained in our greenhouse and thought to be virus free, contained two dsRNA bands of M_r 4.3 and 4.6×10^6 similar to those found in plants infected with known pallidosis isolate Rip 157. Some smaller dsRNAs also occurred in each case. DsRNA bands of M_r 4.3 and 4.6×10^6 were found in Alpine seedlings after leaf grafting with leaflets of our clones of UC-4, UC-5, and UC-6. UC-10 plants grafted with leaflets from these same clones of UC-4, UC-5, and UC-6 plants developed symptoms of leaf distortion and chlorosis that were very mild but typical of pallidosis disease, suggesting that these clones were contaminated with pallidosis agent. Eight additional pallidosis isolates induced leaf distortion, chlorosis, and dwarfing in UC-10 plants but no obvious symptoms in UC-4, UC-5, UC-6, or seedling Alpine plants. All eight pallidosis isolates examined had two to four dsRNAs in the M_r range of 4.3 to 5.2×10^6 and, depending on the isolate, one to 10 dsRNAs ranging from M_r 1.0 to 2.3×10^6. Until now, pallidosis agent has been detected in strawberry plants only by graft indexing in the absence of viruses that would confound or obscure the symptomatology associated with the pallidosis agent. The electrophoretic band patterns of dsRNA associated with pallidosis are sufficiently distinct to permit its tentative identification in strawberry plants with multiple virus infections.

Strawberry pallidosis, a graft-transmissible, viruslike disease, was described by Frazier and Stubbs in 1969 (10). Additional infections have been reported to occur in the United States, Australia, and Canada (5,10–12). Although pallidosis is latent in many commercial strawberry cultivars, it has been suggested that pallidosis can reduce plant vigor when combined with additional infections by certain strawberry viruses (10,11,18). Other than its graft transmissibility, little is known about the causal agent of pallidosis, except for its possible transmission by the leafhopper *Coelidia olitoria* (Say) (8).

Indicator plants of *Fragaria vesca* L., commonly used to detect many strawberry viruses, do not produce obvious symptoms when affected with pallidosis (7). On the contrary, certain indicator plants of *F. virginiana* Duchesne affected with pallidosis develop symptoms (7,11). At present, a graft-transmissible entity producing symptoms of leaf distortion, chlorosis, and/or dwarfing in plants of *F. virginiana* but not in plants of *F. vesca* is assumed to be the pallidosis-inducing agent (7,11).

Double-stranded RNA (dsRNA) from some affected flowering plants has given electrophoretic band patterns that suggest viral etiology in diseased plants in which virus particles cannot be detected easily (6). The technique also has been useful as a detection method for RNA viruses (6,13,15). In this paper, we report the dsRNA found in pallidosis-affected *Fragaria* plants. A portion of this work has appeared in abstract form (21).

MATERIALS AND METHODS

Indicator plants. Seedling line Alpine of *F. vesca* var. *semperflorens* (Duchesne) Ser. and *F. virginiana* 'UC-10' (7), maintained at Oregon State University, were used as indicator plants. The plants grafted with pallidosis isolates were kept in the greenhouse for 2 mo for symptom observation. In addition to these plants, indicator cultivars UC-4, UC-5, and UC-6 of *F. vesca*, cultivars UC-11 and UC-12 of *F. virginiana* (7), and the cultivated strawberry *F.* × *ananassa* Duchesne. 'Northwest,' maintained at Oregon State University, and UC-5 and UC-10 plants, kindly supplied by Driscoll Strawberry Associates in Watsonville, CA, were used as sources for dsRNA analysis.

Eight pallidosis isolates were used in this study: 1) Rip 157, 2) CK 6-1, 3) Old EMK, 4) 119 V.Will., 5) BM-2 (these five isolates were kindly supplied by N. W. Frazier (University of California, Davis) and were maintained in plants of *F. vesca* or *F. virginiana*), 6) G-SO1A, from the strawberry cultivar Guelph SO1 from Ontario, Canada, 7) G-SO2A, from the strawberry cultivar Guelph SO2 from Ontario, Canada, and 8) Ozark Beauty, from an Arkansas clone of this cultivar. The last three pallidosis isolates were identified at Oregon State University, Corvallis.

