

Lettuce Infectious Yellows Virus—A New Type of Whitefly-Transmitted Virus

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ABSTRACT

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A new yellowing disease of lettuce, sugarbeet, carrot, and other crop and weed hosts was found in the desert areas of southwestern United States. The inciting virus (lettuce infectious yellows virus [LIYV]) was transmitted by the sweet potato whitefly (*Bemisia tabaci*) in a semipersistent manner, but it was not mechanically transmissible. The virus was retained by viruliferous whiteflies for 3 days in serial transfers on susceptible hosts. LIYV had a wide host range (45 species in 15 plant families) and caused economically significant losses in a number of important crop plants. The virus was

purified by differential centrifugation and density gradient centrifugation. Purified preparations had an $A_{260}/A_{280\text{nm}}$ ratio of 1.28 and contained long flexuous particles 13–14 nm wide and 1,800–2,000 nm long. An antiserum with a homologous titer of 1/1,024 showed no relationship to beet pseudo-yellows virus and could be used to detect greenhouse- and field-infected plants by the ELISA method. The host range, particle size, insect transmission, and serology clearly distinguished LIYV from previously described viruses.

Additional key words: closterovirus, electron microscopy, luteovirus.

In 1981, extremely large whitefly populations were associated with severe losses in plants in the crop-growing desert areas of California and Arizona. Populations, mainly of the sweet potato whitefly, *Bemisia tabaci* (Gennadius), dramatically increased and induced a series of viruslike disease epidemics on many important crops (6). A whitefly-transmitted virus termed lettuce infectious yellows virus (LIYV) was recognized as being distinct from previously reported viruses that cause yellowing diseases. A preliminary report of this research has been given (7).

The economic impact of LIYV infections in crops grown in the southwest desert regions, based on estimates of comparable crop production in years prior to the widespread dispersal of this virus, is devastating. Lettuce plantings were virtually 100% infected with LIYV. Yield losses, based on estimates of comparable crops in prior growing seasons, were 50–75% lower than expected. Losses of sugarbeets, infected early in their growth cycle with nearly 100% incidence of LIYV, were estimated at 20–30% or about \$9 million. The virtually 100% incidence of LIYV in addition to squash leaf curl virus in cantaloupe, watermelon, other melons, and squash resulted in over an \$8 million loss during this one growing season. The losses caused by this single virus could radically change the desert agriculture if controls are not found.

The purpose of the research on the LIYV agent reported in this paper was to verify evidence of its viral nature, to measure some of its properties, and to investigate its relationship with its whitefly vector, *B. tabaci*.

MATERIALS AND METHODS

Virus source and whitefly maintenance. The virus isolates used for this study were obtained from several commercial lettuce (*Lactuca sativa* L.) plants collected in the Imperial Valley of California and the Yuma area of Arizona during the fall of 1981. The virus was maintained in lettuce and cheeseweed (*Malva parviflora* L.) and transferred from plant to plant via inoculation with the whitefly vector.

B. tabaci collected from field cotton (*Gossypium hirsutum* L.) were transferred to virus-free sweet potato, *Ipomoea batatas* (L.) Lam., grown in muslin-covered cages. The cages were maintained in growth rooms at temperatures that ranged from 26 to 32 C or in an insectary greenhouse. Exhaustive inoculation and recovery tests showed that sweet potato is immune to the virus under study. Virus sources in the various experiments were lettuce plants inoculated 14–21 days earlier. Lettuce plants (cultivar Summer Bibb) at the two-leaf stage were used as test plants. Following inoculation with the LIYV agent via whiteflies, the plants were sprayed with resmethrin (to eliminate the vectors) and placed in greenhouses. All plants were grown in screened greenhouses fumigated at weekly intervals with dichlorvos and resmethrin.

