Lisianthus Necrosis Virus, a New Necrovirus from *Eustoma russellianum*

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ABSTRACT

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A virus isolated from lisianthus, *Eustoma russellianum*, showing systemic necrotic symptoms was designated lisianthus necrosis virus (LNV). The virus was transmitted by sap inoculation and infected 21 species within 10 plant families; it was also soilborne. The virus lost infectivity when heated for 10 min at 95 but not at 90 C, diluted 10^{-10} but not 10^{-9}, or stored at 20 C for more than 9 wk. The virus did not react with antisera to tobacco necrosis, cucumber necrosis, and melon necrotic spot viruses. The virus particles, which were spherical with diameters of 30 ± 0.48 nm, sedimented as a single component with a sedimentation coefficient of 136 S and had a buoyant density in CsCl of 1.333 ± 0.003 g/cm^3. They contained single-stranded RNA with a molecular weight of 1.67×10^6 (estimated by gel electrophoresis of undenatured RNA) and a single polypeptide with a molecular weight of 35,500.

The cultivation of lisianthus plants, *Eustoma russellianum* Griseb, for flower production has increased recently in Japan. Viruslike diseases of these plants cause considerable yield losses. Three distinct virus diseases occur, two of them caused by broad bean wilt and cucumber mosaic viruses (5). The other is caused by a new virus described below.

The necrotic disease was observed on lisianthus plants in Chiba Prefecture in 1984. The soilborne nature of the disease was suspected because it occurred sporadically in fields. Local lesions appeared on inoculated leaves of many plants after transmission by sap inoculation, and infected plants contained spherical particles about 30 nm in diameter. These properties seemed to be similar to those of necroviruses. However, the virus was not serologically related to any of the necroviruses tested, and the sizes of its RNA and protein were distinctively different from those of necroviruses. It was designated lisianthus necrosis virus (LNV). This paper describes biological, physical, and chemical properties of the new soilborne virus.

MATERIALS AND METHODS

Virus source and maintenance. The virus isolate used for characterization was from naturally infected lisianthus plants showing necrotic symptoms and collected in the Chiba Prefecture in 1984. After transmission by sap inoculation to *Nicotiana tabacum* L., the isolate was passed through three successive single-lesion transfers on *Gomphrena globosa* L. It was maintained and propagated in *N. clevelandii* Gray plants. Infected leaves were kept in an ultra-low-temperature freezer (−70 C) until required. Sap inoculations were done by rubbing Carborundum (600 mesh)-dusted leaf surfaces with cotton pieces soaked in the inoculum. The inoculum was prepared by homogenizing diseased leaves with 0.05 M phosphate buffer (about 10 ml/g of leaf) in mortar.

Host range. Plants representing 28 species within 12 families were sap inoculated with homogenates of infected leaves of *N. clevelandii*. Symptomless plants were assayed by sap inoculation to *Chenopodium amaranticolor* Coste and Reyn 7–10 days after inoculation for inoculated leaves and about 28 days after inoculation for emerging leaves. All plant species (two to six plants per species) were tested at least twice in different seasons.

Transmission. Aphid transmission tests were conducted with virus-free aphids of the species *Myzus persicae* Sulz. reared on turnip plants. Diseased lisianthus or *N. clevelandii* plants were the source plants and healthy lisianthus or *N. clevelandii* were the test plants. Aphids were starved for 2 hr in a glass beaker before an acquisition access feeding of 15 min on diseased plants, followed by an inoculation access feeding period of 24 hr on healthy plants. Aphids were removed by spraying with insecticide after the inoculation access feeding. Fifteen aphids were used per test plant.

Soil transmission tests were conducted with soil from the fields in which the disease had occurred and also with virus-free *Olpidium* sp. collected from soil around plants without disease in those same fields. Suspensions (15 ml) of zoospores from healthy lisianthus roots in water were mixed with crude sap (1 ml) from infected *N. clevelandii* leaves, and 1 ml of the mixture was poured immediately onto the soil around each *N. clevelandii* test plant. About 2 wk later, symptoms were observed and sap inoculation tests were made to *C. amaranticolor* with sap from roots of the test plants.

Seed transmission tests were performed by planting in sterilized soil the seeds from diseased lisianthus plants grown in the glasshouse.