DsRNA extraction and gel electrophoresis. DsRNA was extracted from pallidosis-grafted or nongrafted *Fragaria* plants by using a modification of the method of Morris and Dodds (17). A sample of leaf tissue (10 g) frozen at −70 C was ground to a powder in a chilled mortar and pestle. The powder then was mixed with 20 ml of 2 × STE (50 mM Tris, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.0), 12 ml

of water-saturated phenol, 8 ml of chloroform, 0.2 g of sodium dodecyl sulfate, and 0.2 ml of 2-mercaptoethanol using a tissue homogenizer (Polytron, Brinkmann Instruments Co., Westbury, NY). The extract was stirred for 1 hr at 4 C, then centrifuged at 6,500 *g* for 15 min. The aqueous phase was collected, and ethanol was added to a final concentration of 16%. Whatman CF-11 cellulose powder (1.2 g) was added, and the mixture was stirred for 1 hr at 4 C. The cellulose with bound dsRNA was batch washed three times by centrifugation at 2,500 *g* for 5 min with 20 ml of STE-16% ethanol. The resulting suspension was poured into a small column and washed with 200 ml of STE-16% ethanol. The dsRNA was eluted with 20 ml of STE, incubated with 10 units/ml of T1-RNase (Sigma Chemical Co., St. Louis, MO) for 30 min at 37 C, and then digested with 2 μg/ml of DNase I (Sigma) for 45 min at 37 C. The sample was adjusted to 16% ethanol, and CF-11 cellulose powder (1 g) was added. After being stirred for 1 hr at 4 C, the suspension was poured into a small column and washed with 150 ml of STE-16% ethanol. The dsRNA was eluted with 8 ml of STE and concentrated by precipitation with 20 ml of cold ethanol. After centrifugation at 10,000 *g* for 10 min, the pellet was suspended in 0.4 ml of STE and put into an Eppendorf tube, and 1 ml of cold ethanol was added. The sample was centrifuged and dried, and the dsRNA was resuspended in 10 μl of TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4) containing 20% glycerol and 0.1% bromophenol blue.

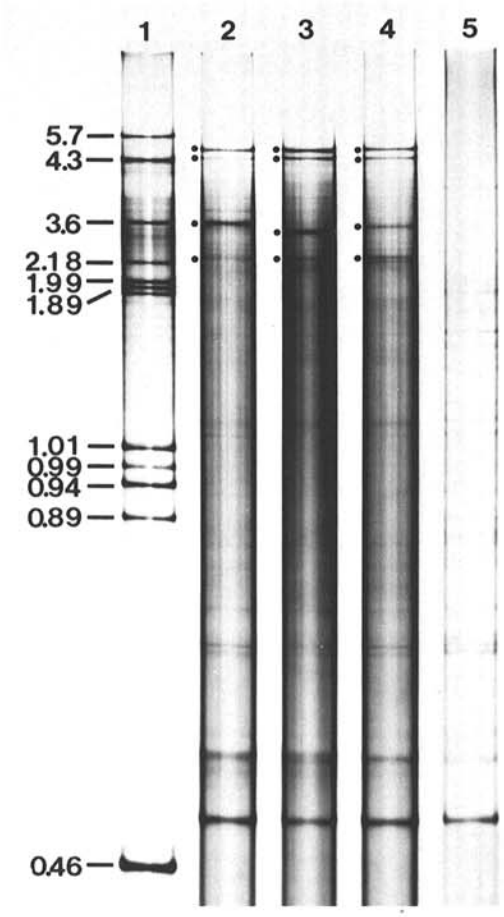

Fig. 1. Electrophoresis in a 5% polyacrylamide gel of dsRNA from nongrafted indicator plants of *Fragaria vesca*. Lane 1, relative molecular weight standards ($M_r \times 10^{-6}$) consisting of a mixture of dsRNA of *Bipolaris maydis*, tobacco mosaic virus, *Geotrichum candidum*, *Penicillium chrysogenum*, and *P. brevicompactum*; lane 2, plants of *F. vesca* 'UC-4'; lane 3, plants of *F. vesca* 'UC-5'; lane 4, plants of *F. vesca* 'UC-6'; and lane 5, plants of *F. vesca* 'Alpine'. • marks the positions of the dsRNA bands found in UC-4, UC-5, and UC-6, but not in Alpine plants. Samples were analyzed by electrophoresis for 17 hr at 100 V and stained with silver nitrate.

The dsRNA from 5 or 10 g of leaf tissue was electrophoresed on a 5% polyacrylamide gel (1.5 mm × 13.5 cm × 14 cm) in a vertical slab gel apparatus in TAE buffer. Electrophoresis was at a constant voltage of 100 V for 17 hr at 4 C, and gels were stained with silver nitrate according to the method of Schumacher et al (19). DsRNA extraction and gel electrophoresis were performed on at least three subsamples in each case.