Host range. The host range was determined by allowing 30–50 viruliferous whiteflies reared on diseased cheeseweed (*Malva parviflora*), to feed on at least 10 seedlings of each species tested for 48 hr. The presence of virus in each plant species tested for susceptibility was determined by whitefly transfer (30–50/plant) to lettuce seedlings 30–45 days after inoculation.

Virus-vector relationships. Transmission tests were made by the leaf cage, or sleeve cage method described previously (3). Five tests (eight plants per replication) were performed in each of the experiments for determination of the transmission by different insect species, vector efficiency, acquisition, inoculation, and transmission threshold periods. Unless otherwise stated, 30 insects per cage were used on each plant in the different tests. In determining transmission by different insect species, nonviruliferous insects were given acquisition and inoculation access periods of 24 and 48 hr, respectively. The ability of viruliferous whiteflies to retain LIYV was determined by two methods. Whiteflies reared on diseased cheeseweed plants were transferred in groups of 30 in daily serial transfers on healthy lettuce seedlings. The ability of viruliferous whiteflies to retain the virus when feeding on an immune host was determined by placing insects reared on diseased cheeseweed on immune sweet potato plants and then testing the whiteflies at intervals by transferring them to lettuce seedlings.

Virus purification. LIYV-infected *Nicotiana clevelandii* Gray plants were harvested in 200-g batches 2–3 wk after inoculation. Plants were homogenized in a meat grinder with cold 0.1 M Tris-HCl plus 5 mM EDTA, 20 mM Na₂SO₃, and 10 mM diethyl-dithiocarbamate (DIECA), pH 7.6 (extraction buffer). The plant material was further homogenized with a ball mill for 2 hr at 4 C. The homogenate was expressed through cotton muslin, and Triton X-100 was added to a final concentration of 4%. The slurry was

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stirred overnight at 4 C, subjected to low speed centrifugation for 3 min at 3,000 rpm (1,500 g) followed by centrifugation 8,000 rpm (10,000 g) for 10 min in a Sorvall GSA rotor. The supernatant was ultracentrifuged for 3 hr at 32,000 rpm (110,000 g) in a Beckman 35 rotor over a 5-ml cushion of 30% sucrose in extraction buffer plus 4% Triton X-100. The supernatant was discarded, and the pellets were carefully rinsed several times with distilled water to remove any remaining pigments and sucrose in the tubes. Pellets were gently resuspended at 4 C with 0.1 M phosphate buffer (Na₂HPO₄-KH₂PO₄), and 5 mM EDTA, 20 mM Na₂SO₃, and 10 mM DIECA, pH 7.0 (resuspension buffer). Tube contents were pooled, Triton X-100 was again added to 4%, and the suspension was stirred overnight at 4 C. The suspension was centrifuged for 10 min at 8,000 rpm (7,800 g) in a Sorvall SS-34 rotor, and the supernatant was ultracentrifuged over a 5-ml cushion of 30% sucrose in resuspension buffer and 4% Triton X-100 for 2.5 hr at 28,000 rpm (66,000 g) in a Beckman 30 rotor. Pellets were rinsed with distilled water; resuspended in 4 ml of 0.1 M phosphate buffer containing 2 mM EDTA, pH 7.0 (PE buffer); and stirred for 5 hr at 4 C. The suspension was centrifuged for 5 min at 8,000 rpm (7,800 g) followed by 5 min at 10,000 rpm (12,000 g) in an SS-34 rotor. The supernatant was brought to a final concentration of 25% Cs₂SO₄ (w/v) and layered over 1 ml of 40% Cs₂SO₄ (w/v) (in PE buffer). The virus-Cs₂SO₄ preparation was ultracentrifuged at 8 C for 15-18 hr at 35,000 rpm (100,000 g) in a Beckman SW 39 rotor. Light-scattering bands were extracted with a bent needle and syringe, dialyzed exhaustively in PE buffer at 4 C, then stored at -20 C after the addition of 5% sucrose.