Stability in sap. The thermal inactivation point, dilution end point, and longevity in vitro of the virus were determined by treating sap from infected *N. clevelandii* leaves and inoculating *C. amaranticolor*. The sap for thermal inactivation point and longevity in vitro tests was diluted 10-fold with 0.05 M phosphate buffer, pH 7.0. Sap for the dilution end point test was diluted with same buffer.

Purification and electron microscopy. Frozen, infected leaves of *N. clevelandii* were homogenized with two volumes (2 ml/g of leaf) of 0.01 M sodium/potassium phosphate buffer (SPPB), pH 7.0, and two volumes of chloroform. Sap was expressed through cheesecloth and centrifuged at 10,000 *g* for 15 min. The aqueous phase was recovered, mixed with 1% Triton X-100 nonionic detergent, and centrifuged at 80,000 *g* for 180 min. The pellet was resuspended in SPPB, and centrifuged at 10,000 *g* for 10 min. The supernatant was subjected to one cycle of differential centrifugation (100,000 *g* for 120 min and 10,000 *g* for 10 min), and the resuspended pellet was layered on 10–40% linear sucrose density gradients. Gradients were centrifuged for 150 min at 74,000 *g*. The opaque, virus-containing zone was removed with a syringe and concentrated by centrifugation at 120,000 *g* for 120 min.

Preparations were made for electron microscopy by grinding small pieces of infected lisianthus leaves in 2% potassium phosphotungstate, pH 6.5. Purified virus was prepared for electron microscopy by mounting a small drop of purified virus on a carbon-stabilized, Formvar-coated grid and staining with 2% potassium phosphotungstate, pH 6.5. Particle size was determined by measuring 100 particles, with particles of tobacco mosaic virus being used as the magnification standard.

Serology. A rabbit was immunized by two intravenous injections of purified virus and two intramuscular injections of the virus emulsified with an equal volume of Freund's complete adjuvant. Antiserum was obtained from blood collected 10 days after the final injection. The titer of the antiserum, determined in agar gel double diffusion, was 1/8,192.

Serological relationships between the virus and four strains or isolates of tobacco necrosis virus (TNV), melon necrotic spot virus (MNSV), and cucumber necrosis virus (CNV) were tested by gel double-diffusion tests in 1% agar containing 0.85% NaCl and 0.01% NaN_3. Antiserum against TNV-HSN was provided by S. Kubo, TNV-Fv by K. Abiko, TNV-D by D. A. Govier, and MNSV and CNV by T. Hibi.

Analytical ultracentrifugation. The sedimentation coefficient (determined at a virus concentration of 1 mg/ml) and buoyant density in CsCl were determined as previously described (4).

Preparation and identification of nucleic acid. Viral nucleic acid was prepared by an SDS-phenol method. Viral nucleic acid was treated with either RNase or DNase for identification as previously described (4).

Electrophoresis. One percent agarose gel electrophoresis for nucleic acid and 10% polyacrylamide gel electrophoresis for protein were done as previously described (4). Tobacco necrosis virus originally isolated from tulip was used for comparison. Molecular weight markers used were bovine serum albumin (molecular weight of 68,000), ovalbumin (45,000), and chymotripsinogen A (25,000).

RESULTS

Symptoms on lisianthus. Lisianthus plants naturally infected with the virus showed necrotic spots and rings on leaves and stems, stunting, and tip necrosis (Fig. 1A). Furthermore, plants with purple-colored flowers sometimes showed white color breaking. Lisianthus plants inoculated with the virus showed necrotic rings on inoculated leaves, and the overall symptom pattern was similar to that observed in the field.

Host range and symptoms. The virus infected 21 species in 10 plant families of the 28 species in 12 families that were tested, indicating a wide host range. The virus caused necrotic local lesions in most of the host plants. Systemic symptoms were produced only in three plant species, *G. globosa, N. clevelandii*, and lisianthus. *G. globosa* showed necrotic local lesions on inoculated leaves and necrotic lesions on newly emerged leaves. *N. clevelandii* developed necrotic local lesions on inoculated leaves 3–4 days after inoculation, and the upper leaves soon became necrotic. Systemically infected plants died 10–14 days later.