Tobacco mosaic virus (TMV) dsRNA (M_r 4.3 × 10^6) and brome

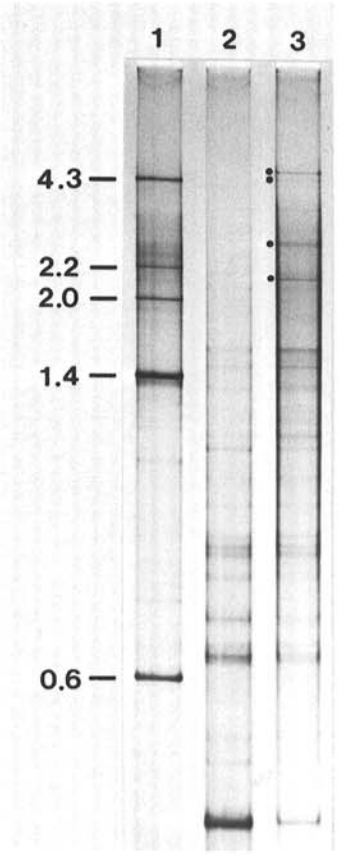

Fig. 2. Graft transmission of dsRNA in plants of *Fragaria vesca* 'UC-4' to plants of *F. vesca* 'Alpine'. Lane 1, relative molecular weight standards ($M_r \times 10^{-6}$) consisting of a mixture of dsRNA of tobacco mosaic virus and brome mosaic virus; lane 2, dsRNA from nongrafted Alpine plants; lane 3, dsRNA from Alpine plants grafted with UC-4 leaflets. • marks the positions of dsRNA bands transmitted by grafting. Samples were electrophoresed in a 5% polyacrylamide gel for 17 hr at 100 V and stained with silver nitrate.

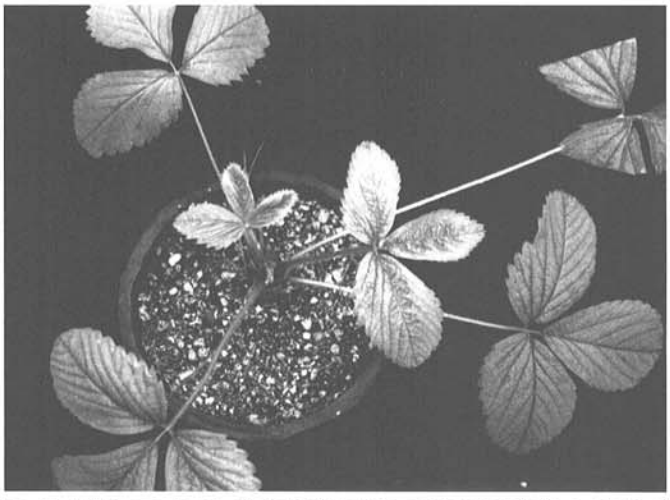

Fig. 3. Mild symptoms of leaf distortion and chlorosis in a plant of *Fragaria virginiana* 'UC-10' grafted with leaflets from a nongrafted clone of *F. vesca* 'UC-5' from Oregon State University.

mosaic virus (BMV) dsRNA (M_r 2.2, 2.0, 1.4, and 0.6 $\times$ 10^6) were extracted from infected tobacco and barley plants, respectively, and used as relative molecular weight standards. The dsRNA of mycoviruses from *Penicillium chrysogenum* Thom (M_r 2.18, 1.99, and 1.89 $\times$ 10^6) (20), *P. brevicompactum* (Dierckx) (= *P. stoloniferum* Thom (M_r 1.01, 0.99, 0.94, 0.89, and 0.46 $\times$ 10^6) (2), *Bipolaris maydis* (Nisikado G. Miyaki) Shoemaker (= *Helminthosporium maydis* Nisiki) (M_r 5.7 $\times$ 10^6) (1), kindly supplied by R. F. Bozarth (Indiana State University, Terre Haute, IN), and *G. candidum* Link (M_r 3.6 $\times$ 10^6) (16) also were used as relative molecular weight standards.