In several other experiments in which the same extraction method was used, viral preparations were layered on sucrose density gradients (10-40% in PE buffer) instead of Cs₂SO₄ gradients and centrifuged at 4 C for 2.5 hr at 25,000 rpm (120,000 g) in a Beckman SW 27 rotor. Gradients were analyzed with an ISCO Model 640 fractionator equipped with a Model UA-5 analyzer.

Light scattering bands from the Cs₂SO₄ preparations and samples from sucrose density gradient peaks were examined for virus particles by placing a drop of the suspension on carbon-coated formvar grids, adding 2% uranyl acetate stain and examining the grids in a Siemens Elmiskop 101. Healthy *N. clevelandii* was also subjected to the same purification method described above as a control.

Infectivity assays. The partially purified virus assayed for infectivity was dialyzed against PE buffer as previously described. Samples removed from sucrose density gradients were not dialyzed. The extracts were adjusted to 25% sucrose and dyed yellow with liquid food coloring before being fed to whiteflies through Parafilm® membranes by using a method described previously (3). Fifty whiteflies per cage and five cages per fraction were used in attempts to detect virus infectivity.

Antiserum production and serology. Purified virus in aliquots of 1 ml containing 325 µg of virus were prepared for four intramuscular injections into a rabbit. Each aliquot was mixed with an equal volume of Freund's complete adjuvant (Difco) just prior to injection. Injections were administered weekly and bleedings were initiated at the fifth week. Microprecipitin tests were used to determine antiserum titer. Enzyme-linked immunosorbent assays (ELISA) were the double antibody sandwich method described by Clark and Adams (2) except that the coating globulin was used at 1 µg/ml and enzyme-conjugated globulin was 1:400.

RESULTS

Host range and symptoms. LIYV induced severe yellowing and/or reddening symptoms and severe stunting, rolling, vein-clearing, and brittleness of affected leaves in a wide range of weed and crop species. Species that naturally show red pigment tended to show intensification of red color in interveinal areas when infected. Symptoms on many of the common hosts were very similar to symptoms induced by beet yellows or beet western yellows (5), which are aphid transmitted, or to beet pseudo-yellows (4), which is transmitted by the greenhouse whitefly (*Trialeurodes vaporariorum* (Westwood)).

The symptoms typical of the yellowing type diseases are virtually identical to those induced by the luteo- and the closteroviruses.

Plants susceptible to LIYV are grouped by family: Apocynaceae — *Vinca minor* L.; Caryophyllaceae — *Dianthus caryophyllus* L.; Chenopodiaceae — *Beta macrocarpa* Guss., *B. vulgaris* L., *Chenopodium capitatum* (L.) Asch., *C. murale* L., *C. quinoa* Wild., *Spinacia oleracea* L.; Compositae — *Carthamus tinctorius* L., *Centaurea cyanus* L., *Cichorium endivia* L., *Lactuca sativa* L., *L. serriola* L., *Helianthus annuus* L., *Sonchus oleraceus* L., *Tagetes patula* L., *Taraxacum officinale* Webber, *Zinnia elegans* Jacq.; Convolvulaceae — *Dichondra repens* Forst., *Ipomoea purpurea* (L.) Lam.; Cruciferae — *Brassica pekinensis* (Lour.) Rupr., *B. rapa* L., *B. septiceps* L., *Capsella bursa-pastoris* (L.) Medic., *Lepidium sativum* L., *Raphanus sativus* L., *Thlaspi arvense* L.; Cucurbitaceae — *Citrullus lanatus* (Thunb.) Mansf., *Cucumis melo* L., *C. sativus* L., *Cucurbita maxima* Dcne., *C. moschata* Dcne., *C. pepo* L.; Geraniaceae — *Erodium cicutarium* (L.) L'Her., *Geranium dissectum* L.; Leguminosae — *Trifolium subterraneum* L.; Malvaceae — *Abutilon theophrastii* Medic., *Anoda abutiloides* A. Gray, *Malva parviflora* L.; Papaveraceae — *Eschscholtzia californica* Cham.; Polygonoaceae — *Rumex crispus* L.; Solanaceae — *Petunia hybrida* Vilm., *Physalis wrightii* Gray, *Nicotiana clevelandii* Gray; Tropaeolaceae — *Tropaeolum majus* L.; and Umbelliferae — *Daucus carota* L.