Another 16 plant species developed necrotic local lesions on inoculated leaves without systemic infection. They were *Chenopodium amaranticolor, C. quinoa* Willd., *Cucumis sativus* L., *C. melo* L. 'Conomon', *Cucurbita maxima* Duch., *C. moschata* Duch., *C. pepo* L., *N. glutinosa* L., *N. tabacum* (Fig. 1C), *Phaseolus vulgaris* L. (Fig. 1B), *Sesamum indicum* L., *Tetragonia expansa* Murr., *Vicia faba* L., *Vigna unguiculata* (L.) Walp. subsp. *sesquipedalis* (L.) Verdc., *V. unguiculata* (L.) Walp. subsp. *unguiculata*, and *Zinnia elegans* Jacq.

Petunia hybrida Vilm. and *Dianthus superbus* L. did not develop any symptoms; however, sap inoculation tests to *C. amaranticolor* indicated that inoculated leaves of these plants contained the virus.

The virus did not infect *Brassica rapa* L., *Capsicum annuum* L., *Dianthus caryophyllus* L., *D. chinensis* L., *Lycopersicon esculentum* Mill., *N. debneyi* Domin., and *Petroselinum sativum* Hoffm.

Transmission. *M. persicae* failed to transmit the virus from diseased lisianthus or *N. clevelandii* plants to lisianthus (0/6) or *N. clevelandii* (0/7) plants. In studies of soil transmission, *N. clevelandii* seedlings were transplanted into field soil from around necrotic diseased lisianthus plants. About 2 wk later these plants (3/3) developed necrotic symptoms, and LNV was recovered by back inoculation to *C. amaranticolor*.

Light microscopy of roots of tobacco, *N. clevelandii*, and lisianthus plants grown in the same soil revealed the presence of zoosporangia and resting spores of *Olpidium* sp. in the roots.

All seven plants of *N. clevelandii* inoculated with the mixture of virus-free zoospore suspension and crude sap from virus-infected plants developed necrotic symptoms about 2 wk after inoculation. Back inoculation from roots of these plants to *C. amaranticolor* indicated that the plants (7/7) contained the virus. After removal of the infected plants, *N. clevelandii* was transplanted again into the same soil. After 2 wk, similar back inoculation proved that the second group of plants (3/3) was infected with the virus.

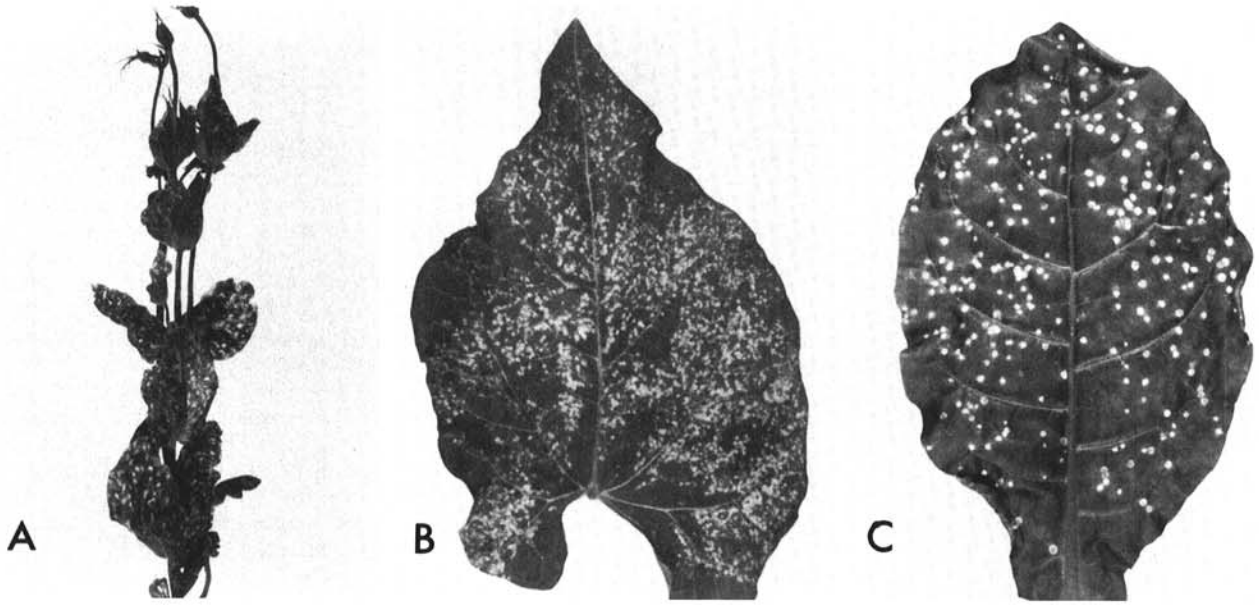

Fig. 1. Symptoms caused by lisianthus necrosis virus. **A,** Systemic necrotic spots on lisianthus. **B,** Necrotic local lesions on inoculated leaf of French bean. **C,** Necrotic local lesions on inoculated leaf of tobacco cultivar Xanthi nc.

