Characterization and Geographical Distribution of a New Ilarvirus from *Fragaria chiloensis*

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ABSTRACT

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A previously undescribed virus, a new member of the ilarvirus group, was isolated from wild *Fragaria chiloensis* plants collected in Chile and imported into the United States during 1990 and 1992. The virus, for which the name fragaria chiloensis ilarvirus (FCIV) is proposed, was detected during the postquarantine period in eight symptomless accessions collected in different locations in Chile. FCIV was transmitted mechanically to *Chenopodium quinoa, C. amaranticolor,* and *Cucumis sativus* but not to other herbaceous plants tested and was also transmitted through seeds collected from naturally infected *F. chiloensis* plants. FCIV particles, purified from inoculated *C. quinoa*, were quasi-isometric with a diameter of 21.4 nm, and bacilliform particles to 54.5 nm in length, containing a single polypeptide with relative molecular mass (M_r) of 28,000 and four RNA molecules of 3,700, 2,700, 2,600, and 1,200 bases, respectively. A polyclonal and a monoclonal antiserum to FCIV were produced. FCIV was related serologically to the ilarviruses asparagus virus II and lilac ring mottle in indirect enzyme-linked immunosorbent but not in immunosorbent electron microscopy assays. Based on tests with *F. chiloensis* accessions collected in the wild in Chile and along the Pacific coast of the United States and Canada, FCIV seems to be geographically limited to Chile.

Fragaria chiloensis L. Duch., a plant native to North and South America, has been used in the breeding of commercial strawberry (*Fragaria* × *ananassa* Duch.) cultivars in North America (15). Attempts to find sources of disease and pest resistance (5) and other desired traits for strawberry breeding programs have resulted in efforts to broaden the genetic diversity of strawberry (12). The realization that the genetic reservoir of native *F. chiloensis* is becoming endangered prompted recent extensive collections of wild accessions of this species in Chile (3), coastal western United States (11), and Canada (6). These germplasm collections, now being maintained in various permanent collections, have considerably expanded the strawberry germplasm base available to breeders in North America (12).

One of the greatest concerns regarding movement of vegetatively propagated plant material is the possible introduction and distribution of viruses and other pests. This concern is magnified when germplasm likely to carry unknown or poorly characterized pathogens is collected in its natural habitat and moved internationally. Virus-infected *Fragaria* plants are often symptomless (4) and are therefore potential carriers of viruses that are spread in vegetatively propagated plant material. Viruses moved in such plant material can subseqently be vectored in their new environment by insects, mites, nematodes, fungi or seeds.

This paper partially characterizes a previously undescribed virus isolated from wild *F. chiloensis* recently imported from Chile into the United States. The virus was detected during the postquarantine period in eight symptomless accessions collected in different locations in Chile but was not found in North American accessions of *F. chiloensis*. The virus, for which the name fragaria chiloensis ilarvirus (FCIV) is proposed, is probably a new member of the ilarvirus group (7). Tobacco streak virus (TSV) is the only member of the ilarvirus group that is known to infect *Fragaria* spp. naturally (16). Prunus necrotic ringspot, prune dwarf, and apple mosaic viruses have been graft-transmitted to *Fragaria vesca* experimentally but have never been detected in *Fragaria* under natural conditions (16).

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MATERIALS AND METHODS

Virus sources. Wild, naturally infected, symptomless *F. chiloensis* plants collected in Chile (3) were the virus sources. FCIV was sap-transmitted to, and maintained in, *Chenopodium quinoa* Willd.

Transmission. Leaves of *F. chiloensis* and infected *C. quinoa* plants were homogenized in 0.05 M potassium phosphate, pH 7.0, containing 2% polyvinyl-pyrrolidone (mol wt 44,000) (PVP) and rubbed onto Carborundum-dusted leaves of three plants each of *C. amaranticolor* Coste & Reyn., *C. quinoa*, *Cucumis sativus* L. 'Straight 8,' *Datura stramonium* L., *Gomphrena globosa* L., *Nicotiana benthamiana* Domin., *N. clevelandii* A. Gray., *N. glutinosa* L., *N. rustica* L., *N. sylvestris* L. Speg. & Comes, *N. tabacum* 'Havana 425', 'Samsun,' 'White Burley,' and 'Xanthi,' *Petunia hybrida* Vilm. 'Coral Satin,' *Phaseolus vulgaris* L. 'Pinto,' 'Red kidney,' and *Vigna unguiculata* L. Walp. Plants mock-inoculated with this buffer served as healthy controls. Plants were grown in a greenhouse with a 16-h day and 8-h night regime, and daytime and nightime temperatures of 22 and 16 C, respec-

tively, and observed for symptom development for a period of 4 wk.

