A Peach Isolate of Prunus Necrotic Ringspot Virus

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ABSTRACT

A multicomponent isolate of *Prunus* necrotic ringspot virus (NRSV-P) was mechanically transmitted from graft-inoculated peach seedlings to cucumber. For purification, NRSV-P was stabilized by the extraction of cucumber tissue in 0.02 M NaDIECA-0.01M Na_2EDTA_2 and the acidification of the extract to pH 5 with 10% HCl. Virus was concentrated by two cycles of differential centrifugation. Rate zonal sedimentation of partially purified NRSV-P preparations in sucrose density gradients resolved three or four components. Infectivity was associated with the most rapidly sedimenting portion of a major 70-80 S component. This component consists of at least two sedimenting species. The faster-sedimenting species was predominant in NRSV-P preparations 5 days after infection, whereas the slower sedimenting species was predominant 7 days after infection. The average ribonucleic acid (RNA) content of this centrifugally heterogenous component is ca. 18-19% RNA as determined spectrophotometrically. Electron microscopic examination of NRSV-P negatively stained with potassium phosphotungstate, uranyl acetate, and uranyl oxalate, or fixed with 2% glutaraldehyde, mainly revealed irregularly shaped particles and only a few particles resembling virus particles with well-defined structure.

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Additional key words: Prunus persica, peach virus, stone fruit virus, virus purification, virus characterization.

In our studies of the nature of the causal agent(s) of peach stem-pitting disease (11), graft-inoculated peach seedlings often exhibit leaf symptoms similar to those caused by *Prunus* necrotic ringspot virus (NRSV). We mechanically transmitted several isolates of NRSV (NRSV-P) to cucumber from peach seedlings graft-inoculated with buds or root chips from naturally pitted peach trees. Our NRSV-P isolate formed a single precipitin line in agar gel double-diffusion tests against NRSV antiserum (R. W. Fulton, *personal communication*). Several variant forms of NRSV are described (1, 2, 6, 7, 14, 17, 18, 19). This paper describes the purification and some in vitro properties of NRSV-P, and discusses the relationship of this isolate to other NRSV variants.

MATERIALS AND METHODS.—The NRSV-P isolate described herein was mechanically transmitted initially from leaves of a graft-inoculated peach (*Prunus persica* [L.] Batsch 'Halford') seedling to cucumber (*Cucumis sativus* L. 'National Pickling') (11). Since no local lesion host is available for passage through single-lesion transfers, the NRSV-P culture was started from infectious virus purified by differential and sucrose density-gradient centrifugation to minimize the possibility of contamination. The culture was subsequently maintained in cucumber by weekly transfer with 0.03 M potassium phosphate buffer (pH 7.2) as diluent.

Since no local lesion assay host for this NRSV isolate was available to quantitatively estimate relative virus concentration, an assay based on systemic infection of cucumber seedlings was used. Relative infectivities of 2-fold serial dilutions of partially purified NRSV-P preparations are expressed as the ratio of plants infected to plants inoculated times the A_{260} times the reciprocal of the dilution for each sample tested.

NRSV-P was purified from fresh cucumber cotyledons and leaves 5 days after inoculation. Tissue was homogenized in a freshly prepared solution containing 0.02 M sodium diethyldithiocarbamate and 0.01 M disodium ethylenediaminetetraacetate (g:ml, 1:1.5) at 3 C. The extract was clarified by filtering the homogenate through cheesecloth, titrating the filtrate to pH 4.9-5.0 (12) with 10% (ca. 0.3 N) HCl and centrifuging at 10,000 *g* for 20 min. Virus in the low-speed centrifugation supernatant was concentrated by two cycles of differential centrifugation, and high-speed centrifugation pellets were resuspended in demineralized water. Further purification was achieved by centrifugation at 24,000 rpm (Spinco SW 25.1 rotor) for 4 hr in 5-40% linear sucrose density gradients. Centrifuged density gradients were fractionated with an ISCO Model D density-gradient fractionator. Successive 1-ml fractions were collected and diluted with 1 ml demineralized water. Absorbance at 260 nm was determined for each fraction before inoculating cucumber seedlings.

