Detection and Comparison of Electrophorotypes
of Hibiscus Chlorotic Ringspot Virus

S. S. Hurtt

U.S. Department of Agriculture, Agricultural Research Service, Florist and Nursery Crops Laboratory, Beltsville, MD 20705.

ABSTRACT

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Isolates of hibiscus chlorotic ringspot virus (HCRSV) were obtained from *Hibiscus rosa-sinensis* by mechanical inoculation to kenaf and *Chenopodium quinoa*. After isolation and serial passage in *C. quinoa*, the virus was avirulent on kenaf but reacted with HCRSV antiserum. Therefore, virus from *H. rosa-sinensis*, kenaf, and *C. quinoa* was purified and compared. Virions from the three hosts were indistinguishable by buoyant density in CsCl (1.34–1.35 g/cm³) and coat protein size in sodium dodecylsulfate polyacrylamide gel electrophoresis (34–35.5 kDa). Each host also contained three major double-stranded RNAs (molecular weights of 3.0, 1.4, and 1.1×10^6). However, the electrophoretic profiles of purified preparations from each host were different when subjected to electrophoresis in agarose slab gels. Virions purified from *H. rosa-sinensis* separated into one major fast-migrating and one or two slower-migrating components, designated electrophorotypes. Virus from kenaf or from *C. quinoa*, after a prior passage in kenaf, migrated as one major electrophorotype (HCRSV-K) with a mobility like that of the major component from *H. rosa-sinensis*. Virus serially passed in *C. quinoa* was a mixture of several unique electrophorotypes. The fastest (HCRSV-F) and slowest (HCRSV-S) electrophorotypes were eluted and propagated in *C. quinoa*. Antibodies raised to HCRSV-F and -S were used to demonstrate that the antigens were serologically related but unique. Amino acid analysis showed that the aspartic acid and threonine content of HCRSV-F and -S also differed. This is the first report of host-associated variants of HCRSV.

Additional key words: ammonium sulfate precipitation, carnation mottle-like virus, host passage effects, intragel absorption serology.

Hibiscus chlorotic ringspot virus (HCRSV) is an unclassified, icosahedral virus that occurs worldwide in ornamental hibiscus (*Hibiscus rosa-sinensis* L.). The virus was partially characterized by Waterworth et al (20). While characterizing a virus (later shown to be HCRSV) from a naturally infected ornamental hibiscus in this laboratory, we found that after isolating and serially passing the virus in *Chenopodium quinoa* Willd. it reacted with antiserum to HCRSV but failed to infect kenaf (*H. cannabinus* L.), a systemic host for HCRSV (20). Virus was subsequently purified from (i) the naturally infected hibiscus; (ii) kenaf inoculated with crude sap from *H. rosa-sinensis*; (iii) *C. quinoa* after transfer from *H. rosa-sinensis* and serial passage in *C. quinoa*; and (iv) *C. quinoa* after one passage of the virus from *H. rosa-sinensis* to kenaf and then one or more transfers in *C. quinoa*. These preparations, referred to as wild-type virus, kenaf-type virus, *C. quinoa*-type virus, and kenaf-*C. quinoa*-type virus, respectively, were compared for serological, biochemical, biophysical, or electrophoretic differences. This paper describes the purification procedure and the results of comparing HCRSV propagated in different hosts.

MATERIALS AND METHODS

Propagation of virus isolates. Wild-type HCRSV was obtained from a naturally infected, variegated, ornamental hibiscus with leaf distortion, rugosity, and abaxial cupping. Crude sap inoculations were made in 0.05 M phosphate buffer, pH 7, or in 0.1 M sodium acetate-acetic acid buffer (NaAc), pH 5.1–6.2. All plants were maintained under ambient greenhouse conditions (15–30 C). Inoculated seedlings received 16 hr/day of supplemental cool-white light from November through April. Cultivars E41 and E71 of kenaf from Belle Glade, FL, and cultivar G48 from Guatemala were used interchangeably (seeds from T. A. Campbell, USDA, ARS, Beltsville, MD 20705).

Crude sap from ground leaves of the ornamental hibiscus was inoculated to kenaf and *C. quinoa*. The virus in *C. quinoa* was then transferred every 2–3 wk during a 2-yr period and purified from *C. quinoa* (*C. quinoa*-type virus). Inoculated kenaf became systemically infected and was used for virus purification (kenaf-type virus) and inoculations to *C. quinoa* (kenaf-*C. quinoa*-type virus).

For comparative purposes, carnation mottle (CarMV) and turnip crinkle (TCV) viruses (from T. J. Morris, University of California, Berkeley 94720) and type-strain tomato bushy stunt virus (TBSV)(from R. L. Steere, USDA, ARS, Beltsville, MD 20705) were propagated in this laboratory and purified as described for HCRSV.