Virus purification. Systemically infected leaves of *C. quinoa* harvested 12–14 days after inoculation were used for virus purification. Tissue was blended (2 ml/g of tissue) in 0.1 M dibasic potassium phosphate, pH 8, containing 10 mM diethyl dithiocarbamic acid, and 10 mM sodium thioglycolic acid. The homogenate was centrifuged at 16,000 *g* for 20 min (low-speed centrifugation); the supernatant was adjusted to pH 4.5 with glacial acetic acid while stirring. This preparation was then left at room temperature for 30 min; subjected to low-speed centrifugation, and the supernatant centrifuged for 2 h at 146,000 *g* through a 20% sucrose cushion made in 0.02 M potassium phosphate, pH 8, (phosphate buffer). The pellets were resuspended in phosphate buffer (1 ml per 12-ml tube) and, after a low-speed centrifugation, the virus was pelleted by centrifugation at 171,000 *g* for 1.5 h. For further purification, resuspended pellets were layered onto 10–40% sucrose density gradients in phosphate buffer and centrifuged in a swinging-bucket rotor for 2 h at 180,000 *g*. Gradients were scanned with an ISCO UA-5 absorbance-fluorescence detector (ISCO, Inc. Lincoln, NE) fitted with a 254-nm filter, fractions with peak absorbance were collected, diluted with phosphate buffer, and virus was pelleted by high-speed centrifugation. Alternatively after the first high-speed centrifugation, pellets were resuspended in phosphate buffer, and CsCl (4.72 g/ 10 ml final volume, based on the buoyant density of tobacco streak virus (TSV) in CsCl) (8) was added. The preparation was then centrifuged at 207,000 *g* for 16 h at 10 C, the opalescent band was collected, diluted with phosphate buffer, and pelleted as above. Purified virus preparations were stained with 2% uranyl acetate (UA) on nickel grids for examination with a 600 Hitachi electron microscope.

Electrophoresis of virus coat protein and RNA. The relative molecular mass (M_r) of the coat protein was determined by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a vertical 12% gel with a 4% stacking gel (10) in a Bio-Rad MiniProtean II system (Bio-Rad, Richmond, CA). Purified virus, diluted in 0.05 M Tris-Cl, pH 6.8, containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.00125% bromophenol blue, was boiled for 3 min before electrophoresis. Electrophoresis was at 10 mA per gel until the dye front moved into the separating gel and then at 20 mA until the dye front was 0.5 cm from the bottom. Gels were fixed and stained with 0.2% Coomassie brilliant blue R 250 in 25% methanol and 7% acetic acid, and then destained with 25% methanol and 7% acetic acid.

For nucleic acid extraction, purified virus was resuspended in 0.5 ml of 0.2 M Tris-Cl, pH 7.5, containing 0.025 M EDTA, 0.3 M NaCl, 2% SDS, and 250 μg/ml of proteinase K and incubated at 37 C for 30 min. RNA was extracted twice with 0.5 ml of phenol/chloroform (1:1, v/v) and once with 0.5 ml of chloroform/isoamyl alcohol (24:1, v/v). The nucleic acid was precipitated at −20 C overnight with 1/10 volume of 3 M sodium acetate, pH 5, and 2.5 volumes of absolute ethanol. RNA was separated by electrophoresis at 8 V/cm for 1 h in a 1% denaturing agarose gel containing 5 mM methylmercuric hydroxide. The gel was stained with ethidium bromide after the addition of 2-mercaptoethanol (13). The nature of the nucleic acid was determined by digestion with RNase T1 (5 μg/ml) (Sigma, St. Louis, MO) in enzyme buffer (50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol) or RNase-free DNase (Promega, Madison, WI) in buffer (40 mM Tris-Cl, pH 7.9, 10 mM NaCl, and 6 mM $MgCl_2$) for 10 min at 37 C before electrophoresis. Controls included viral nucleic acid in each of the buffers without enzyme and plasmid DNA digested with each of the enzymes.

Serology. For production of a polyclonal antiserum, a New Zealand white rabbit was immunized with one subcutaneous injection of 0.2 mg of purified virus emulsified with an equal volume of Freund's complete adjuvant followed by three intramuscular injections of purified virus emulsified with Freund's incomplete adjuvant at 3-wk intervals. Blood was collected 12 days after the final injection. The titer of the antiserum was determined in agar gel double-diffusion tests (1).