RESULTS.—Cucumber reacted variously to NRSV-P. This is probably due, in part at least, to variations in physiological condition of the plants inoculated and to fluctuating environmental conditions (19). Generally, NRSV-P-infected cucumber seedlings are severely stunted. Faint chlorotic spots may appear on inoculated cotyledons. Developing leaves are distorted, due to cupping or puckering, and show varying amounts of chlorosis. Terminal growth of infected plants is severely stunted. Infected squash (*Cucurbita pepo* L. 'Cocozelle') seedlings develop a systemic mosaic with dwarfed and puckered or cupped leaves. *Momordica balsamina* L. exhibits varying degrees of local necrosis on inoculated cotyledons followed by general chlorosis and systemic chlorotic spots. No symptoms were expressed in the following species: *Chenopodium amaranticolor* Coste & Reyn., *C. quinoa* Willd., *Gomphrena globosa* L., *Nicotiana tabacum* L. 'Samsun', *Phaseolus vulgaris* L. 'Pinto' and 'Cherokee Wax', and *Vigna unguiculata* L. Walp. 'Early Ramshorn'. A seed-transmitted isolate of

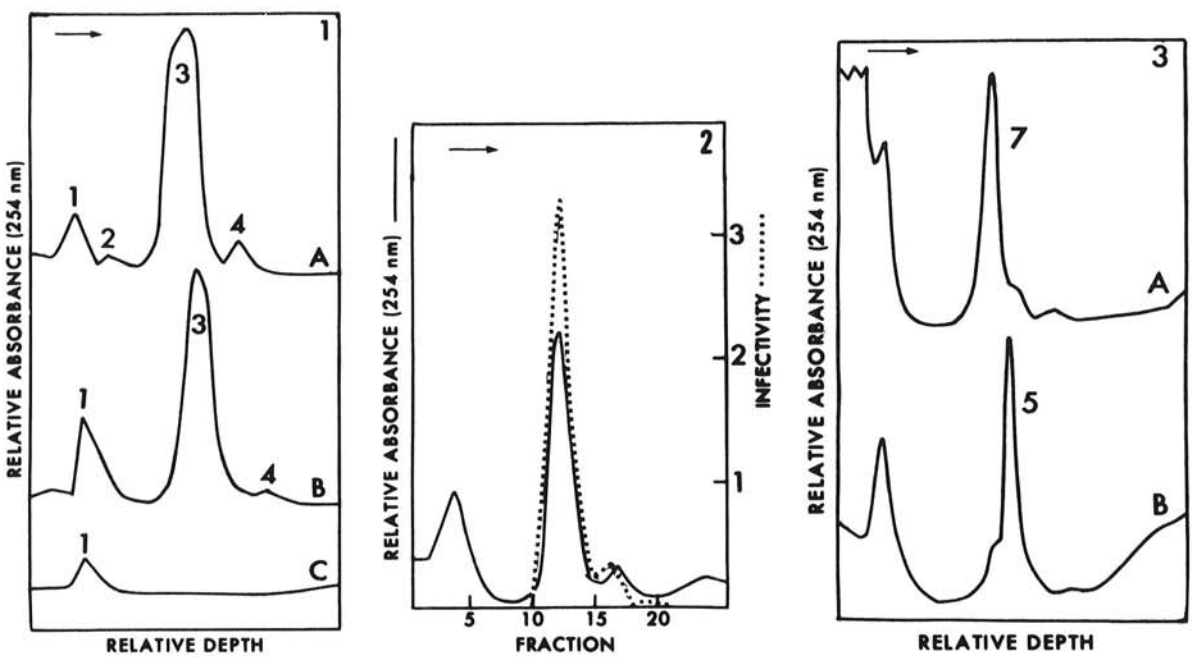

Fig. 1-3. 1) Sedimentation of (A) a concentrated sample of NRSV-P; and (B) a similar preparation of NRSV-P centrifuged at same time as (C) a preparation from healthy cucumber. Samples were centrifuged in 50-400 mg/ml sucrose gradients in demineralized water for 4 hr at 24,000 rpm in an SW 25.1 rotor. Arrow indicates direction of sedimentation. **2)** Sedimentation and distribution of infectivity of NRSV-P in a density gradient. Sample was centrifuged in a 50-400 mg/ml sucrose gradient in demineralized water for 4 hr at 24,000 rpm in an SW 25.1 rotor. Arrow indicates direction of sedimentation. Successive 1-ml fractions were collected and diluted with an equal volume of demineralized water before determining A_{260} of each fraction. Six cucumber seedlings were inoculated with each diluted fraction. Relative infectivity of each fraction was determined as described in the text. **3)** Sedimentation of dilute samples of NRSV-P purified from cucumber (A) 7 and (B) 5 days after infection. Samples were centrifuged in 50-400 mg/ml sucrose gradients in demineralized water for 4 hr at 24,000 rpm in an SW 25.1 rotor. Arrow indicates direction of sedimentation.

NRSV from peach was recently reported to be restricted to species of Cucurbitaceae (7). Infectivity of NRSV-P was not lost after acidification to pH 4.9-5.0, whereas infectivity of a seed-transmitted NRSV isolate from peach was lost after acidification to pH 5 (7). This could reflect intrinsic differences in the nature of the structural forces, such as protein-ribonucleic acid (RNA) interactions, among different NRSV isolates.

