Soybean Chlorotic Mottle, a New Caulimovirus on Soybean

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ABSTRACT

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A virus with major properties similar to those of caulimoviruses induced chlorotic mottling and stunting in soybean (*Glycine max*). The virus was transmitted by mechanical inoculation to soybean, bean, lablab bean, and cowpea but was not transmitted by five species of aphids. The virus, which consisted of isometric particles 50 nm in diameter, was not related serologically to cauliflower mosaic virus.

Many virus diseases that occur in soybean plants worldwide cause considerable loss in soybean yields. More than 50 viruses have been reported in soybean (11), of which eight viruses have been observed in Japan (4–6,12,13). During surveys on virus diseases of soybean (*Glycine max* (L.) Merr.) in Aichi Prefecture in 1981, we collected some soybean plants that showed mosaic symptoms and isolated soybean mosaic, alfalfa mosaic, and cucumber mosaic viruses as well as an unknown virus. The fourth virus, which was found to consist of spherical particles about 50 nm in diameter, had not been reported previously on soybean.

This report describes some properties of the virus, designated soybean chlorotic mottle virus, that was isolated from soybean and is considered to be a new member of the caulimovirus group.

MATERIALS AND METHODS

Virus source and maintenance. The virus was isolated from naturally infected soybean plants collected in Aichi, Japan, in 1981 and maintained on soybean or bean plants by mechanical inoculation. Inoculum for mechanical inoculation was prepared by grinding systemically infected soybean (*G. max* cv. Tsurunoko) or bean (*Phaseolus vulgaris* cv. Kintoki) leaves in 0.05 M potassium and sodium phosphate buffer, pH 7.0, containing 0.01 M sodium diethyldithiocarbamate and 1 mM L-cysteine. All test plants were grown in a glasshouse. Mechanical inoculation was carried out by rubbing a cotton piece soaked in inoculum on leaves previously dusted with Carborundum.

Host range and properties. Plants representing 42 species belonging to 12 families were mechanically inoculated with sap from infected Kintoki bean plants. Symptomless plants were assayed by back-inoculation to Kintoki beans using sap extracted from inoculated leaves 14 days after inoculation and from newly emerged leaves about 28 days after inoculation. Dilution end point, thermal inactivation point (TIP), and longevity in vitro (LIV) of the virus were determined using Kintoki bean as the assay host. Sap for TIP and LIV tests was diluted 10-fold in 0.05 M potassium and sodium phosphate buffer, pH 7.0.

Transmission. Virus-free aphids *Acyrthosiphon pisum* Harris, *Aphis craccivora* Koch, *A. glycines* Matsumura, *Aulacorthum solani* Kaltenbach, and *Myzus persicae* Sulzer were reared on alfalfa (*Medicago sativa*), cowpea (*Vigna unguiculata*), soybean (*G. max*), soybean, and turnip (*Brassica rapa*), respectively. In the aphid transmission tests, aphids were starved for 1–2 hr and given acquisition access periods of 15 min to 48 hr on diseased soybean or bean followed by inoculation access periods of 1–48 hr on healthy soybean or bean plants. Ten to 20 aphids were used per test plant.

Seed transmission tests were carried out using seeds collected from infected Tamahikari and Akasaya soybeans and Kintoki and Top Crop beans grown in a glasshouse.

Purification. The virus was purified according to a modification of the procedure described by Hull and Shepherd (3) for cauliflower mosaic virus. Frozen, infected leaves of Kintoki bean were ground in a homogenizer with 0.5 M potassium phosphate buffer (3–4 ml/g of leaf) containing 0.75% sodium sulfite, pH 7.2, and the homogenate was expressed through cheesecloth. The expressed sap was mixed with 2.5% Triton X-100 and 1 M urea for 60 min. Mixed juice was centrifuged at 124,800 *g* for 90 min, and the pellets were resuspended in distilled water. They were mixed with 1:5 (v/v) of chloroform and 1:20 (v/v) of *n*-butanol for 1 min and the emulsion was broken by high-speed centrifugation (7,000 *g* for 10 min). The aqueous phase was placed on 10–40% sucrose density gradients. Gradients were centrifuged at 74,700 *g* for 150 min with a Hitachi RPS 27-2 swinging-bucket rotor. After centrifugation, the opaque zone containing the virus was removed with a syringe and centrifuged at 126,800 *g* for 90 min. The pellets were resuspended in distilled water and used for rabbit immunization and electron microscopy.

Serology. A rabbit was immunized by four intramuscular injections of partially purified virus emulsified with an equal volume of Freund's complete adjuvant at intervals of 3 wk. Antiserum obtained from blood collected 30 days after the final injection reacted with purified virus in agar gel diffusion tests at a dilution of 1:512 and in ring tests at a dilution of 1:32. The antiserum also reacted with juice of healthy bean leaves; therefore, the antiserum was used in serological tests after neutralization with an equal volume of juice of healthy bean leaves.