Purification. Virus was purified by homogenizing frozen leaves in a Waring Blendor in two to four volumes (grams per milliliter) of 0.2 M NaAc buffer, pH 5.1–5.5, containing 0.1% 2-mercaptoethanol. The slurry was centrifuged at 12,000 g for 15 min in a Sorvall GSA rotor at 4–10 C. The supernatant fluid was decanted through a disposable tissue (Kimwipe) and recentrifuged. One volume of saturated ammonium sulfate solution was stirred into the clarified extract, and the mixture was incubated on ice for 1–2 hr. The precipitate was collected by centrifugation at 12,000 g for 15–20 min at 4 C and allowed to resuspend overnight in 0.05 M NaAc buffer containing 2-mercaptoethanol. The virus preparation was then subjected to two cycles of differential centrifugation. The resulting preparations were diluted, and 0.5–0.75 ml of ≤0.5 mg of virus per milliliter was layered onto continuous-density gradients of 15–45% CsCl in 0.05 M NaAc buffer, pH 5.4 or 7.2. Gradients were centrifuged in a Beckman SW 41 Ti rotor at 34,000 rpm for 5 hr at 20 C and fractionated on an ISCO Model 640 density-gradient fractionator equipped with a Model UA-5 monitor. Refractive indexes of fractions were measured with an Abbe refractometer. Selected fractions were dialyzed overnight against 0.02 M NaAc buffer, pH 5.2, and the contents analyzed by ultraviolet absorbance from 310 to 210 nm. Viral concentrations were calculated using an extinction coefficient of $5(mg/ml)^{-1}cm^{-1}$ at 260 nm (20).

Agarose gel electrophoresis of virions. Agarose slab gels were cast by pouring 13 ml of 0.8% agarose in water (DNA pure agarose, Bio-Rad Laboratories, Richmond, CA 94804) into a horizontal slab mold measuring 6.5 × 10.2 cm. Electrophoresis buffer was 0.075 M 2(N-morpholino)ethanesulfonic acid or 0.05–0.1 M NaAc buffer, pH 5.2–6.4. Gels were soaked in buffer or subjected to electrophoresis for about 15 min before application of samples. Sucrose and tracking bromophenol blue dye were added to purified virus preparations (0.5–2.0 mg/ml in 0.02 NaAc buffer), and 9- to 11-μl aliquots were placed into wells of the gel. Electrophoresis was carried out at a constant 50 V for 1–2 hr at room temperature in a minisub electrophoresis cell (Bio-Rad Laboratories). Buffers in the anode and cathode chambers were mixed by hand when electrophoresis exceeded 1 hr. Virus bands were stained for 5–10 min in aqueous ethidium bromide (10 μg/ml), destained briefly in distilled water, and photographed on an ultraviolet transilluminator. Gels were then stained with 0.1% Coomassie brilliant blue R250 in water:methanol:acetic acid, destained in the solvent only, and rephotographed or copied on electrophoresis duplicating paper according to the manufacturer's recommendations (Eastman Kodak Co., Rochester, NY 14650).

Purified C. quinoa-type virus was subjected to electrophoresis in multiple wells of one gel. One lane of the gel was excised from the gel and stained with ethidium bromide to identify areas that contained fluorescing bands. Areas corresponding to the positions of the bands were excised from neighboring, unstained lanes and eluted in distilled water. The remaining gel was stained with ethidium bromide to ensure that the virus-containing zone had been removed by the excision. Eluates of the crushed agarose blocks were bioassayed on C. quinoa. The eluted virions with the greatest mobility (HCRSV-F) and those with the least mobility (HCRSV-S) were maintained and multiplied by serial transfers in C. quinoa. Each was purified from C. quinoa and reexamined by electrophoresis in agarose.

Serology. Antisera to purified HCRSV-F (5 mg/ml) and HCRSV-S (3.2 mg/ml) were prepared at Cocalico Biologicals, Inc. (Reamstown, PA 17567) in 11- to 13-kg, female, New Zealand white rabbits by injection of 1 ml of antigen emulsified with 1 ml of Freund's complete adjuvant into multiple hip sites. Intravenous booster injections were made 2 wk later with 1 ml of antigen without adjuvant. Rabbits were test bled 1 wk after intravenous injections and exsanguinated 30 days after the initial intramuscular injections. Antiserum to HCRSV was kindly supplied by H. E. Waterworth, Beltsville, MD 20705; homologous virus was not available.

Gel double-diffusion tests were performed in 0.6 or 0.8% agarose in distilled water. Serial dilutions of antisera were made in phosphate-buffered saline (PBS). Purified antigens were diluted to 0.5–1.0 mg/ml in PBS or NaAc buffer. Intragel absorption tests were performed in a similar manner except that 20–25 μl of purified virus (0.5 mg/ml) was incubated in the central well for several hours before 20–25 μl of antiserum was dispensed into the same well. Test results were recorded after incubating the plates overnight at room temperature and 1–3 days of incubation at 4 C. Plates were rinsed repeatedly with PBS and stained with Coomassie brilliant blue R250 as described for agarose slab gels. Reaction lines were photographed or copied with electrophoresis duplicating paper.

SDS-PAGE. Purified virions (1 mg/ml) were mixed with an equal volume of disruption buffer (8 M urea, 1% sodium dodecylsulfate [SDS], and 5% 2-mercaptoethanol) and boiled in a water bath for 2–3 min. Molecular weight standards were treated similarly. Standards were mixtures of α-lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase b (Pharmacia Inc., Piscataway, NJ 08854). CarMV and TCV were also disrupted and used as standard markers on some gels.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed in polyacrylamide slab gels measuring 10 × 14 × 0.15 cm using tris-borate buffer containing EDTA and SDS (8). Electrophoresis of proteins was at a constant 90–100 V for 3–6 hr (according to gel concentration) at room temperature. Protein

zones were visualized with Coomassie brilliant blue R250 staining. For molecular weight estimations, the log molecular weight of each protein standard was plotted against distance migrated. The molecular weights of the capsid proteins of isolates of HCRSV were extrapolated from the graphs.