RESULTS

DsRNA in nongrafted indicator plants. We evaluated dsRNA from our local clones of nongrafted, apparently healthy indicator clones of *F. vesca* and *F. virginiana*. Unexpectedly, our local clones of plants of *F. vesca* 'UC-4,' 'UC-5,' and 'UC-6,' but not Alpine seedling plants, contained two dsRNA with M_r of 4.3 and 4.6 $\times$ 10^6, similar to those found in pallidosis-infected plants, and some smaller dsRNA (Fig. 1). All of these dsRNA were found in Alpine seedlings after leaf grafting with leaflets from our local, nongrafted clone of UC-5 (Fig. 2).

When leaves from our local, nongrafted UC-4, UC-5, or UC-6 plants were grafted to plants of *F. virginiana* 'UC-10,' mild symptoms of leaf distortion and chlorosis appeared on them (Fig. 3). UC-10 plants grafted with Alpine leaves showed no obvious symptoms. These results indicate that our nongrafted local clones of UC-4, UC-5, and UC-6 were contaminated with pallidosis agent, although the symptoms induced by the isolates from these plants were much milder than those of known pallidosis isolates. In contrast to our indicator clones of *F. vesca*, our local clones of *F. virginiana* 'UC-10,' 'UC-11,' and 'UC-12' contained no dsRNA in the M_r range of 4.3 to 5.2 $\times$ 10^6 (Fig. 4). These dsRNA could not be detected in UC-5 and UC-10 plants from Driscoll Strawberry Associates (Fig. 4).

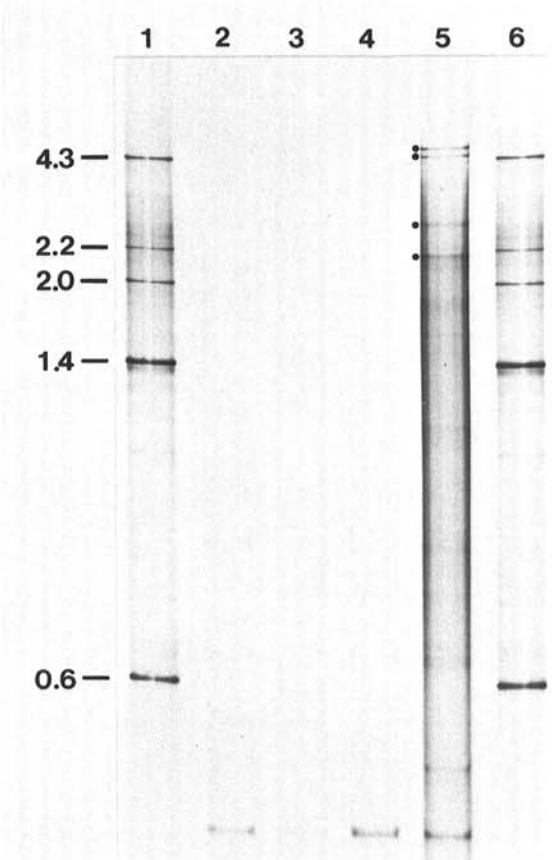

Fig. 4. Electrophoresis in a 5% polyacrylamide gel of dsRNA from nongrafted *Fragaria* plants. Lanes 1 and 6, relative molecular weight standards (M_r $\times$ 10^{-6}) consisting of a mixture of dsRNA of tobacco mosaic virus and brome mosaic virus; lanes 2 and 3, plants of *F. virginiana* 'UC-10' from Driscoll Strawberry Associates and from Oregon State University, respectively; lanes 4 and 5, plants of *F. vesca* 'UC-5' from Driscoll Strawberry Associates and from Oregon State University, respectively. • marks the position of the dsRNA associated with pallidosis. Samples were analyzed by electrophoresis for 17 hr at 100 V and stained with silver nitrate.

A number of faint dsRNA bands with M_r <3 $\times$ 10^6 were found in several nongrafted *Fragaria* clones, *F. vesca* 'Alpine' (Fig 5, lane 2), clone UC-10 of *F. virginiana* (Fig. 5, lane 3), and *F.* $\times$ *ananassa* 'Northwest' (Fig. 5, lane 4). Because these bands had comparatively low relative molecular weights, it was easy to distinguish them from pallidosis-associated dsRNA bands.