The serological relationship of the virus to cauliflower mosaic virus was analyzed in agar gel double-diffusion and ring tests. Antiserum to cauliflower mosaic virus was provided by A. A. Brunt, Glasshouse Crops Research Institute, England. Cauliflower mosaic virus used in serological tests was purified from infected turnip by the procedure of Hull and Shepherd (3). Double-diffusion tests were conducted in 0.8% agar prepared in water containing 0.85% sodium chloride and 0.04% sodium azide. Antiserum was added in each well 6 hr after supplying the antigens.

Light microscopy. Epidermal strips were removed with forceps from the undersides of midribs of infected Kintoki bean leaves. Samples were dipped and stained in 1% (w/v) phloxine for 5 min. After washing off excess phloxine with distilled water, the samples were mounted in water and examined under an Olympus H microscope.

Electron microscopy. Dip preparations for electron microscopy were prepared by

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Fig. 1. Symptoms caused by soybean chlorotic mottle virus. **(A)** Mottle symptom in Tamahikari soybean, **(B)** stunting symptom in Tamahikari soybean plant, and **(C)** veinclearing, mottling, and leaf curl symptoms in Akasaya soybean.

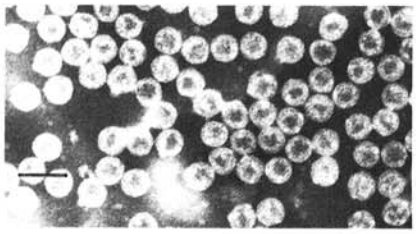

Fig. 2. Particles of soybean chlorotic mottle virus. Bar = 100 nm.

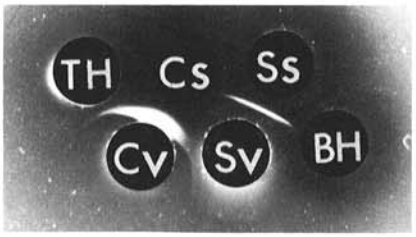

Fig. 3. Agar gel double diffusion test of soybean chlorotic mottle virus. Sv = soybean chlorotic mottle virus, Cv = cauliflower mosaic virus, Ss = antiserum to soybean chlorotic mottle virus, Cs = antiserum to cauliflower mosaic virus, and BH and TH = juice from healthy bean and turnip leaves, respectively.

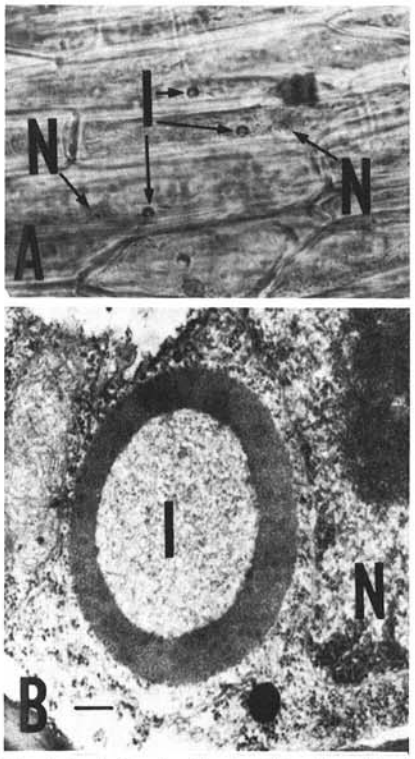

Fig. 4. Inclusion bodies in **(A)** epidermis of bean under light microscope and **(B)** soybean tissues under electron microscope. I = inclusion and N = nucleus. Bar = 200 nm.

grinding the small pieces of infected soybean leaves in 2% potassium phosphotungstic acid, pH 6.5. Preparations of purified virus for electron microscopy were prepared by mounting a small drop of purified virus on a carbon-stabilized, Formvar-coated grid and staining with 2% potassium phosphotungstic acid, pH 6.5.

For ultrathin sections, small pieces of diseased soybean and bean leaves were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, for 1 hr, then postfixed with 1% osmium tetroxide in the same buffer for 2 hr. Fixed samples were dehydrated in an acetone series and embedded in epoxy resin. Ultrathin sections were cut with an LKB Ultrotome 8800. Sections were stained with uranyl acetate and lead citrate and observed in a Hitachi Model H-500 electron microscope.

RESULTS

Host range and symptoms. The virus infected only four plant species, *G. max, Dolichos lablab, P. vulgaris,* and *V. unguiculata*, which belong to the family Leguminosae among the 42 species within 12 families that had been inoculated with sap.