Amino acid analysis. Amino acid composition of purified TCV, HCRSV-F, and HCRSV-S was determined (18) with a Beckman 119 BL automatic amino acid analyzer. Lyophilized virus (0.5–1.0 mg) was hydrolyzed for 22 hr at 110 C in sealed ampules under nitrogen atmosphere with 1.5 ml of constant-boiling 6 N HCl containing 0.75 μl of 2-mercaptoethanol. The HCl was bubbled with high-purity nitrogen for 20 min before the ampules were sealed. Excess HCl was removed in vacuo over NaOH pellets and samples were dissolved in 0.5 ml of citrate buffer. Aliquots of 0.1 ml were injected for analysis. Threonine and serine were automatically corrected for 5% and 10% losses, respectively. Amino acid values for three different purified preparations of HCRSV-F and -S each were averaged and statistically compared using a t test and four degrees of freedom.

Double-stranded RNA analysis. Nucleic acid was extracted from 2–10 g of leaves from uninoculated or mechanically inoculated plants of C. quinoa and from systemically infected leaves of kenaf or H. rosa-sinensis. Double-stranded (ds) RNAs were recovered by CF-11 cellulose chromatography (12,13). Electrophoresis of dsRNA extracts was performed in 6% polyacrylamide minislab gels (Idea Scientific, Corvallis OR 97339) at a constant 30–35 mA for 4 hr at room temperature or in 0.8% agarose gels at 100 V for 3 hr. Nucleic acid bands were stained with ethidium bromide and visualized on an ultraviolet transilluminator. Some gels were subsequently incubated in RNase (50 μg/ml in 0.3 M NaCl for 30 min at 37 C) and restained in ethidium bromide.

RESULTS

Virus propagation and purification. Crude sap preparations of the naturally infected H. rosa-sinensis gave local lesions on kenaf followed by systemic symptoms as described by Waterworth et al (20). The same preparations gave chlorotic local lesions in C. quinoa in 7–12 days. Crude sap preparations of the inoculated kenaf also produced similar local lesions in C. quinoa (Fig. 1A). However, virus in C. quinoa gave decreasing numbers of local lesions on kenaf with successive serial passage (Fig. 1B). After five or more transfers, virus purified from C. quinoa without passage in kenaf did not infect kenaf.

Virus purified from H. rosa-sinensis, kenaf, and C. quinoa behaved similarly upon centrifugation in CsCl density gradients. Virus had a buoyant density of about 1.34–1.35 g/cm³ (Fig. 2), which varied slightly with the pH of the gradient. The $A_{260/280nm}$ of purified virus from acidic CsCl gradients was typically 1.44 (uncorrected for light scattering). The CsCl gradients contained variable amounts of a component with a density of 1.28 g/cm³. Larger amounts of this component were detected when partially purified virus was centrifuged in pH 7.2, as compared with pH 5.4, gradients (Fig. 2). The component had an $A_{260/280nm}$ of about 1.1 and showed low infectivity when bioassayed. The low-density particles were penetrated by phosphotunstate in negatively stained preparations examined by electron microscopy (Fig. 3A), whereas the high-density particles were not (Fig. 3B). The low-density particles were considered to be virions devoid of most of their nucleic acid. Virus preparations centrifuged in gradients at pH 7.2 gave lower yields of infectious virus as compared with those centrifuged in gradients at pH 5.4, and the gradients at pH 7.2 had an additional ultraviolet-absorbing zone ($A_{260/280nm}$ = 1.9–2.0) near the bottom of the gradient (Fig. 2). This was evidence of virion degradation and release of RNA. Virus for additional studies was, therefore, routinely prepared in CsCl density gradients adjusted to pH 5.0–5.4.