For the control experiments, 1 ml of crude sap containing virus or 1 ml of a zoospore suspension of virus-free *Olpidium* sp. was poured onto the soil around each *N. clevelandii* plant, and subsequent back inoculation showed that none of these plants (0/6 and 0/8, respectively) was infected with the virus.

In studies of seed transmission, none of the 178 seedlings emerging from seeds harvested from infected lisianthus plants showed any symptoms. Back inoculation from roots of these plants to *C. amaranticolor* indicated that the plants did not contain the virus.

Stability in sap. The virus lost infectivity when heated for 10 min at 95 but not 90 C, diluted 10^{-10} but not 10^{-9}, or stored at 20 C for more than 9 wk.

Purification and electron microscopy. A single, opaque band typical of a virus-containing zone was observed in sucrose density gradients. Purified virus preparations had an ultraviolet absorption spectrum typical of nucleoprotein, with maximum absorbance at 260 nm and minimum at 244 nm. The A_{260nm}/A_{280nm} ratio was 1.61 ± 0.04, and the A_{max}/A_{min} was 1.26 ± 0.06.

The purified preparations contained large numbers of spherical particles 30 ± 0.48 nm in diameter (Fig. 2). Electron microscopy of dip preparations from infected lisianthus and *N. clevelandii* revealed similar particles.

Serology. The purified virus did not react with antisera against TNV-tulip isolate (11) (homologous titer 1:4096), TNV-HSN (14) (1:128), TNV-strawberry isolate (Fv) (6) (no data), TNV-D (1) (1:2048), MNSV (8) (1:1280), and CNV (10) (1:1024). Antiserum against LNV did not react with purified preparations of TNV-tulip isolate, TNV-HSN, TNV-strawberry isolate, and MNSV.

Properties and composition of particles. Virus particles from purified preparations sedimented as a single peak during analytical ultracentrifugation. The sedimentation coefficient of the virus particles at 20 C was calculated to be 136 S at 1 mg/ml in 0.05 M citrate buffer, pH 6.8. Virus particles were stable in CsCl, and at equilibrium the particles sedimented as a single band corresponding to a buoyant density of 1.333 ± 0.003 g/cm^3.

In agarose gels, the nucleic acid of LNV migrated as a single peak with a molecular weight of $1.67 \pm 0.04 \times 10^6$ (mean of three determinations) in nondenaturing conditions (Fig. 3). When RNA of TNV was run under the same conditions, TNV-RNA migrated faster than nucleic acid of LNV. The molecular weight of TNV-RNA was estimated to be 1.5×10^6. Phenol-extracted nucleic acid of LNV was infectious. Infectivity of viral nucleic acid was abolished completely in the presence of RNase under high salt ($1\times$ SSC) and low salt ($0.1\times$ SSC) conditions, whereas the infectivity was almost unaffected after DNase treatment. These results indicated that nucleic acid of LNV is single-stranded RNA.

Protein of LNV migrated as a single component in 10% polyacrylamide gels with molecular weight of $35{,}500 \pm 500$ (mean of four determinations) (Fig. 4). Under the same conditions, protein of TNV migrated with molecular weight of 27,000. Protein of LNV also migrated more slowly than protein of TNV in 5%, 7.5%, and 12.5% polyacrylamide gels.

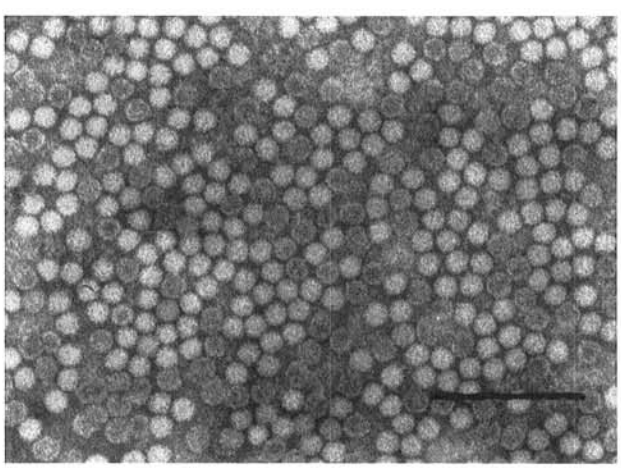

Fig. 2. Lisianthus necrosis virus particles from purified preparation. Bar represents 200 nm.