For production of monoclonal antibodies, a BALB/c mouse was immunized with four injections of 0.04 mg of purified virus emulsified with an equal volume of Freund's complete adjuvant for the first injection and incomplete adjuvant for subsequent injections. The injections were at 3-wk intervals, with the first administered subcutaneously and the following three intraperitoneally. A final intraperitoneal injection (without adjuvant) was administered 5 days before fusion (9).

Serological relationships with other ilarviruses were determined by an indirect ELISA and by immunosorbent electron microscopy (ISEM) tests. The antisera listed in Table 1 were a gift from G. I. Mink, Prosser, WA, except for the prune dwarf virus (PDV) antiserum, which was supplied by M.F. Clark, East Malling, U. K.; apple mosaic virus (ApMV) and TSV antisera, which were supplied by R. H. Converse, USDA-ARS, Corvallis, OR; and lilac ring mottle virus (LRMV) antiserum, which was supplied by G. Leone, Wageningen, The Netherlands. The antibodies were compared in tests done simultaneously on the same virus preparation. In the indirect ELISA, plates were washed extensively after each step except for blocking and all reagents were used at 100 μl per well in Linbro flat-bottom microtiter plates (Flow Laboratories, McLean, VA) except for the blocking step which was 200 μl per well. Plates were coated with purified FCIV at 1 μg/ml in PBS (127 mM sodium chloride, 2.6 mM potassium chloride, 8.5 mM disodium phosphate, and 1.1 mM potassium dihydroxyphosphate) for 2 h at room temperature. Sap of uninoculated *C. quinoa* diluted 1:50 in PBS was also coated on ELISA plates and incubated as above. Plates were blocked with PBS-Tween containing 0.1% nonfat dried milk powder (blocking buffer) for 1 h at room temperature. Eight fivefold serial dilutions (starting at 1:200) of the various antisera in blocking buffer were done directly in the microtiter plates. After incubation for 2 h at 37 C, alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immuno-Research Laboratories, Inc., West Grove, PA) was added at a dilution of 1:5,000. The plates were incubated for 2 h at 37 C and substrate (*p*-nitrophenyl phosphate, Sigma, St. Louis, MO) at 0.5 mg/ml in 10% diethanol-amine, pH 9.8, was added. The absorbance (A_{405nm}) of each well was recorded after 30 min and 1 h with an ELISA microplate reader (MR5000

TABLE 1. Serological reactivity of ilarvirus antisera with purified fragaria chiloensis ilarvirus by indirect enzyme-linked immunosorbent assay and immunosorbent electron microscopy

Antiserum[a]	Endpoint with fragaria chiloensis ilarvirus	No. of particles trapped[b]
Fragaria chiloensis ilarvirus	6.25×10^5	611 a
Asparagus virus II	5.00×10^3	165 b
Lilac ring mottle	5.00×10^3	153 b
Tobacco streak	ND[c]	...[d]
Prune dwarf	ND	88 b
Apple mosaic	ND	55 b
Blueberry shock	ND	...
Citrus leaf rugose	ND	...
Elm mosaic	ND	...
Elm mottle	ND	...
Prunus nectoric ringspot	ND	...
Spinach latent	ND	...
Tulare apple mosaic	ND	...
Normal goat antiserum	ND	110 b

[a] Eight fivefold serial dilutions (starting with crude antisera diluted 1:200) of the various antisera in blocking buffer were done directly in the microtiter plates.

[b] Purified virus was diluted to one or two particles per field. For each antiserum, four grids were used. Figures represent counts of trapped particles in a total of 50 fields. One field = screen area viewed through the binocular at 20,000× selected at random. Analysis of variance followed by Duncan's new multiple range test was used to detect differences in number of particles trapped by the different antisera. Values followed by the same letter are not significantly different.

[c] ND = not determined. $A_{(405nm)}$ value of zero was recorded at the 1:200 starting dilution of antiserum, therefore no reaction was detected between FCIV and this antiserum diluted 1:200.

[d] Test not done.

Dynatech, Chantilly, VA). The A_{405nm} for each antiserum at each dilution for control sap samples was subtracted from the respective absorbance obtained with FCIV coated directly onto the plates.