Agarose gel electrophoresis. Wild-type, kenaf-type, and C. quinoa-type viruses gave different banding patterns upon electrophoresis in agarose slab gels (Fig. 4). However, each zone (considered a viral electrophorotype) stained with both Coomassie
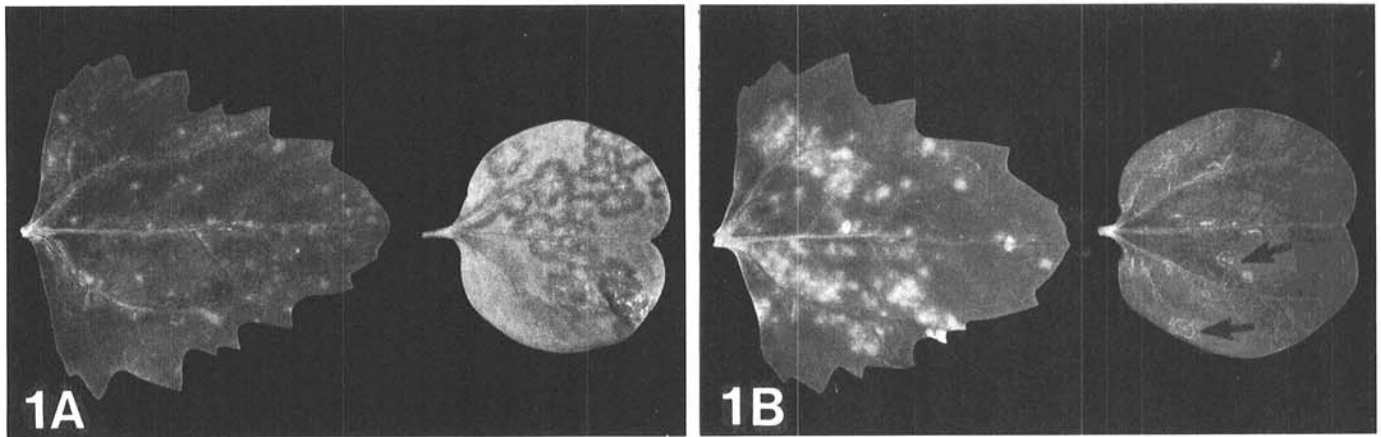
Fig. 1. Local lesions in *Chenopodium quinoa* (left) and kenaf (right) after inoculation with hibiscus chlorotic ringspot virus. **A,** Crude sap from kenaf after four serial passages of virus in this host (kenaf-type virus) produced local lesions in kenaf and *C. quinoa*. **B,** Crude sap from *C. quinoa* after four serial passages of virus from *H. rosa-sinensis* in *C. quinoa* (*C. quinoa*-type virus) produced numerous local lesions in *C. quinoa* but only two or three abnormal lesions in kenaf (arrows).
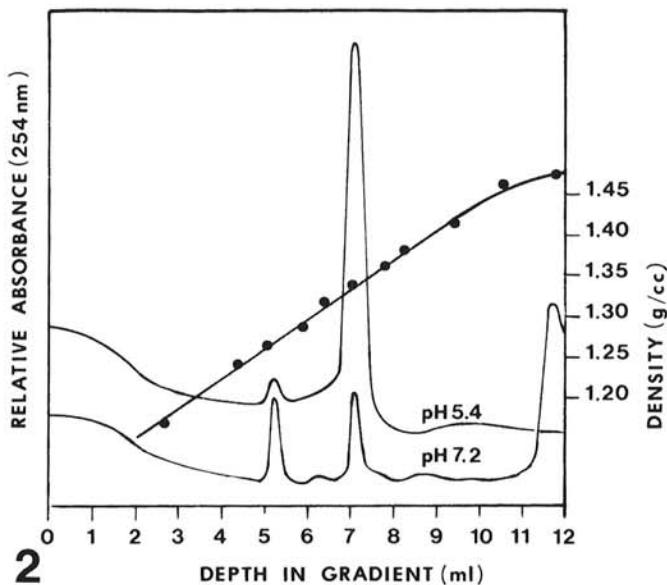

Fig. 2. Ultraviolet-absorbance profiles of 15–45% CsCl density gradients at pH 5.4 or 7.2. Purified *Chenopodium quinoa*-type virus was layered on gradients and centrifuged at 34,000 rpm for 5 hr at 20 C in Beckman SW 41 Ti rotor. Virions had buoyant density of 1.34–1.35 g/cm³. Viral degradation occurred in CsCl at pH 7.2, giving rise to light component (1.28 g/cm³) containing empty capsids and heavy, nucleic-acid-rich component near bottom of gradient.
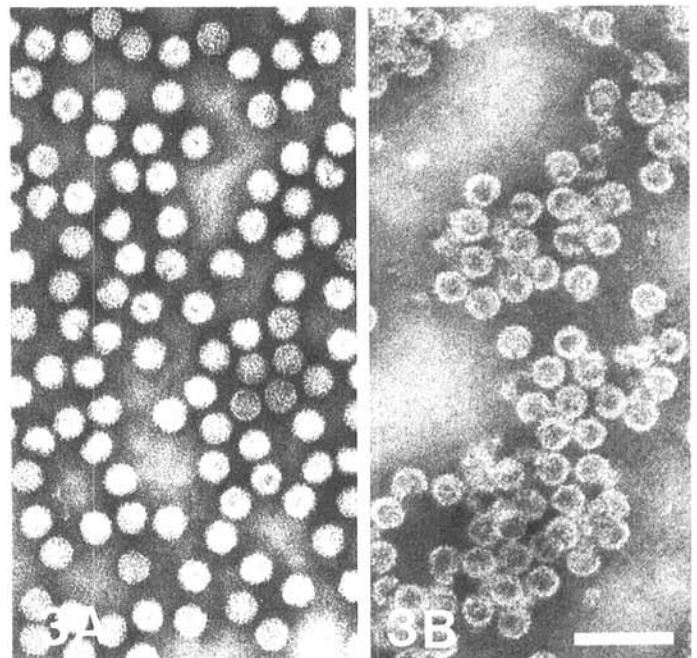

Fig. 3. *Chenopodium quinoa*-type hibiscus chlorotic ringspot virus from CsCl density gradients after dialysis and negative staining with phosphotunstate, pH 5.4. **A,** Virus from gradient of pH 5.4. **B,** Light component from gradient of pH 7.2. Scale bar = 100 nm.

brilliant blue R250 (Fig. 4A) and ethidium bromide (Fig. 4B) and thereby was believed to contain intact virions.

A major, rapidly migrating electrophorotype was always detected in wild-type virus preparations (Fig. 4A, lane 2; 4B, lane 3). One or two additional, slower-migrating components were detected, but their concentrations varied among three different preparations. Virus yields from *H. rosa-sinensis* were usually low, and these isolates were not further characterized.

A single electrophorotype component (HCRSV-K) with a mobility equal to that of the major component from *H. rosa-sinensis* was observed in kenaf-type (Fig. 4B, lanes 1 and 2) and kenaf-*C. quinoa*-type virus (*not shown*) preparations.