Plants not susceptible to LIYV under the conditions of this test are: Amaryllidaceae — *Allium cepa* L.; Chenopodiaceae — *Atriplex lentiformis* (Torr.) S. Wats; Compositae — *Bellis perennis* L.; Convolvulaceae — *Convolvulus arvensis* L., *Ipomoea batatas* (L.) Lam.; Cruciferae — *Brassica napus* L., *B. oleracea* var. *botrytis* L., *B. oleracea* var. *gemmifera* DC.; Cucurbitaceae — *Lageraria siceraria* Standl.; Labiatae — *Ocimum basilicum* L.; Leguminosae — *Cyamopsis tetragonoloba* (L.) Taub., *Medicago sativa* L., *Phaseolus vulgaris* L., *Pisum sativum* L., *Vicia faba* L.; Malvaceae — *Althea rosea* (L.) Cav., *Gossypium hirsutum* L., *Hibiscus esculentus* L., *Sida hederacea* (Dougl.) Torr.; Pedaliaceae — *Sesamum indicum* L.; Solanaceae — *Capsicum annum* L., *Datura metel* L., *D. stramonium* L., *Lycopersicon esculentum* Mill., *Nicotiana glauca* Grah., *Nicotiana glauca* Grah., *N. tabacum* L., *Physalis floridana* Rybd.; and Umbelliferae — *Coriandrum sativum* L.

Transmission tests. Mechanical. Numerous attempts were made to transmit the LIYV mechanically by techniques that included the use of an abrasive, phosphate buffer, and sodium sulfite. The virus sources included sugarbeet, lettuce, and *N. clevelandii*. The plants inoculated included these and a number of other species found to be susceptible when inoculated via the whitefly vector. The results were negative in all tests.

Insects. Insect collections taken during the initial outbreak of the viruslike disease epidemic in the desert crop-growing areas had indicated the occurrence of three major whitefly species, *B. tabaci*, *Trialeurodes vaporariorum* (Westwood), and *Trialeurodes abutilonea* (Hald.). Preliminary studies indicated that LIYV was readily transmitted by *B. tabaci*. However, because of the prevalence of the other two whitefly species and their role in the transmission of other yellowing-type viruses (*unpublished*, [4]), and the similarity in symptoms induced by the aphid-transmitted yellowing viruses, it was important to determine whether these other whiteflies and the green peach aphid, *Myzus persicae* (Sulzer), could transmit LIYV. Under conditions of these tests, LIYV was transmitted by *B. tabaci*, but not by *T. vaporariorum*, *T. abutilonea*, or *M. persicae*.

Transmission efficiency. Single whiteflies reared on diseased plants of *M. parviflora* are capable of transmitting LIYV. Transmission rates established by 1, 5, 10, 20, and 40 whiteflies per plant were: 22.2, 66.6, 77.5, 92.1, and 97.4%, respectively.

Minimum acquisition access period. LIYV was sometimes acquired by whiteflies after a 10-min feed but was transmitted more efficiently after longer feeding periods—68.4, 89.5, 81.6, 80.0, and 86.6% after 1-, 3-, 6-, 24-, and 48-hr acquisition feedings, respectively.

Minimum inoculation access period. Whiteflies reared on diseased plants of *M. parviflora* transmitted to 7.5, 60.0, 71.0, 92.3, and 96.7% of plants after inoculation feeding periods of 1, 3, 6, 24 and 48 hr, respectively.