For the ISEM test, purified virus was diluted until one or two particles were viewed in one field (= screen area viewed through the binocular at 20,000× magnification selected at random). Nickel grids were coated for 1 h with each of the crude antisera diluted 1:1,000 in PBS, washed twice with two drops of PBS, and floated on the diluted FCIV preparation for 1 h at room temperature, washed with 0.1% bacitracin, stained with UA, and viewed in the electron microscope. For each antiserum, four grids were prepared and counts were taken in a total of 50 fields. Analysis of variance followed by Duncan's new multiple range test was used to detect differences among antisera in the number of virus particles trapped per microscope field. Counts were transformed to square root for analysis.

Detection of virus in plants. Double antibody sandwich (DAS)-ELISA was used to detect FCIV in *Fragaria* plants. Two leaflets from each plant were homogenized (1:40, w/v) in blocking buffer containing 2% PVP. Tests were done in duplicate wells. Polyclonal antiserum was used for coating at 1 μg/ml in coating buffer. A monoclonal antibody made against FCIV was used as the secondary antibody; cell culture fluid precipitated with ammonium sulphate was diluted 1:500 in blocking buffer and incubated for 2 h at 37 C. Alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson Immuno-Research Laboratories, Inc. West Grove, PA) was added at a dilution of 1:5,000. The plates were incubated for 2 h at 37 C followed by addition of substrate. A_{405nm} of healthy controls ranged from 0 to 0.02, and values above 0.25 were considered positive for FCIV.

Transmission through seed. Seeds collected from the *F. chiloensis* accessions that had been found to be naturally infected with FCIV were either tested directly or germinated. Seedlings were transplanted to pasteurized potting medium with one plant per pot and grown for 2 mo. Leaf tissue samples from each plant were homogenized and assayed for FCIV by ELISA.

RESULTS

Mechanical transmission. Inoculated leaves of *C. quinoa* developed circular, chlorotic local lesions about 8 days after inoculation with leaf homogenates of symptomless, naturally infected *F. chiloensis* plants. These lesions later expanded and became necrotic. Systemic symptoms consisting of yellow mottling appeared on young expanding leaves within 14 days postinoculation. These symptoms were not present on the mock-inoculated plants. Of all the plant species tested, only *C. quinoa, C. amaranticolor,* and *C. sativus* expressed symptoms. FCIV was transmitted mechanically from symptom-expressing *C. quinoa* to *C. quinoa, C. amaranticolor,* and *C. sativus* but was not transmitted to any of the other test plants. These results were confirmed by ELISA. Mechanical transmission was found to be season-dependent and was best achieved in the spring.

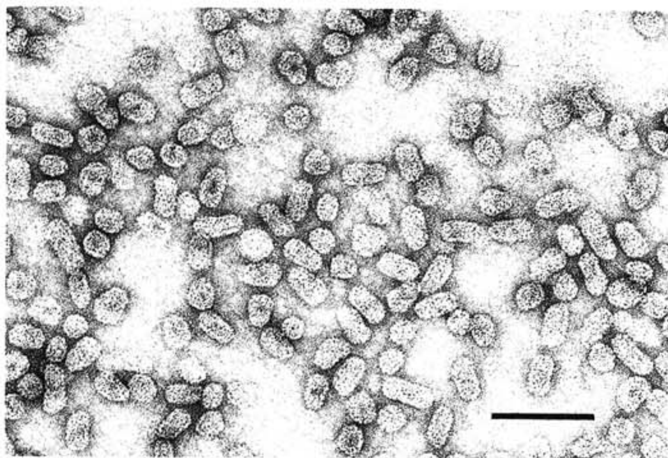

Fig. 1. Electron micrograph of negatively stained quasi-isometric and bacilliform particles from a purified preparation of fragaria chiloensis ilarvirus (bar = 100 nm).

Virus purification. Two ultraviolet absorbing bands were observed in the gradients after sucrose rate-zonal density-gradient centrifugation. Fractions collected at the peak absorbance of each band, stained with UA, and examined in the electron microscope, were found to contain both quasi-isometric and bacilliform particles (Fig. 1). The diameter of the quasi-isometric particles ranged from 18.0 to 24.3 nm, with an average diameter of 21.4 nm (based on the measurement of 100 particles), and lengths of bacilliform particles were up to 54.5 nm. Yields of purified virus ranged from 0.8 to 1.0 mg per 100 g of infected *C. quinoa* calculated from extinction coefficient of TSV $A_{260nm} = 5.1$ at 1 mg/ml (8). FCIV particles were also purified on CsCl gradients. When the suspension derived from the first high-speed pellet was layered on CsCl gradients an opalescent band formed near the middle of the gradient following centrifugation. Purified virus had an ultraviolet absorption spectrum typical of nucleoprotein, with absorption maxima and minima at 259–260 and 240–242 nm, respectively; the $A_{260/280nm}$ ratio for purified virus ranged between 1.57 and 1.77 without correction for light scattering.