C. quinoa-type virus was resolved into several electrophorotypes (three to four, depending on the preparation) (Fig. 4). The fastest-migrating component, designated HCRSV-F (Fig. 4B, lanes 4 and 6), migrated slightly faster than HCRSV-K. The slowest-migrating component, designated HCRSV-S (Fig 4A, lanes 5 and 6; 4B, lane 5), migrated at about two-thirds the rate of HCRSV-F (Fig. 4B, lanes 4 and 6) and slightly faster than the slowest electrophorotype

wild-type virus preparations. Minor electrophorotypes with mobilities intermediate to HCRSV-F and -S were also detected in *C. quinoa*-type virus.

When eluted from the gels, HCRSV-S and -F were infectious on *C. quinoa* but not on kenaf. Local lesions on *C. quinoa* developed 1–2 days sooner when inoculated with HCRSV-S than with HCRSV-F in all subsequent transfers of the isolates. After serial transfer in *C. quinoa*, each isolate consisted of virions with an electrophoretic migration like that of the gel-eluted parent electrophorotype. Trace amounts of virions that migrated slightly slower than HCRSV-S or -F were detected in some purifications of HCRSV-S and -F, respectively, but these components were not investigated further. Trace amounts of HCRSV-S were also present in some preparations of HCRSV-F after serial passage in *C. quinoa*. It is not known whether HCRSV-S arose from mutation of HCRSV-F or represented incomplete separation of the components. Since HCRSV-S has a shorter latent period in *C. quinoa*, its multiplication is favored over that of HCRSV-F in serial transfers. Contamination of HCRSV-F in the -S
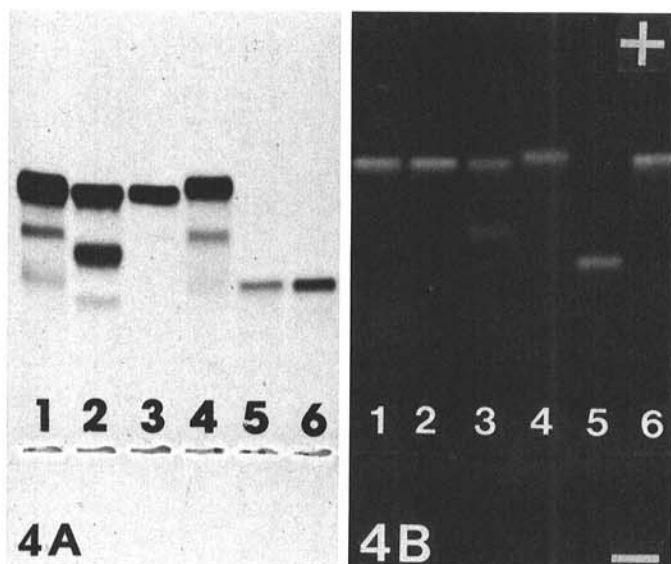
Fig. 4. Agarose slab gels showing electrophorotypes of hibiscus chlorotic ringspot virus (HCRSV). **A,** *Chenopodium quinoa*-type virus (lanes 1 and 4), wild-type virus (lane 2), kenaf-*C. quinoa*-type virus (lane 3), and HCRSV-S (lanes 5 and 6) after electrophoresis and staining with Coomassie brilliant blue R250. Gel copied on electrophoresis duplicating paper. **B,** Kenaf-type virus (lanes 1 and 2), wild-type virus (lane 3) (because of concentration, slowest component visible only with longer exposure), HCRSV-F (lanes 4 and 6), and HCRSV-S (lane 5). Virus location was visualized with ethidium bromide staining and ultraviolet illumination.

Fig. 5. Ouchterlony gel double-diffusion and intragel absorption tests in 0.6 or 0.8% agarose. Antiserum to hibiscus chlorotic ringspot virus (HCRSV) -S (1), HCRSV-F (2), or HCRSV (from H. E. Waterworth et al) (3) was diluted in phosphate-buffered saline. Antigens in outer wells were purified HCRSV-S (S), HCRSV-F (F), kenaf-type virus (K), wild-type virus (H), or *Chenopodium quinoa*-type virus (M). Virus was diluted to 0.5–1.0 mg/ml in phosphate-buffered saline or sodium acetate buffer. **A,** Antigens K and H reacted with antiserum to HCRSV-S and reactions spurred over HCRSV-S reaction. Spur between HCRSV-S and -F was small in this test and not discernible in photograph. **B,** HCRSV-S antiserum absorbed with HCRSV-F reacted only with HCRSV-S and M. **C,** HCRSV-S antiserum absorbed with K reacted with HCRSV-S and -F. **D,** Antigens HCRSV-F, K, and H reacted with antiserum against HCRSV-F and reactions spurred over HCRSV-S reaction. **E,** Antigens HCRSV-F, K, and H reacted with antiserum to HCRSV-Waterworth and reactions spurred over HCRSV-S reaction. **F,** HCRSV-Waterworth antiserum absorbed with HCRSV-F showed faint residual activity with K and H at 0.8 mg/ml but not at 0.4 mg/ml.

preparations was not detected.

Serology. Antisera raised against HCRSV-S and -F each had titers in gel double-diffusion tests of 1/256. Each antiserum reacted equally well with the heterologous antigen and with wild- and kenaf-type virus (Fig. 5). In a test with antisera to HCRSV-S, a reaction of partial fusion formed between the HCRSV-S and either HCRSV-F, kenaf-type, or wild-type virus (Fig. 5A). The reaction line of HCRSV-S spurred over that of the other preparations. After absorption of HCRSV-S antiserum with either of the last three antigens, HCRSV-S antigen still gave a precipitin line with its homologous antiserum (Fig. 5B). HCRSV-S was concluded to be serologically related to the other test antigens, but also to possess at least one unique epitope. Absorption of HCRSV-S antiserum with kenaf-type virus left residual antibodies to HCRSV-F and -S (Fig. 5C), indicating that HCRSV-S and -F shared epitopes not found on -K.