Transmission threshold period. Acquisition access feeding of 1, 3, and 6 hr followed by inoculation access periods of 1, 2, 3, 6, 12, 24, and 27 hr indicated transmission in a minimum period of 6 hr. A latent period in the vector, if any, is less than 6 hr.

Persistence. The ability of viruliferous *B. tabaci* to retain LIYV was determined by daily serial transfers to healthy plants or by testing whiteflies feeding on immune sweet potato. Insects in both groups lost transmitting ability in 3 days or less.

Virus purification. Rate zonal centrifugation of partially purified virus in sucrose density gradients resulted in preparations with a small peak of apparent virus particles at about 20 mm below the meniscus. This band was absent in healthy material subjected to the same purification technique. The bands from diseased plants contained long flexuous particles but we were unable to demonstrate infectivity in membrane feeding tests.

A single band at 32 mm from the meniscus was observed in Cs_2SO_4 gradients of partially purified virus. This band was absent in gradients of healthy material subjected to the same purification technique. The band from LIYV-infected plants was usually so tightly aggregated that the bent needle would not penetrate it

during extraction attempts. The band, after dialysis, had an absorption spectrum typical of viral nucleoprotein. The $A_{260/280\text{nm}}$ value for the purified virus was 1.28. Virus yields ranged from 1.2 to 1.5 mg/kg of leaf tissue using an extinction coefficient of $2 \text{ (mg/ml)}^{-1} \cdot \text{cm}^{-1}$ at 260 nm derived for beet yellows virus (1). Nonviruliferous whiteflies allowed access to the purified preparation failed to transmit LIYV to test plants.

Electron microscopy. Purified preparations from sucrose and Cs_2SO_4 gradients showed large numbers of flexuous filamentous particles of variable length. In some instances, ring-shaped particles were observed (Fig. 1). End-to-end aggregation of broken particles is thought to be the explanation of these structures (Fig. 1). Measurements of over 150 particles indicated a continuous decreasing distribution from the shorter particles (33 particles in the 400–500 nm range) to the longest particles (21 particles in the 1,800–2,000 nm range) with no intermediate modal lengths. The intact particles are estimated at 13–14 nm wide and 1,800–2,000 nm long.

Serology. In microprecipitin serology tests, LIYV ($5 \mu\text{g}$) formed white flocculent precipitates with its homologous antiserum diluted up to 1/1,024. ELISA tests demonstrated LIYV antisera reacted with purified LIYV and reacted to LIYV-infected tissue from sugarbeet, lettuce, melon, and a number of weed species from naturally infected and greenhouse-grown (artificially inoculated)