DISCUSSION

Necroviruses infect most plants only locally and have a wide host range. In addition, these viruses also infect a limited number of

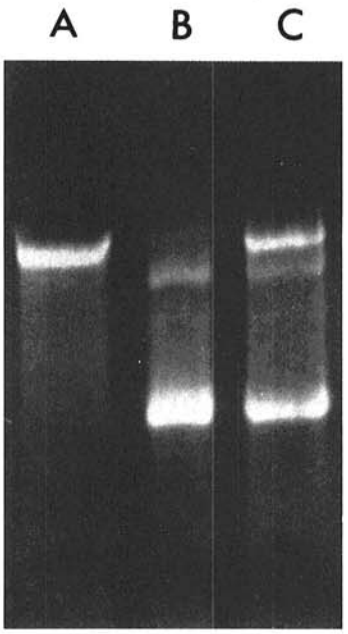

Fig. 3. Electrophoretic patterns of RNA of lisianthus necrosis virus (LNV). Electrophoresis was conducted in 2% acrylamide-0.5% agarose composite gel at 40 mA for 2 hr under nondenaturing conditions. After electrophoresis, gel was stained with ethidium bromide. Lane A, LNV; B, tobacco necrosis virus containing satellite virus; C, LNV + tobacco necrosis virus.

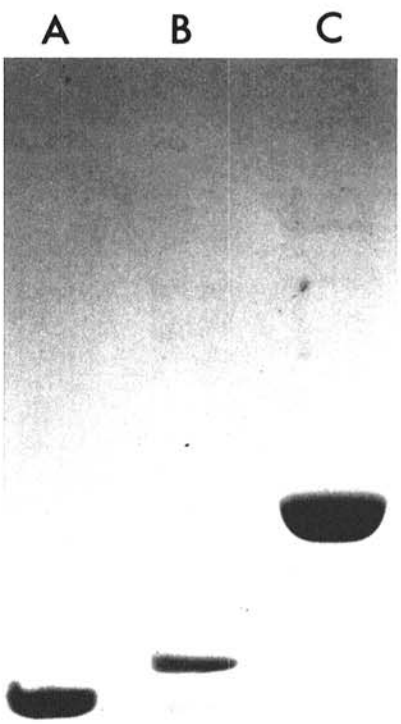

Fig. 4. Electrophoretic patterns of protein of lisianthus necrosis virus (LNV). Electrophoresis was conducted in 10% polyacrylamide gel at 30 mA for 3 hr. After electrophoresis, gel was fixed and stained with Coomassie brilliant blue R-250. Lane A, cucumber mosaic virus; B, tobacco necrosis virus; C, LNV.

plant species systemically, causing severe symptoms—for example, TNV in tulip (7), French bean (2), and potato (12); MNSV (8) in melon; and CNV (10) in cucumber. Like these viruses, LNV induced local lesions in many plants but infected lisianthus, *N. clevelandii*, and *G. globosa* systemically.

Particle morphology, transmission mode, and stability in sap of LNV were similar to those described for necroviruses. However, no serological relationship was detected between LNV and any isolate of TNV, MNSV, or CNV tested so far. Host range properties of these viruses would indicate certain similarity between LNV and TNV, since they did not infect Cucurbitaceae plants systemically. However, these two viruses were distinct in biochemical properties. LNV-RNA migrated more slowly than TNV-RNA by electrophoresis under nondenaturing conditions. The molecular weight of TNV coat protein has been reported to be 22,600–27,000 for three distinct isolates (3,13,15). These values were much smaller than that found for LNV. The Urbama isolate of TNV was exceptional in that its coat protein was larger, a molecular weight of 33,000 (9), and rather close to that of LNV. Host range properties or serological relationship between the Urbama isolate and other TNV isolates have not been reported.

From these results, LNV appears to be a new member of the necrovirus group. The isolates of LNV tested to date have not contained an associated satellite virus.

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