Sedimentation of NRSV-P-containing preparations in linear 50-400 mg/ml sucrose density gradients is shown in Fig. 1-A and B. Depending on the preparation, three or four sedimenting, ultraviolet-absorbing components are detectable. Component 1 is present in preparations from both healthy and NRSV-P-infected cucumber tissue. Heterogeneity of component 1 is indicated by the broadening of the ultraviolet-absorbing peak on the right side (Fig. 1-B). This material may consist of normal plant protein such as 18-19 S fraction 1 protein, and possibly virus-related products (3, 20). The relative amount of component 1 from NRSV-infected cucumber tissue varied with the preparation. In several preparations, a second component (component 2) was resolved, sedimenting immediately below component 1 (Fig. 1-A). Component 2 was not present in preparations from healthy cucumber. Components presumably representing two forms of the Q antigen and sedimenting at 22 and 35 S were associated with infection by sour cherry isolates of NRSV (3, 20). Component 1 of NRSV-P could contain the 22 S component sedimenting close to fraction 1 protein, while component 2 could represent the previously described 35 S component.

Components 3 and 4 are not present in preparations from healthy cucumber plants. Component 3 sediments to the region between the top and middle components of a soil isolate of tobacco ringspot virus (TobRSV) sedimented under identical conditions. This is equivalent to an estimated sedimentation constant of 70-80 S, assuming that the top, middle, and bottom components of TobRSV have sedimentation coefficients of 53, 91, and 126 S, respectively (15). Component 4 has an estimated sedimentation coefficient of ca. 100 S.

Infectivity is associated primarily with component 3 (Fig. 2). However, in several experiments, infectivity was associated with the most rapidly sedimenting portion of this component, suggesting that it is centrifugally heterogeneous. With dilute samples of partially purified NRSV-P, component 3 is resolved into at least two closely sedimenting species (Fig. 3-A, B). Infectivity is associated with the faster sedimenting material. Some fractions below component 3 were also infectious. However, infectivity in these fractions was not consistently associated with any specific component.

There is an apparent change in the relative proportions of the sedimenting species comprising component 3 with increasing age of infection in cucumber (Fig. 3-A, B). Whereas the faster-sedimenting species was consistently predominant in NRSV-P cultures 5 days after infection (Fig. 3-B), the slower sedimenting species was predominant 7 days after infection (Fig. 3-A). The slower-sedimenting species was not detected in NRSV-P preparations from cucumber 4 days after infection. The nature of this change and the significance of this phenomenon to NRSV-P infection of *Prunus* spp. is unknown. This phenomenon was observed when NRSV-P was extracted from tissue consisting of both inoculated cotyledons and systemically infected leaves.