Coat protein and nucleic acids. A single major polypeptide was detected following SDS-PAGE of protein preparations from purified particles (Fig. 2). The relative molecular weight of the single polypeptide was estimated to be 28,000 ± 142 (mean of

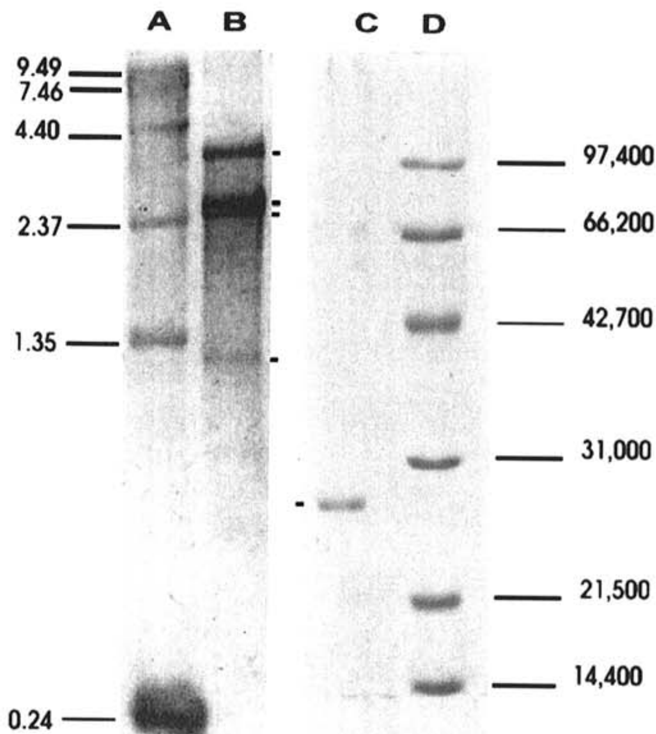

Fig. 2. Electrophoresis of fragaria chiloensis ilarvirus (FCIV) protein and RNA. **A, B,** Gel electrophoresis of fragaria chiloensis ilarvirus RNA under denaturing conditions in a 1% agarose gel containing 5 mM methylmercuric hydroxide. A, an RNA ladder of 0.24–9.5-kb fragments (Bethesda Research Laboratories, Gaithersburg, MD) was used as RNA standards (300 ng per lane). B, FCIV RNA (200 ng per lane). This is a negative print. **C, D,** Polyacrylamide gel electrophoresis of FCIV coat protein. **D,** markers (500 ng per lane) with relative molecular masses indicated on the left. **C,** Purified FCIV (1 μg per lane). The low-range molecular weight protein markers (Bio-Rad, Richmond, CA) include: phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400 daltons. The protein gel was scanned directly and is a positive print, the RNA gel was photographed and then scanned and a negative of the image printed.

five determinations ± standard deviation). The nucleic acid of the virus particles migrated as four bands of 3,700, 2,650, 2,600, and 1,200 bases (Fig. 2). The nucleic acid was RNA, since it was degraded by RNase, and not affected by RNase-free DNase. Plasmid DNA used as a control was digested by the DNase but not by the RNase.

Serology. Antiserum produced against FCIV had a dilution endpoint of 1/320 in agar gel double-diffusion tests. In the electron microscope, virus particles were trapped, decorated, and gold-labeled using the antiserum produced against the virus (not shown). Antisera to 14 other ilarviruses were tested in indirect ELISA for reactivity against FCIV. The reciprocal dilution end-points of these antisera when reacted with purified FCIV (1 μg/ml) coated directly on microtiter plates are shown in Table 1. Purified FCIV reacted with antisera to asparagus virus II, lilac ring mottle virus, and FCIV in these tests but not with any of the other ilarvirus antisera tested. In ISEM the number of virus particles trapped by antiserum to FCIV was significantly greater than the number of particles trapped by antiserum to asparagus virus II, lilac ring mottle, prune dwarf, or apple mosaic viruses. The latter four antisera did not trap significantly more virus particles than normal goat antiserum. Results of the ISEM tests are presented in Table 1.