Naturally infected soybean plants showed mosaic and stunting symptoms. Symptoms on soybean inoculated mechanically in the laboratory with the virus varied with the cultivars. Cultivar Tamahikari at first showed veinclearing and chlorosis on young leaves, which were reduced in size compared with leaves of uninfected plants. Thereafter, the leaves showed mottle symptoms (Fig. 1A). Also, infected plants showed stunting with shortened internodes (Fig. 1B). Lateral buds sometimes grew vigorously and showed mottle symptoms in the late stages of infection. Cultivar Akasaya showed veinclearing, mottling, and leaf curling on young leaves (Fig. 1C). Infected plants were slightly stunted compared with healthy plants. Incubation period of the virus in plants varied with the season. Symptoms appeared after an incubation period of about 30 days in winter (22–27 C) and about 20 days in summer (24–30 C). *D. lablab* showed veinclearing on the upper leaves. *P. vulgaris* showed chlorotic local lesions in the inoculated leaves 7–14 days after inoculation, then chlorotic spots on the upper leaves. Young leaves showed veinclearing, mottling, and leaf curling. Inoculated leaves of *V. unguiculata* failed to develop symptoms.

The following plant species did not become infected with the virus: *Apium graveolens, Arachis hypogaea, Beta vulgaris* var. *cicla, Brassica oleracea, B. rapa, Cajanus cajan, Calendula arvensis, Capsicum annuum, Cassia tora, Chenopodium amaranticolor, C. quinoa, Cucumis sativus, Cucurbita moschata, Datura stramonium, Dianthus caryophyllus, Gomphrena globosa, Hibiscus esculentus, Lactuca sativa, Lycopersicon esculentum, Nicotiana clevelandii, N. debneyi, N. glutinosa, N. tabacum, Petroselinum sativum, Petunia hybrida, Phaseolus angularis, Pisum sativum, Sesamum indicum, Spinacia oleracea, Tetragonia expansa, Trifolium pratense, T. repens, Vicia faba, Vigna mungo, V. radiata, V. sesquipedalis,* and *Zinnia elegans.*

Stability in crude sap. Crude sap of infected soybean leaves remained infective after dilution of 10^{-3} but not 10^{-4}, after heating for 10 min at 85 C but not at 90 C, and after storage for 1 day at 20 C but not 3 days.

Transmission. The virus was transmitted easily by sap inoculation but not by the aphids *Acyrthosiphon pisum* (0/14, total used aphid number = 280), *Aphis craccivora* (0/125, total used aphid number = 1,545), *A. glycines* (0/67, total used aphid number = 945), *Aulacorthum solani* (0/16, total used aphid number = 400) and *M. persicae* (0/9, total used aphid number = 180) in a nonpersistent or persistent manner.

The virus was not transmitted through

seeds of Tamahikari soybean (0/574), Akasaya soybean (0/262), Kintoki bean (0/19), and Top Crop bean (0/91).

Particle morphology. Expressed sap from infected soybean and bean plants and partially purified preparations contained spherical particles about 50 nm in diameter (Fig. 2).

Serology. In agar gel diffusion tests, the virus and cauliflower mosaic virus yielded a single precipitin line with each homologous antiserum but did not with another antiserum (Fig. 3). In ring tests also, the virus and cauliflower mosaic virus reacted wtih homologous antiserum at a dilution of 1:32 but did not with another antiserum.

Inclusion bodies in light microscopy. Roundish inclusion bodies similar to those produced by caulimoviruses (10) were observed in the epidermis cells of the undersides of infected bean leaves (Fig. 4A).

Inclusion bodies in electron microscopy. Inclusions were easily found in the cytoplasm of infected soybean and bean tissues examined in an electron microscope. The inclusions were frequently ovoid or elliptical and consisted of an amorphus, vacuolated, electron-dense matrix. These inclusions were similar to those induced by caulimoviruses (10). Spherical virus particles about 45 nm in diameter were observed only in the inclusion bodies (Fig. 4B).

DISCUSSION

Six definitive and four possible members of the caulimoviruses (8) have been reported previously; however, infection of soybean with these viruses has not been reported.

The properties of the virus isolated from soybean were similar to those of caulimoviruses with regard to the morphology of particles, inclusion bodies under the light microscope and electron microscope, and physical properties, although the virus was not transmitted by the aphids tested.

Some caulimoviruses show serological relationships among their members (1,7,9). The virus isolated from soybean, however, was not related to cauliflower mosaic virus in the serological tests. Moreover, the virus contained dsDNA as reported separately (2). These results suggest that the virus is a new member of caulimovirus.

Because the virus was detected only in a few samples in one field, its distribution appears to be limited. However, the presence of the virus may be overlooked because the symptoms and host range of the virus are similar to those of soybean mosaic virus occurring in many soybean fields.

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