Purified NRSV-P in demineralized water stored at 3 C has remained infectious for at least 2 weeks without detectable loss of infectivity, as determined by the cucumber bioassay.

The RNA content of component 3 of NRSV-P from centrifuged density gradients was estimated spectrophotometrically (13) in four independent trials. The average corrected $A_{280/260}$ was 0.62, and corresponds to ca. 18-19% RNA. This value is slightly higher than the 16% RNA value estimated for NRSV-H by the same method (4). NRSV isolates from cherry and a peach seedling contain about 20% RNA as determined spectrophotometrically (7). Degradation of NRSV-P in 0.02 M phosphate, pH 7.2, by variations of phenol extraction (8), or in the presence of 3 M KCl (10), resulted in the release of low molecular-weight, polydisperse, noninfectious RNA.

Electron-microscopic examination of purified NRSV-P, negatively stained in potassium phosphotungstate, uranyl acetate, or uranyl oxalate, mainly revealed irregularly shaped particles and only a few particles resembling virus particles with well-defined structure. The irregularly shaped particles in NRSV-P preparations may represent intact virus particles, and aggregates of intact or disrupted particles or virus subunits. Fixing samples with 2% glutaraldehyde did not alter the appearance of particles in NRSV-P preparations.

DISCUSSION.—NRSV-P was readily isolated from several peach seedlings graft-inoculated with buds or root chips from naturally infected, stem-pitted peach trees. We recently reported that there was no correlation between the presence of NRSV-P and occurrence of stem-pitting in artificially or naturally infected peach trees (11).

Multicomponent NRSV-P exhibits certain characteristics not described for other NRSV variants. Infectivity of NRSV-P is associated with the most rapidly sedimenting portion of a major 70-80 S component. Infectivity of a NRSV isolate from hops was associated with a minor 107 S component; while a major 79 S component was only slightly or not at all infectious (6). The 102 S component of a seed-transmitted NRSV isolate from peach seedlings was infectious, but a 70 S component was not (7). Both virus-specific 71 S and 105 S components of viruses from plum trees infected with either decline, line-pattern, or ringspot diseases were infectious (14). Infectious components of *Prunus* recurrent necrotic ringspot virus sediment at 95 and 117 S (16).

The major 70-80 S component of NRSV-P apparently consists of at least two sedimenting species. Preparations of at least one other NRSV isolate (N.5 from sour cherry) contain three or four sedimenting components (20). The predominant component of this isolate has a sedimentation constant of 72 S. Infectivity of four NRSV and five cherry yellow virus isolates was associated with 66 to 75 S components (20). Thus, several types of particles have been associated with NRSV infection. Additionally, infectivity has been associated with several sedimenting components ranging from about 66 to 117 S (6, 7, 14, 16, 18) in different NRSV preparations. Although NRSV variants may possess characteristic components, the role of these structures is not clearly understood.

The nature and significance of the apparent change in the relative proportions of the two sedimenting species of the major 70-80 S component in NRSV-P preparations are not known. No data are available regarding the in vivo interconversion of NRSV-specific components. A change in the relative proportions of *Prunus* recurrent, necrotic ringspot virus-associated 95 and 117 S particles with continued culture in cucumber may have resulted in specific selection of mutations induced by culturing the virus in cucumber (16).

Heterogeneity in particle size and shape has been described for a sour cherry NRSV isolate (18), rose mosaic virus (RMV) (5), and three virus isolates from plum infected with decline, line-pattern, or ringspot disease (14). This heterogeneity may be due to structural artifacts resulting from preparation for electron microscopy, and it may be related to the structural lability of the protein coat or to the nature of the association between protein and RNA. Appearance of particles in purified preparations of a sour cherry isolate of NRSV suggested that intact virus particles consist of loosely packed subunits (18), accounting for the inhibition of infectivity of NRSV by ribonuclease in crude extracts of infected cucumber leaves (9).

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