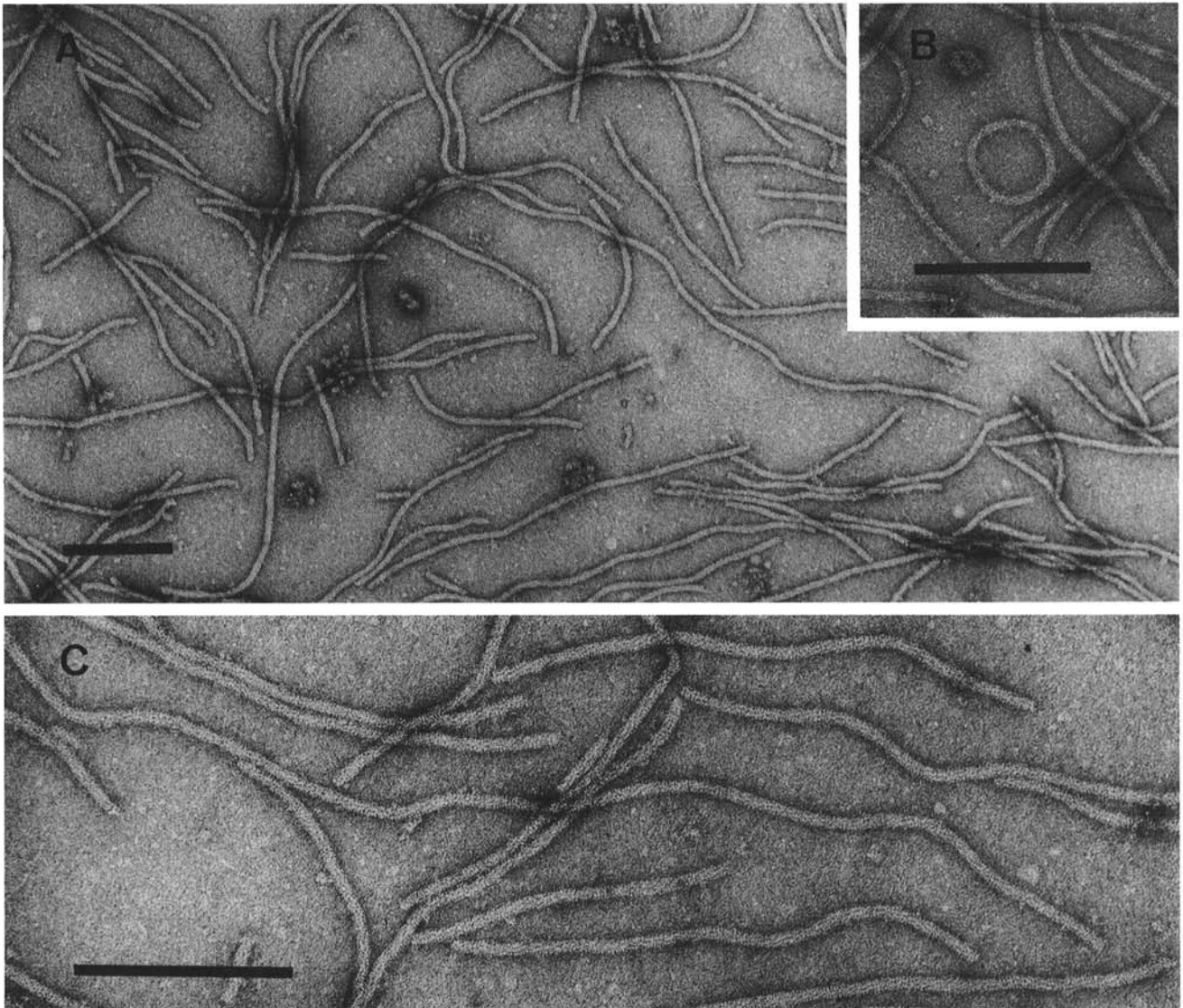


Fig. 1. Electron micrographs of purified lettuce infectious yellows virus particles stained in 2% uranyl acetate. Bars represent 250 nm. A, $\times 64,000$. B, $\times 102,400$. C, $\times 128,000$.

infected plants but not with healthy plants of the same species. The antisera did not react with these same plant species infected with the beet pseudo yellows virus.

Epidemiology and control. The disease induced by LIYV, when first distinguished from other yellowing diseases in 1981, occurred in epidemic proportions in the desert southwest of the United States. Every crop now known to be susceptible to the disease was virtually 100% infected. This extensive distribution and incidence, of course, implies that the disease had been present for a number of years and was well established in the weed and crop plants of the region. Whitefly populations (*Bemisia*) have been increasing in the area since 1975. Reasons for the increasing whitefly populations are thought to be related to higher summer and winter temperatures and the increased use of insecticides on cotton and alfalfa. Symptoms of LIYV in most hosts are almost identical to those caused by aphid-transmitted viruses of the yellows complex and was probably confused with them in recent years.