When HCRSV-F (Fig. 5D) antiserum or HCRSV-Waterworth antiserum (Fig. 5E) was reacted with the antigens, the precipitin lines formed by HCRSV-F, kenaf-type, and wild-type virus were confluent with each other but formed spurs that extended beyond the precipitin line formed by HCRSV-S. HCRSV-F, kenaf-type, and wild-type virus were therefore shown to be serologically similar to each other but to possess at least one epitope not present in HCRSV-S. As expected, HCRSV-F and HCRSV-Waterworth antisera absorbed with HCRSV-S showed residual activity with HCRSV-F, kenaf-type, and wild-type virus. However, if HCRSV-Waterworth antiserum was absorbed with HCRSV-F, a small residual activity was detected to kenaf-type and wild-type virus (Fig. 5F). HCRSV-K and HCRSV-F therefore were not serologically identical.

SDS-PAGE. CarMV and TCV were found to have major capsid proteins that were larger than those of HCRSV isolates. The major capsid proteins of HCRSV-S, -F, kenaf-type, and wild-type virus migrated similarly in more then six comparisons in SDS-PAGE and were calculated to be 34–35.5 kDa (Fig. 6). This size estimate is less than earlier ones (9), which were based on molecular weights of 41–43 kDa for CarMV, TBSV, and TCV. TCV and CarMV capsid proteins were recently determined to be 38.3 and 38.1 kDa, respectively (7; J. C. Carrington et al, *in preparation*). The value herein reported for HCRSV is smaller than that reported for a
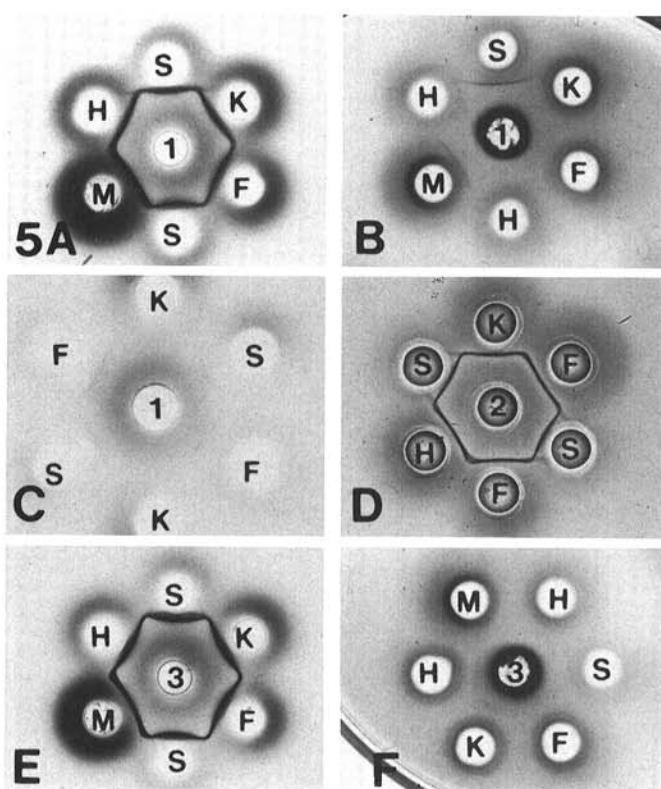
Fijian isolate of HCRSV (39.6 kDa) (3).

Amino acid composition. Based on an intermediate size value from SDS-PAGE analysis of 34.5 kDa and results of the amino acid analysis (Table 1), the capsids of both HCRSV-F and -S were each estimated to contain 330 amino acid residues. The isolates had similar percentage amino acid composition profiles. Statistically significant differences occurred in the quantities of four amino acids at the 10% probability level and in only two amino acids at the 5% probability level, using a *t* test with four degrees of freedom. HCRSV-S contained fewer glutamic and aspartic amino acids (dicarboxylic amino acids) and more threonine (neutral amino acid) and arginine (basic amino acid) than did HCRSV-F. The amino acid profile of HCRSV-S showed three minor peaks, representing unidentified substances, that were absent from the profiles of HCRSV-F (*data not shown*). Two peaks represented materials that eluted from the column before aspartic acid and one represented a material that eluted immediately before threonine. No estimates were made for quantities of tryptophan, which occurs in low amounts in most plant viruses. By the methods used here, glutamic and aspartic acid residues could not be differentiated from glutamate and aspartate residues, respectively.