Identity of the pallidosis agent. Eight additional pallidosis isolates were grafted to indicator plants to determine that they were identified correctly and to reduce the probability that they were contaminated with other known strawberry viruses (3,4). The reactions of indicator plants to each pallidosis isolate are summarized in Table 1. All eight pallidosis isolates induced leaf distortion, chlorosis, and dwarfing on plants of *F. virginiana* 'UC-10,' as exemplified by the isolate G-SO2A in Figure 6. In contrast, plants of *F. vesca* 'UC-4,' 'UC-5,' 'UC-6,' and 'Alpine' when grafted with these pallidosis isolates produced no obvious symptoms (Table 1). The types and severities of symptoms on UC-10 plants were similar for all isolates. These results indicate that all eight of these isolates fit the definition of the pallidosis agent (10,11) and are free from other viruses reported in strawberry (3,4,7).

DsRNA in pallidosis-infected plants. We extracted dsRNA from UC-10 plants infected with three different pallidosis isolates (Rip 157, G-SO2A, and Ozark Beauty) and then electrophoresed them on 5% polyacrylamide gel. Several dsRNA bands in the >M_r 4.3 $\times$ 10^6 range, which could not be found in uninfected UC-10 plants, were detected from pallidosis-infected plants (Fig. 7, lanes 3–5). The slowest-migrating doublet bands were in common in these three different isolates, with an M_r of 4.6 $\times$ 10^6, as determined from their mobility relative to marker dsRNA (Fig. 8). Several smaller dsRNA bands were not shared among these three isolates, although such bands occurred reproducibly for any given isolate. For example, two bands (M_r 2.2 and 2.1 $\times$ 10^6 were found for Rip 157, five bands (M_r 1.7, 1.65, 1.45, 1.25, and 1.0 $\times$ 10^6) for G-SO2A, and three bands (M_r 1.7, 1.55, and 1.45 $\times$ 10^6) for Ozark Beauty (Fig. 7, lanes 3, 4, and 5, respectively).

The dsRNA samples from all eight pallidosis isolates were electrophoresed in the same polyacrylamide gel (Fig. 5). All pallidosis isolates had two to four dsRNA bands with M_r ranging from 4.3 to 5.2 $\times$ 10^6 (Fig. 5, lanes 6–13). As was the case in Figure 7, smaller dsRNA bands ranging from M_r 1.0 to 2.3 $\times$ 10^6 also showed great variation in number and size, depending on the isolate (Fig. 5, lanes 6–13).

DISCUSSION

Frazier and Stubbs (10) reported that pallidosis spread in the greenhouse in California. Frazier (9) also reported that all clones

TABLE 1. Reactions of indicator plants to eight pallidosis isolates

	Reaction of indicator plants[a]				
	Fragaria vesca				F. virginiana
Pallidosis isolates	UC-4	UC-5	UC-6	Alpine	UC-10
Rip 157	–	–	–	–	+++
CK 6-1	–	–	–	–	+++
Old EMK	–	–	–	–	+++
119 V.Will.	–	–	–	–	+++
BM-2	–	–	–	–	+++
G-SO1A	–	–	–	–	+++
G-SO2A	–	–	–	–	+++
Ozark Beauty	–	–	–	–	+++

[a]– = no obvious symptoms; +++ = severe leaf distortion, chlorosis, and dwarfing.