Detection of virus in plants. FCIV has been detected by ELISA in leaf samples and seeds collected from wild accessions of *F. chiloensis* imported from Chile. A_{405nm} values for infected plants and seeds were above 0.25, whereas those of healthy samples were below 0.02. The ELISA tests were done during the postquarantine period in which plant material has been maintained in the germplasm repository at Corvallis, OR. FCIV was detected in eight accessions of the 210 collected in 42 locations in Chile (Table 2). Three FCIV-infected plants were from the 14 accessions collected in the Lake Conguillio area and two from the 22 in Lonquimay. Seeds collected from these plants (five seeds pooled for one test sample) tested positive for FCIV. The two locations from which these plants were collected are in the general area of the Andes Mountains about 30 km apart (approximately 71°30′W, 38°30′S). At the Lonquimay site, *F. chiloensis* plants were collected from volcanic ashes deposited only a year before the collection was made (3). It is not known whether the various accessions collected in each location represent distinct clones.

FCIV was also detected in two seedlings germinated from seeds of the cultivated *F. chiloensis* (known as White Chilean strawberry) collected in Chile. One infected seedling was from seeds originating at Futaleufú (approximately 71°50′W, 43°10′S) in the valley of the Futaleufú River and the other from seeds in fresh fruit purchased in a local market in the village of Pata De Gallina near Mt. Contulmo (approximately 73°15′W, 38°04′S), where White Chilean strawberry has been cultivated for many years (3). FCIV was not detected in leaf samples from 1978 wild *F. chiloensis* accessions which had been collected along the Pacific coast of California, Oregon, Washington, and British Columbia and which are currently maintained in permanent germplasm collections in the United States and Canada (Table 2).

Seed transmission. Seeds collected from the *F. chiloensis* accessions found to be naturally infected with FCIV were germinated and seedlings were allowed to grow for about 2 mo before being tested for seed transmission. Twenty-three of the 42 seedlings, though symptomless, were found to be infected with FCIV, indicating a rather high rate of seed-transmission.

TABLE 2. Results of tests for fragaria chiloensis ilarvirus in wild *F. chiloensis* accessions collected in Chile and coastal West North America

Collection area	Accessions (no.)	Sites (no.)	Positives/ tested (no.)
Chile	210	42	8/210
California	153	9	0/153
Oregon	269	16	0/269
Washington	21	9	0/21
British Columbia	1,535	118	0/1,535

DISCUSSION

FCIV shares several properties with other members of the ilarvirus group, including bacilliform and quasi-isometric particle morphology; virus diameter of approximately 21 nm; a single peptide of M_r 28,000; four species of single-stranded RNA; mechanical transmissibility to a narrow herbaceous host range, at least two particle types are separable by rate zonal centrifugation; and seed transmission. FCIV is proposed as a new member of the ilarvirus group. It has only a distant serological relatedness to asparagus virus II an ilarvirus included in subgroup 2 and LRMV (not subgrouped) (7). In ISEM studies, FCIV was not trapped by any of the other antisera tested, suggesting a very distant relationship. The only other member of the ilarvirus known to infect *Fragaria* spp. naturally is TSV. FCIV did not react with TSV in two-way gel diffusion tests (data not shown) or in indirect ELISA tests.

FCIV has been detected in wild accessions and the cultivated White Chilean *F. chiloensis* plants in various locations in Chile associated with different topography, vegetation, and climatic conditions. Detection of FCIV in the White Chilean strawberry indicates that the virus may be widely spread in these plants since they have been cultivated in Chile for many years and most likely propagated vegetatively.

Based on tests performed in this study with plant samples from *F. chiloensis* accessions collected in the wild in Chile and along the Pacific coast of the United States and Canada, FCIV seems to be geographically limited to Chile. The natural host range and vectors of FCIV are currently unknown. Moreover, it is also not clear whether the virus is a potentially important pathogen of cultivated strawberry.

Detection of FCIV in symptomless plants and in seeds during a postentry quarantine period clearly demonstrates the potential for accidental introduction and spread of a new virus as a result of germplasm transfer. It emphasizes that postquarantine measures must be taken to minimize the risk of introducing new pathogens before any germplasm is released to the public. However, the increased international movement of wild relatives of various plant species requires that virus detection procedures appropriate for germplasm evaluation be established for poorly characterized and unknown viruses. This will ensure conservation of valuable and sometimes endangered germplasm with minimum phytopathological risk.

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