Our estimate of the capsid composition of TCV was in good agreement with that found by nucleic acid sequencing techniques (5A) (Table 1). Discrepancies occurred between our data and those
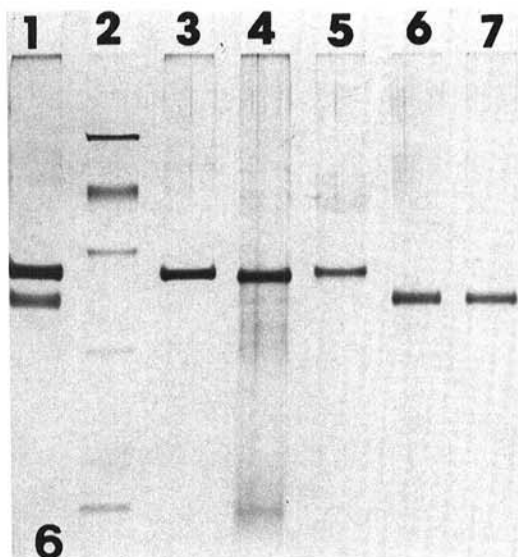
Fig. 6. Sodium dodecylsulfate polyacrylamide gel electrophoresis of hibiscus chlorotic ringspot virus (HCRSV) -F and -S and molecular weight standards in 10% polyacrylamide gel. Samples were turnip crinkle virus and HCRSV-S (lane 1); molecular weight standards (from top to bottom) phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) (lane 2); turnip crinkle virus (lanes 3 and 5); carnation mottle virus (lane 4); HCRSV-S (lane 6); and HCRSV-F (lane 7). Electrophoresis was performed in tris-borate buffer with 10 mM EDTA and 0.1% sodium dodecylsulfate at constant 90 V for 4 hr at room temperature. Proteins were fixed for 0.5 hr in 12.5% trichloroacetic acid and stained with Coomassie brilliant blue.

TABLE 1. Amino acid composition of capsid proteins of two isolates of hibiscus chlorotic ringspot virus (HCRSV-F and -S) and turnip crinkle virus (TCV)

Amino acid	Number of residues per polypeptide		
	TCV[a]	HCRSV-S[b]	HCRSV-F[b]
Aspartic acid	30 (29)	32**[c]	35**
Threonine	30 (31)	32***	27***
Serine	26 (28)	29	26
Glutamic acid	34 (34)	18*	22*
Proline	20 (19)	14	15
Cysteine	0 (2)	0	1
Glycine	33 (30)	35	36
Alanine	37 (36)	32	33
Valine	27 (26)	28	27
Methionine	4 (5)	6	6
Isoleucine	11 (11)	15	14
Leucine	25 (24)	27	27
Tyrosine	9 (9)	8	8
Phenylalanine	13 (13)	13	13
Histidine	2 (2)	4	4
Lysine	24 (25)	16	17
Arginine	18 (19)	17*	16*
Tryptophan	ND[d] (8)	ND	ND
Total	343 (351)	326	327

[a] Values found in one analysis in these experiments followed by value in parentheses as determined by Carrington et al (5A).
[b] Average of three experiments rounded to nearest whole number.
[c] * = Difference between pairs is significant at 0.1 level, ** = significant at 0.05 level, and *** = significant at 0.01 level.
[d] Not determined.

of Carrington et al in the quantitation of serine and glycine. These two amino acids showed the largest standard deviations from the mean in the analysis of HCRSV-S and -F. Thus, the data herein provide approximate values for these two amino acids. Hydrolysis of viral nucleic acid may have contributed to the larger-than-expected glycine values (17).

Analysis of dsRNA. Extracts of HCRSV-infected *C. quinoa* gave high yields of dsRNA that migrated as three species with estimated molecular weights of 2.8–3.0, 1.4, and 1.1×10^6 daltons (Fig. 7). Double-stranded RNAs of tobacco mosaic virus (TMV) (4, 2.4, 1.2, and 0.54×10^6 daltons) (gift from P. Hunst, St. Louis, MO 63167), TBSV (3.3, 1.5, and 0.7×10^6 daltons), and CarMV (2.6, 1.16, and 1.06×10^6 daltons) were used as standards for constructing nonlinear standard curves of $\log_{10}$ molecular weights vs. distance migrated. The dsRNA profiles of *C. quinoa* infected with *C. quinoa*-type virus (*not shown*), HCRSV-S (Fig. 7, lane 4), and -F (Fig. 7, lane 5) were indistinguishable. The double-genomic dsRNAs of HCRSV isolates migrated slower than the double-genomic dsRNAs of CarMV (Fig. 7, lane 1) and TCV (Fig. 7, lane 3) and slightly faster than that of TBSV (Fig. 7, lane 2). The two presumed subgenomic dsRNA species of HCRSV were also slightly larger than the corresponding subgenomic dsRNAs of CarMV.

Although high yields of dsRNA were obtained from *C. quinoa*, yields from kenaf were low and a slimy substance plugged the CF-11 cellulose columns. Similar difficulties were encountered with extracts of leaves and flowers of *H. rosa-sinensis*. Roots from pot-bound plants did not contain the slimy material, but dsRNA was not detected in extracts of these plant parts.

DISCUSSION

Biological, serological, biophysical, biochemical, or electrophoretic heterogeneity among particles of a virus is common (1,2,4,5,6,10,11,14,15,19,21). The origin of the variants or mutants is often poorly understood. In this study, HCRSV variants were first detected by differences in their biological properties. That is,
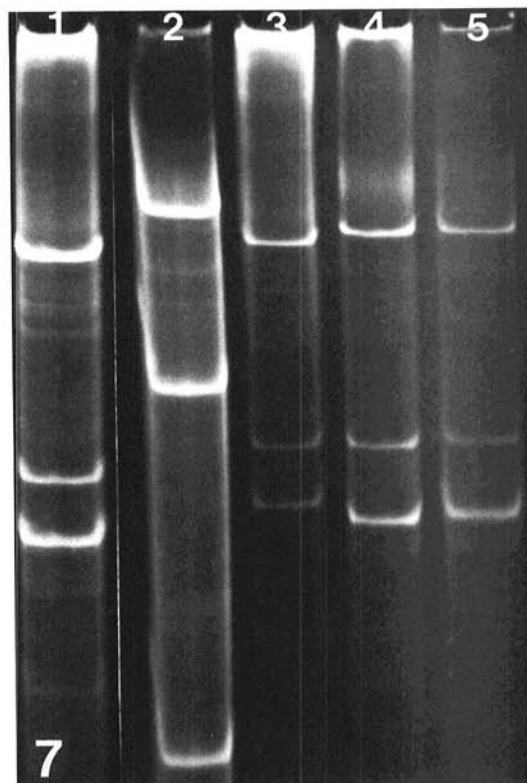