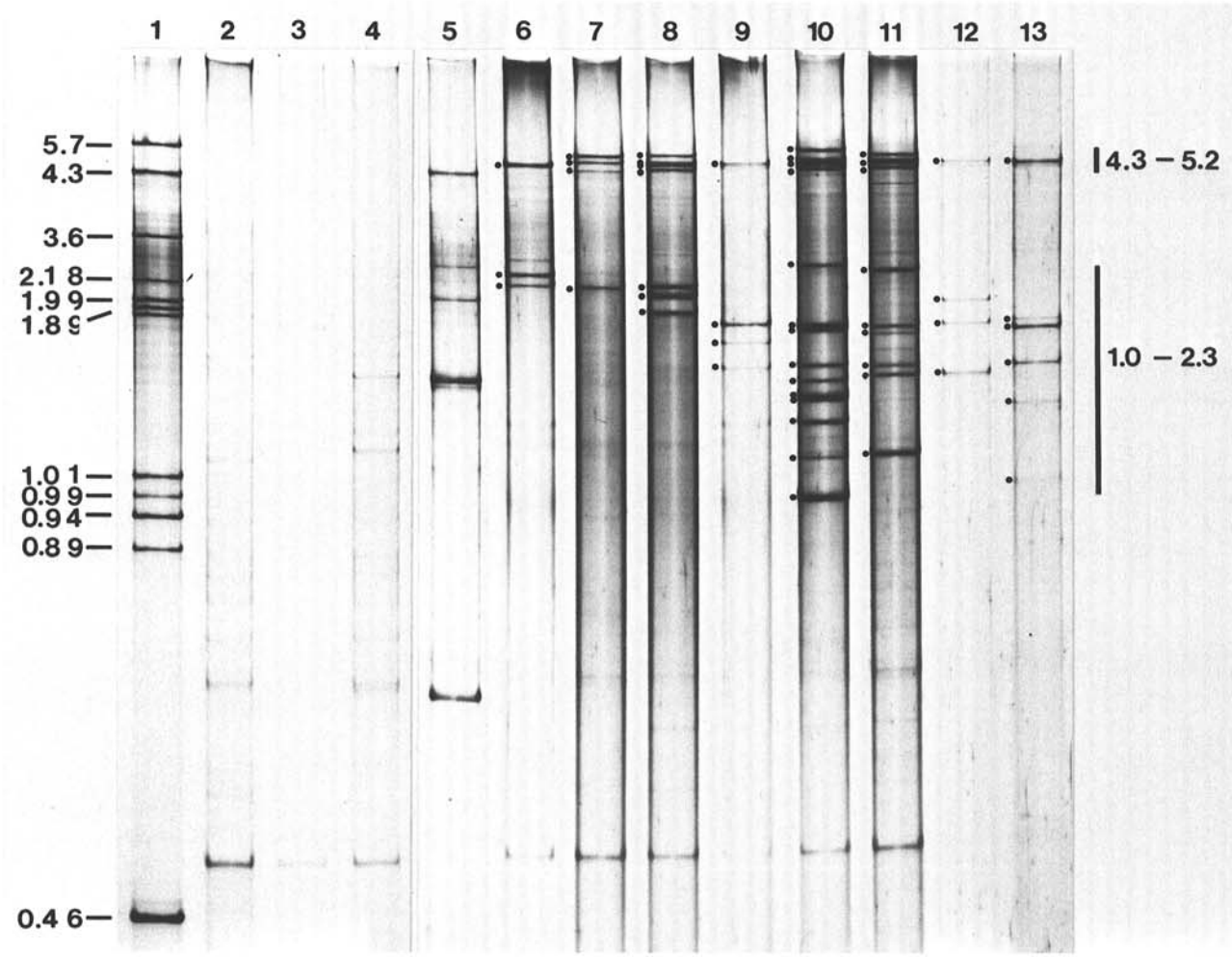

Fig. 5. Electrophoresis in a 5% polyacrylamide gel of dsRNA from plants infected with eight different pallidosis isolates. Lane 1, relative molecular weight standards ($M_r \times 10^{-6}$) consisting of a mixture of dsRNA of *Bipolaris maydis*, tobacco mosaic virus (TMV), *Geotrichum candidum*, *Penicillium chrysogenum*, and *P. brevicompactum*; lane 2, nongrafted plants of *Fragaria vesca* 'Alpine'; lane 3, nongrafted plants of *F. virginiana* 'UC-10'; lane 4, nongrafted plants of *F.* × *ananassa* 'Northwest'; lane 5, size standards (a mixture of dsRNA of TMV and brome mosaic virus); lane 6, UC-10 plant infected with isolate Rip 157; lane 7, plant of *F. vesca* 'UC-6' infected with isolate CK 6-1; lane 8, plant of *F. vesca* 'EMK' infected with isolate Old EMK; lane 9, UC-10 plant infected with isolate Ozark Beauty; lane 10, plant of *F. vesca* 'UC-5' infected with isolate BM-2; lane 11, plant of *F. vesca* infected with isolate 119 V.Will.; lane 12, plant of *F.* × *ananassa* 'Guelph SO1' infected with isolate G-SO1A; and lane 13, plant of *F.* × *ananassa* 'Guelph SO2' infected with isolate G-SO2A. • marks the positions of the reproducible dsRNA bands found in pallidosis-infected plants. Samples were analyzed by electrophoresis for 17 hr at 100 V and stained with silver nitrate.

