Double-stranded RNA Associated with the Rose Rosette Disease of Multiflora Rose

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ABSTRACT

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Four double-stranded RNAs (dsRNAs) of M_r of 2.9, 1.2, 1.0, and 0.93×10^6 were associated with tissues of multiflora rose showing symptoms of the rose rosette disease. Similar dsRNAs were not detected in tissue from a healthy rose. The dsRNAs were consistently associated with both transmission of the causal agent by grafting and with mechanical transmission using minutens. No evidence was obtained for transmission of the causal agent through seed or fruit harvested from infected plants.

The multiflora rose (*Rosa multiflora* Thunb.) is a vigorous, prickly shrub native to Japan that has been widely planted as an aid to soil conservation, as wildlife cover, and as a "living fence." The plant has been more aggressive than first recognized and can now be found in many areas of the United States. In several states, *R. multiflora* has been designated a noxious weed.

A disease of *R. multiflora,* termed rosette or witches' broom of rose, was first reported from Manitoba in 1941 (4) and has occurred periodically since that time (5). Electron microscopy of thin sections from symptomatic leaf tissue has not produced convincing evidence for the identity of the etiologic agent of the disease, which remains unidentified (5,6). Rose wilt, a disease first described in Australia in 1931 and later in New Zealand, the United Kingdom, and Bulgaria, is reported to have symptoms similar to those of rose rosette (12). It is not known whether the two diseases are caused by the same agent.

Rose rosette is lethal to infected *R. multiflora* within 3 yr after the first observation of symptoms (Di, Hill & Epstein, *unpublished*). Although successful mechanical transmission of the agent has not been reported previously, the disease can be transmitted by grafting (13) and by the eriophyid mite *Phyllocoptes fructiphilus* K. (1).

We here report the association of double-stranded RNA (dsRNA) with tissue from infected *R. multiflora* and its apparent absence in uninfected tissue. We also report the successful mechanical transmission of the disease agent.

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MATERIALS AND METHODS

Source of experimental material. *R. multiflora* plants with symptoms of rose rosette disease were transported from pastureland in southern Iowa and established in the greenhouse. Nonsymptomatic plants from areas where disease symptoms did not occur were also established in the greenhouse. In addition, nonsymptomatic plants were obtained from commercial sources. Plants were maintained in steam-pasteurized soil. Control of potential insect vectors was achieved by regular insecticide (2% avermectin B_1) application.

In order to evaluate the potential for seed transmission, seed and fruits were collected in the field from plants showing symptoms of the disease. After vernalization, seed was planted in pasteurized soil in the greenhouse.

Mechanical inoculation of multiflora plants. Inoculum used for mechanical inoculation of nonsymptomatic *R. multiflora* plants was prepared by grinding symptomatic leaf tissue in 0.05 M phosphate (pH 7.0) supplemented with 1 unit per milliliter of human placental ribonuclease inhibitor RNAsin (Promega Corp., Madison, WI). Inoculation was performed by applying drops of the inoculum to the vegetative shoot of a nonsymptomatic plant and then using minutens inserted into the end of a small wooden dowel to abrade the plant tissue through the inoculum droplets.

Analysis of dsRNA from plant tissue. The procedures used were similar to those described by Morris and Dodds (8) and Jordan et al (7). Approximately 0.5 g of leaf tissue was ground in liquid nitrogen and extracted with 1.3 ml of 2X STE buffer (1X buffer = 0.1 M NaCl, 0.05 M TRIS-HCl, 0.001 M EDTA, pH 6.8) containing 10% dodecyl sodium sulfate, 3mg/ml bentonite, and 0.5% 2 mercaptoethanol; 0.7 ml water-saturated phenol (redistilled); and 0.7 ml chloroform:isoamyl alcohol (24:1 v/v). The emulsion was broken by centrifugation at 3,000 $\times$ *g* for 15 min. The aqueous phase was transferred to a microfuge tube containing 50 mg Whatman CF-11 cellulose powder (1.0 ml wet volume) in STE:16.5% ethanol (v/v). After the emulsion was mixed and incubated for 20 min at 20 C, the tube contents were centrifuged at 8740 $\times$ *g* for 5 min at 4 C. The cellulose powder was washed three times (5 min each time with centrifugation as above) with 1 ml of STE:16.5% ethanol. DsRNA was eluted by the addition of 500 μl of STE followed by centrifugation for 5 min. The samples were treated with DNase 1 (10 μg/ml), 5mM $MgCl_2$ and proteinase K (50 μg/ml) for 30 min at 30 C. DsRNA was concentrated from the supernatant by ethanol precipitation and centrifugation. The dsRNA was resuspended in electrophoresis buffer (0.04 M TRIS, 0.02 M sodium acetate, 0.01 M Na_2EDTA, pH 7.8) containing 20% glycerol. Electrophoresis was carried out in 6% polyacrylamide slab gels (7 $\times$ 8 cm $\times$ 0.8 mm) for 4 hr at 30 mA after which the gel was stained with 30 ng/ml ethidium bromide. After destaining, the gel was soaked with RNase A (10 μg/ml) prepared either in 0.3 M NaCl or distilled water for 2 hr at 30 C to determine the nature of the RNA. Alternatively, the gel was stained with acridine orange (30 μg/ml). The apparent molecular weight of the RNA bands was determined by the method of Bozarth and Harley (3) with reference to the dsRNAs of wound tumor virus.