Early observations by field representatives and growers suggest that cotton played an important role in the LIYV disease cycle on lettuce and sugarbeets. Our evidence indicates that cotton serves as the major source of the whitefly, but is not a host, or source of the virus. The LIYV infects a wide range of crop and weed plants and has been isolated from them during all seasons of the year. Among wild plants, *Malva parviflora*, *Physalis wrightii*, and *Chenopodium murale* seem to play a major role in the incidence of the virus. It would be difficult to design a simple crop management strategy that would eliminate or greatly reduce the incidence of the virus in wild species. The cucurbits, cantaloupe, watermelon, other melons and squash appear to play an important role in the epidemiology of LIYV. Fields of these crops are continuously grown through the year in the desert vegetable- growing areas. The cucurbits are a breeding host of the whitefly following the defoliation of cotton; they also serve as a source of LIYV for newly emerging crops of carrot, lettuce, and sugarbeet in early September. It is apparent that a 2- to 3-wk period free of cucurbits in late July or August would greatly reduce the incidence of LIYV and reduce populations of *Bemisia*.

Reservoirs of LIYV are widespread in the southwestern desert; under present cropping procedures, populations of the whitefly vector seem to be the critical factor in the incidence of this destructive disease.

DISCUSSION

The yellowing-type virus diseases, which are characterized by stunting of infected plants, accompanied by rolling, yellowing, reddening, and brittleness of affected leaves, are probably the most important artificial group of plant virus diseases based on crop losses. Currently, the viruses that induce these diseases have been classified into two recognized virus groups: the luteoviruses, persistent aphid-transmitted viruses with isometric particles, 23–27 nm; and the closteroviruses, semipersistent aphid-transmitted viruses with flexuous, filamentous particles. A third separate group (perhaps a subgroup of the closteroviruses) which also causes yellowing diseases indistinguishable from the aphid-transmitted viruses, includes the whitefly-transmitted viruses such as LIYV. Preliminary data (*unpublished*) indicate two possible other members of the whitefly-transmitted yellowing viruses with flexuous, filamentous particles (beet pseudo-yellows, transmitted by *T. vaporariorum* and Abutilon yellows, transmitted by *T. abutilonea*). A yellows-inducing virus of cucumber (cucumber yellows virus [9], transmitted by *T. vaporariorum*) and a yellow-vein-inducing virus of *Diodea* ([R. C. Larsen and K. S. Kim

unpublished] transmitted by *T. abutilonea*) would also fall in this group.

We have no direct evidence, at this point, that these whitefly-transmitted flexuous rods are closteroviruses. The particles, however, are of similar size and flexibility. Analysis of LIYV-infected tissue indicates that the virus is an RNA virus (*unpublished*). Cytopathological examination of infected tissues of two of the viruses indicate that infections are associated with intracellular inclusions including vesicles which are very similar to those associated with infection by known closteroviruses (8, and R. C. Larsen and K. S. Kim [*unpublished*]).

Although we have not demonstrated the direct infectivity of the long flexuous, filamentous particles associated with LIY, there seems to be no doubt that these are the virus particles. Whiteflies feeding on plants containing the particles readily transmit them to healthy plants. Antisera made against purified preparations of these particles react only with plants showing symptoms of LIYV and which contain the particles.

Ultrastructure studies have shown flexuous rods essentially the same size as the isolated LIYV particles in vascular parenchyma cells, sieve elements, plasmodesmata, and sieve plates of infected leaves but not in control plants (8).

It is not too surprising that we have not as yet demonstrated the direct infectivity of the isolated particles. The virus is not mechanically transmitted and thus far only one whitefly-transmitted virus (squash leaf curl, a geminivirus) has been transmitted by whitefly feeding (3) through membranes. As far as the authors are aware, there is no evidence of the transmission of any long flexuous rod-shaped virus similar to closteroviruses from purified preparations through membranes by any insect.

Although none of the other whitefly-transmitted yellowing viruses has been purified to the degree of LIYV, preliminary results have shown that they have long flexuous, filamentous particles (*unpublished*). LIYV, however, differs from these viruses in being transmitted by different whitefly species and appears to be serologically unrelated to them.

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