of *F. vesca* 'UC-4,' 'UC-5,' 'UC-6,' and several Alpine plants, but not *F. virginiana* 'UC-10,' 'UC-11,' and 'UC-12,' used at six California laboratories contained pallidosis. Our results on dsRNA analysis of nongrafted *Fragaria* indicator plants were consistent with these reports; that is, our nongrafted clones of UC-4, UC-5, and UC-6 used in this study were contaminated with pallidosis agent and contained two dsRNA with M_r of 4.3 and 4.6×10^6 similar to those found in the other eight pallidosis isolates studied. Conversely, clones of UC-10, UC-11, and UC-12 did not have dsRNA in that same M_r range. The clones of UC-4, UC-5, and UC-6 maintained in our greenhouse at Oregon State University may already have had pallidosis when we received them. It is noteworthy that the UC-5 plants that we received from Driscoll Strawberry Associates were free from dsRNA associated with pallidosis disease but did not differ in their symptomatology from UC-5 plants containing the pallidosis agent when graft inoculated in our greenhouse with common aphid-borne strawberry viruses.

All eight pallidosis isolates used in this study fit Frazier's original definition of pallidosis (10,11), in that they do not produce symptoms on UC-4, UC-5, UC-6, and Alpine but produce severe symptoms on UC-10 plants. These results also indicate that the pallidosis isolates that we studied were not contaminated with known strawberry viruses (3,4,7). However, each of these pal-

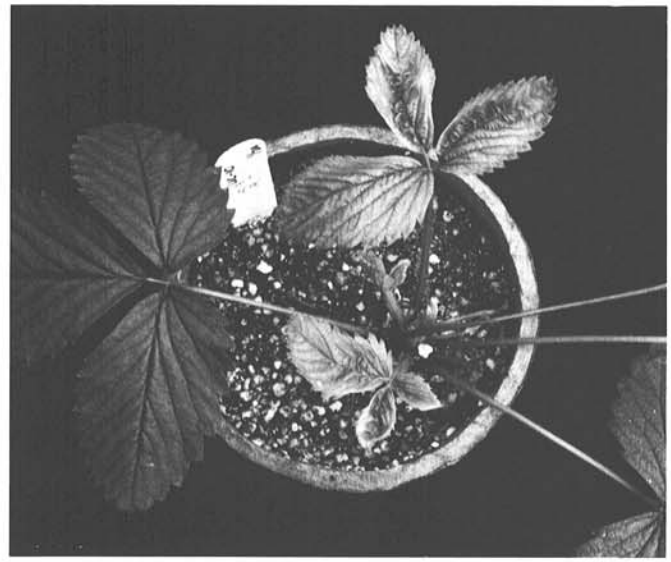

Fig. 6. Symptoms in a plant of *Fragaria virginiana* 'UC-10' infected with pallidosis (isolate G-SO2A) showing leaf distortion, chlorosis, and dwarfing.