RESULTS AND DISCUSSION

Independent extractions were taken from *R. multiflora* plants transplanted from the field and showing symptoms of rose rosette in the greenhouse. In each of the 13 extractions, we consistently found four dsRNA bands of molecular weights 2.9, 1.2, 1.0, and 0.93 $\times 10^6$ (Fig. 1, lane 2). No such bands were found in tissue samples obtained from nonsymptomatic control plants obtained from commercial sources or from the field (Fig. 1, lane 3). The double-stranded nature of the RNA was demonstrated by a characteristic green stain with acridine orange and treatment of the gel with RNase A prepared in high salt or distilled water. The banding pattern was not altered by treating the gel with RNase in high salt, but was eliminated after treating the gel with RNase dissolved in distilled water. The pattern of Lambda DNA Hind III fragments applied to an adjacent lane was not altered by treating the gel with RNase A dissolved in high salt or distilled water (data not shown).

The unique dsRNA bands were also detected in symptomatic tissue obtained from 10 multiflora rose plants to which the apparent pathogen had been transmitted by grafting. The recipient plants did not originally show disease symptoms or the dsRNAs (data not shown). Grafting into recently formed succulent stems often caused disease symptoms in about 6 wk. However, symptoms often appeared after 1 yr when grafts were made to older woody stems. The unique bands were also found in samples collected in Arkansas from plants showing similar symptoms (Fig. 1, lane 6). The dsRNAs were not detected in fruits harvested from plants showing symptoms in the field. None of the plants grown from seed collected from symptomatic plants showed symptoms of the disease.

The four dsRNAs were also found in symptomatic and nonsymptomatic tissue collected from four of 20 plants to which the pathogen was mechanically transmitted (Fig. 1, lanes 4 and 5). Although transmission efficiency was low (about 20%), this is the first report of mechanical transmission of the etiologic agent. Efforts are in progress to increase transmission efficiency.

The M_r 2.9 $\times 10^6$ RNA was detected in extracts of tissue from both symptomatic and inoculated (both symptomatic and nonsymptomatic) plants growing in the greenhouse (Fig 1, lanes 2, 4, 5). It did not appear in extracts of tissue collected from symptomatic plants in Arkansas (Fig 1, lane 6). The M_r of this RNA may underestimate the real value; the relationship between mobility on gels and molecular weight is a curve, particularly at high molecular weight values, and extrapolation may lead to erroneous values. The pattern of four dsRNAs is similar to that found in avacado infected with a virus complex (9).

Three of the dsRNAs associated with the rosette disease have molecular weight values similar to the 0.8–1.6 $\times 10^6$ range reported for the two or three size classes of dsRNAs associated with the cryptic viruses (2). The cryptic viruses are reported to be seed-transmissible but not graft-transmissible, however, and are not known to induce disease symptoms in plants (2).

Although dsRNAs have occasionally been reported in plants (7, 11, 14), the dsRNAs associated with the rosette disease are disease-specific and are not found in nonsymptomatic plant tissue. At this point, we cannot demonstrate that the RNAs associated with the rosette disease are associated with the etiologic agent of the disease and not with a plant response to the agent. The former is more likely, though, as all known cases of

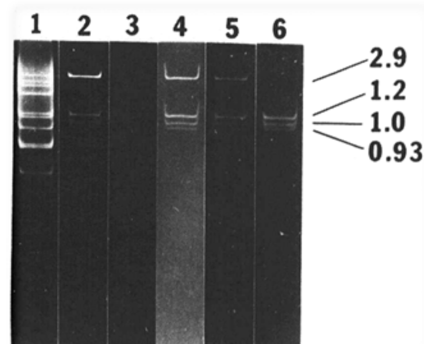

Fig. 1. Electrophoresis in 6% polyacrylamide gels of double-stranded RNA extracted from multiflora rose tissue: symptomatic tissue (lane 2); healthy tissue (lane 3); inoculated, symptomatic tissue (lane 4); inoculated, nonsymptomatic tissue (lane 5); and symptomatic tissue from Arkansas (lane 6). The M_r of double-stranded wound tumor virus genomic RNAs used as standards (lane 1) were (top to bottom) 2.9 (segment 1), 2.45 (segment 2), 2.20 (segment 3), 1.80 (segments 4 and 5), 1.20 (segment 6), 1.10 (segment 7), 0.9 (segment 8), 0.6 (segments 9–11), and 0.33 $\times 10^6$ (segment 12). Values used are from Reddy and Black (10). The same RNA pattern was present after treating the gel with RNase prepared in 0.3 M NaCl, but the banding pattern was eliminated after treating the gel with RNase prepared in distilled water.

dsRNAs associated with an apparent disease are a form of, or associated with, the agent itself. No case is known in which dsRNA is a host response to infection. However, dsRNAs have been associated with a pathogen which itself is not a virus, such as *Endothia parasitica* (Murrill) P. J. & H. W. Anderson. On the basis of these results, further investigations will be targeted to clarify the role of the RNAs in the disease syndrome.

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