Fig. 7. Double-stranded RNA extracts from *Chenopodium quinoa* infected with carnation mottle virus (lane 1), tomato bushy stunt virus (lane 2), turnip crinkle virus (lane 3), hibiscus chlorotic ringspot virus (HCRSV) -S (lane 4), and HCRSV-F (lane 5). Electrophoresis was in 6% polyacrylamide gel at constant 90 V for 4 hr at room temperature. Nucleic acids were stained with ethidium bromide.

isolates of the virus in *C. quinoa* that reacted with antiserum to HCRSV failed to infect kenaf, a reported host for the virus (20). HCRSV from all hosts, irrespective of passage history, was similar in particle size, in capsid protein size, in buoyant density in CsCl, and in genome size. However, the populations were heterogeneous with respect to capsid structure as reflected in the number and migration rate of electrophoretes and in serological properties. The variants arose as a consequence of host passage and thereby seemed to reflect host passage effects (21). The mechanism of change brought about by host passage is unknown, but certain observations seem salient in reaching such an understanding.

The wild-type virus contained three electrophoretes of HCRSV. However, when it was inoculated to kenaf, only one electrophorete, HCRSV-K, was detected. The presence of only one electrophorete in kenaf may be the result of host selection (11,21). Inoculations of virus from nine other naturally infected *H. rosa-sinensis* plants to kenaf also gave HCRSV virions with mobilities identical to those of HCRSV-K (S. S. Hurtt, *unpublished data*). Although the composition of the HCRSV electrophoretes of these nine hibiscus plants was unknown, it was evident that HCRSV typically replicated in kenaf with less variability than in *C. quinoa*.

When wild-type virus was passed in *C. quinoa*, several electrophoretes were detected in the purified preparations, but their mobilities differed from those of the electrophoretes from *H. rosa-sinensis*. In three experiments conducted more than a year apart, the transfer and passages of wild-type inoculum from hibiscus to *C. quinoa* produced virus with the HCRSV-F and -S electrophoretic components and minor electrophoretes with migration rates intermediate to those of HCRSV-F and -S. The repetitive nature of the phenomenon is inconsistent with the theory that the components arose by random mutation or environmentally induced mutation; host selection or host-directed mutation seems more probable. But if the phenomenon was the result of host selection, it is unclear why the HCRSV-K variant was not detected after passage of wild-type virus in *C. quinoa*. HCRSV-K multiplied to high titers in *C. quinoa* after transfer from kenaf, and the production of HCRSV-F or -S was not detected even after three serial passages of kenaf-type virus in *C. quinoa*. Perhaps HCRSV-K and the similar component in *H. rosa-sinensis* possessed genetic differences that were not expressed in their electrophoretic properties, but differences that bridged a gap needed for stable HCRSV-K replication in *C. quinoa*. Comparisons of nucleic acid sequences are needed to address this possibility.

HCRSV-F and -S do not infect kenaf, and attempts to reinoculate seedlings of *H. rosa-sinensis* with these variants have failed (S. S. Hurtt and B. C. Raju, *unpublished data*). It has been impossible thereby to determine whether the variants in *C. quinoa* would revert to wild-type virus in *H. rosa-sinensis*. However, if strain selection had caused the change in the electrophoretic profile of the virus in *C. quinoa*, the *C. quinoa*-type virus should infect the hibiscus at least at low levels. The initial data indicating a lack of virulence of the *C. quinoa* electrophoretes in hibiscus favor the concept of host (*C. quinoa*)-directed mutation over the concept of host selection. This system therefore may provide a new model for the study of host-induced, adaptive mutation—i.e., Lamarckism, or the inheritance of acquired characteristics (21).

Since the HCRSV electrophoretes were similar in size and density, the differences in mobility were attributed to differences in virion surface charge (19). Two electrophoretes were further examined for differences in serological properties and amino acid composition. Antisera raised against HCRSV-F and -S and antiserum to HCRSV from Waterworth (probably similar to HCRSV-K since the antigen was purified from kenaf) were used to show that differences in the virion net surface charge were associated with differences in epitopes. Electrophoretes with similar mobilities (HCRSV-K and -F) were also more closely related serologically than those with greater differences in mobilities (HCRSV-F or -K and -S). The amino acid composition of HCRSV-F and -S were different, but the differences were small. Therefore, electrophoretes with smaller differences in migration

and serological properties were not examined for amino acid composition. The data did indicate, however, that some neutral amino acids on the surface of the HCRSV-S component were replaced conformationally or sequentially by acidic amino acids in HCRSV-F.

In addition to reporting and comparing host-associated variants of HCRSV, this paper describes an improved purification method for HCRSV based on its improved stability in moderately acidic buffers. It also presents additional characteristics of HCRSV that are important for taxonomic classification of the virus. HCRSV resembles TCV and CarMV in many of its biochemical and biophysical properties (16). These and additional properties of HCRSV should be compared with CarMV-like viruses in the future.

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