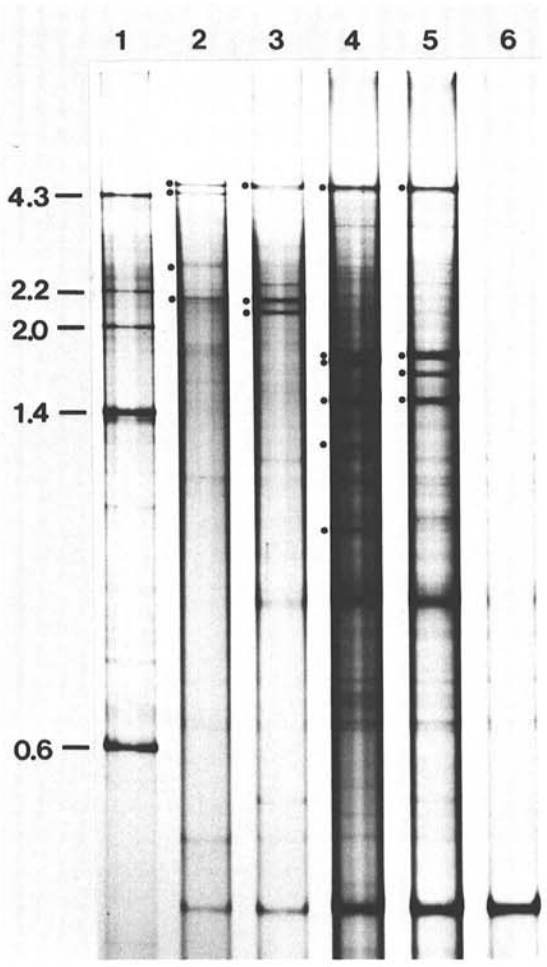

Fig. 7. Electrophoresis in a 5% polyacrylamide gel of dsRNA from three plants of *Fragaria virginiana* 'UC-10,' each infected with a different pallidosis isolate. Lane 1, relative molecular weight standards ($M_r \times 10^{-6}$) consisting of a mixture of dsRNA of tobacco mosaic virus and brome mosaic virus; lane 2, dsRNA from nongrafted plants of *F. vesca* 'UC-4'; lanes 3, 4, and 5, dsRNA from UC-10 plants infected with isolates Rip 157, GSO2A, and Ozark Beauty, respectively; and lane 6, dsRNA from nongrafted UC-10 plants. • marks the positions of the reproducible dsRNA bands found in pallidosis-affected plants. Samples were analyzed by electrophoresis for 17 hr at 100 V in 40 mM Tris, 20 mM sodium acetate, and 2 mM ethylenediaminetetraacetic acid, pH 7.4, and stained with silver nitrate.

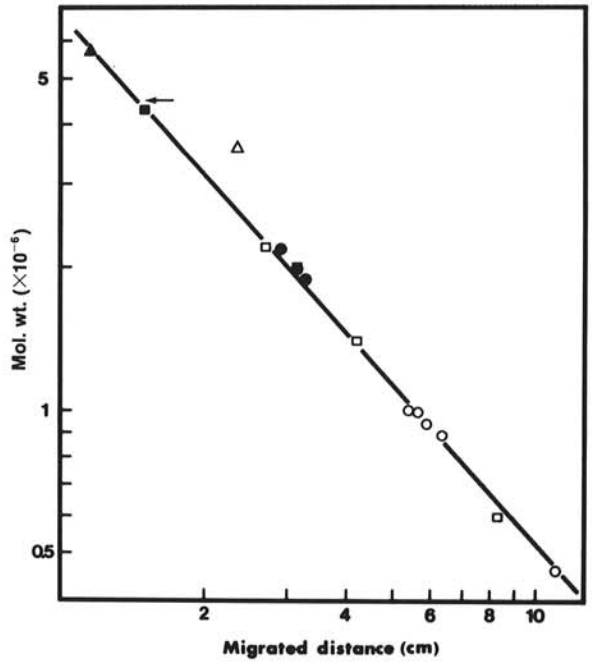

Fig. 8. Relative molecular weight determinations of dsRNA from pallidosis-infected plants. The logarithms of relative molecular weights are plotted versus the logarithms of the electrophoretic mobilities of dsRNA. △ = *Bipolaris maydis* (M_r 5.7 × 10^6); ■ = tobacco mosaic virus (M_r 4.3 × 10^6); △ = *Geotrichum candidum* (M_r 3.6 × 10^6); • = *Penicillium chrysogenum* (M_r 2.18, 1.99, and 1.89 × 10^6); □ = brome mosaic virus (M_r 2.2, 2.0, 1.4, and 0.6 × 10^6); and ○ = *P. brevicompactum* (M_r 1.01, 0.99, 0.94, 0.89, and 0.46 × 10^6). Arrow indicates the M_r of dsRNA common to the three pallidosis isolates in Figure 7.

lidosis isolates produced similar symptoms on leaf-grafted UC-10, making it difficult for us to classify them by their symptom production on this indicator plant.

Although strawberry pallidosis is thought to be a virus disease because it is graft transmissible, there are few reports about the causal agent. Henriques and Schlegel (14) reported long, flexuous, viruslike particles in the phloem cells of a pallidosis-infected strawberry. On the other hand, Fulton (11) reported that electron microscopy generally has failed to demonstrate any viruslike particles or any ultrastructural changes that would suggest virus etiology in pallidosis-affected plants. Our finding that two to four dsRNA ranging from M_r 4.3 to 5.2 × 10^6 are associated consistently with all of the pallidosis isolates we studied provides evidence that pallidosis disease may have viral etiology.

We have no explanation for the great variability of dsRNA, ranging from M_r 1.0 to 2.3 × 10^6, among the pallidosis isolates we tested. It is unlikely that this variability reflects the association of different viruses, cryptic or otherwise, with each isolate, although there is a possibility that more than one agent may be associated with pallidosis disease. Further investigations using nucleic acid hybridization techniques are needed to clarify the relationships of dsRNA to each other among pallidosis isolates and also within isolates.

Because the symptoms on UC-10 plants infected with pallidosis are not diagnostic, no means were heretofore available to detect pallidosis when other strawberry viruses were present in the plants (11). The dsRNA analysis reported here may be useful for detection of the pallidosis agent in complex with other viruses.

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