

## Whitefly Transmission and Some Properties of Cowpea Mild Mottle Virus on Soybean in Thailand

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### ABSTRACT

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A rod-shaped virus was isolated from naturally infected soybean (*Glycine max*) plants collected at Phitsanulok, northern Thailand, in 1979. The virus caused mild mottle symptoms in soybean and was transmitted by whiteflies, *Bemisia tabaci*, in a semipersistent manner, by sap inoculation and through soybean seed; it was not transmitted by the aphids *Aphis craccivora* or *A. glycines*. Among 26 plant species tested by mechanical inoculation, the virus systemically infected mainly plants of the family Leguminosae, and it produced poorly defined local lesions on inoculated leaves of *Chenopodium amaranticolor*. Virus particle dimensions (10–15 × 650–700 nm) were in the range of those of carlaviruses. In tube precipitin tests, the virus reacted with antiserum to cowpea mild mottle virus, a previously reported virus infecting legumes in Africa, and showed distant serologic relationships with carnation latent virus and chrysanthemum virus B but not with other morphologically similar viruses. Infectivity of the virus was retained in sap diluted to 10<sup>-5</sup> but not 10<sup>-6</sup>, heated for 10 min at 70 C but not 75 C, or held at 20 C for 21 days but not 28 days. The virus contained single-stranded ribonucleic acid and was identified as cowpea mild mottle virus on the basis of host range, symptomatology, particle morphology, and serologic relationships.

During surveys of virus diseases of soybean (*Glycine max* (L.) Merr.) areas in Thailand in 1979, we observed many plants showing crinkle leaf symptoms and vein enations. Preliminary tests indicated that some of these plants were doubly infected with presumably two viruses borne by whiteflies. One was rod-shaped and caused mild mottle in soybeans and was mechanically transmissible; the other caused crinkle leaf symptoms with vein enations on the undersurface of leaves, was not mechanically transmissible, and no particles were detected in infected tissues.

We present results from studies of the mechanically transmissible virus that indicated that it had some properties of the carlavirus group, but that it was transmitted by whiteflies instead of aphids. The virus appeared to be cowpea

mild mottle virus (CMMV), which has been known to occur on cowpea and tomato in Africa (2,4), on soybean in Thailand (3), and on peanut in India (D. V. R. Reddy and N. Iizuka, *personal communication*).

### MATERIALS AND METHODS

**Virus source and maintenance.** The virus was isolated from naturally infected soybean plants collected at Phitsanulok, northern Thailand, in 1979 and maintained in soybean plants by mechanical inoculations. Sap for inoculation was prepared by grinding leaves from infected plants in 0.05 M potassium and sodium phosphate buffer, pH 7.0, containing 0.01 M sodium diethylthiocarbamate and 1 mM L-cysteine. All test plants were grown in a glasshouse at Ibaraki, Japan, or in an insect-proof house at Bangkok, Thailand.

**Host range and properties.** Plants representing 26 species in nine families were mechanically inoculated with sap from soybean plants infected via whiteflies. Symptomless plants were assayed by back inoculation to Tsurunashi Kintoki bean, using sap extracted from inoculated leaves 7–10 days after inoculation and from newly emerged leaves about 21 days after inoculation. All plant species were tested at least twice in different seasons.

Dilution end point (DEP), thermal

inactivation point (TIP), and longevity in vitro (LIV) for the virus were determined using Tsurunashi Kintoki bean as the assay host. Sap for TIP and LIV tests was diluted tenfold in 0.05 M potassium and sodium phosphate buffer, pH 7.0.

### Electron microscopy and purification.

Samples for electron microscopy were prepared by dipping a piece of infected soybean leaf into a small drop of 2% potassium phosphotungstate, pH 6.5, on a carbon-stabilized, Formvar-coated grid. Grids were air-dried and observed with a Hitachi H 300 or H 500 electron microscope.

Frozen, infected leaves of Shirotsurunoko soybean or Tsurunashi Kintoki bean were homogenized with three volumes of 0.2 M potassium phosphate buffer, pH 8.0, containing 0.1% 2-mercaptoethanol and 0.01 M sodium ethylenediaminetetraacetate. Sap was expressed through cheesecloth and mixed with a half volume of a chloroform:carbon-tetrachloride mixture (1:1, v/v); the emulsion was broken by high-speed centrifugation (9,000 g, 15 min). The aqueous phase was recovered, mixed with 0.5% Triton X-100, and centrifuged at 100,000 g for 70 min.

Pellets were resuspended in 0.2 M potassium phosphate buffer (pH 8.0) containing 0.1% 2-mercaptoethanol and 2 mM sodium ethylenediaminetetraacetate and centrifuged at 9,000 g for 10 min. The supernatant was subjected to one cycle of differential centrifugation as above, and resuspended pellets were layered on 10–40% linear sucrose density gradients. Gradients were centrifuged for 120 min at 60,000 g using a Hitachi RPS-25 swinging-bucket rotor. The opaque, virus-containing zone was removed with a syringe and concentrated by centrifugation at 100,000 g for 70 min.

**Serology.** Antiserum was obtained by injecting rabbits twice intravenously with purified virus and twice intramuscularly with the virus mixed with Freund's complete adjuvant. Antiserum obtained 10 days after the final injection reacted with purified virus in tube precipitin tests at a dilution of 1:2,048. Serologic relationships between the virus and five morphologically similar viruses were

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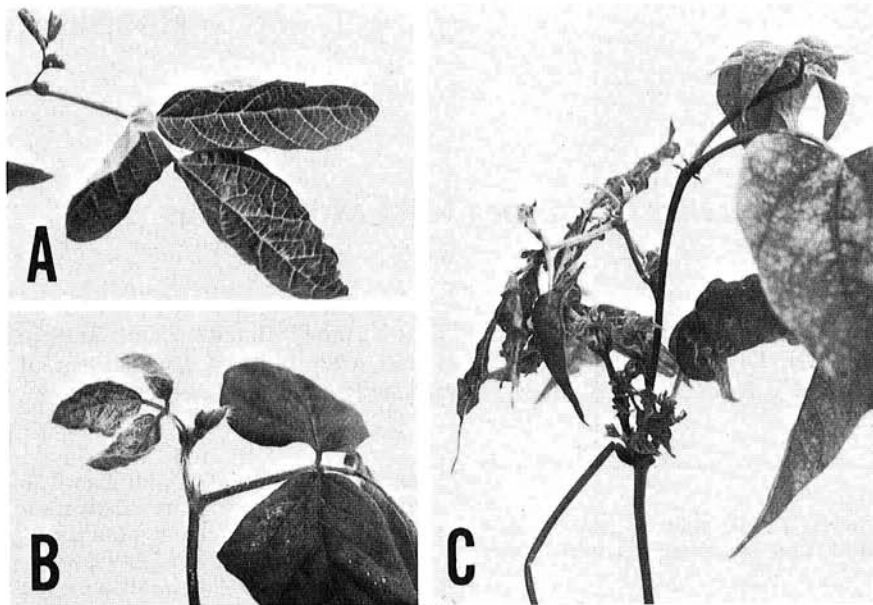


Fig. 1. Symptoms of infection with cowpea mild mottle virus: (A) Mild mottle symptom in Shirotsurunoko soybean. (B) Mosaic and necrotic symptoms in Toyosuzu soybean. (C) Mottle and stunt symptoms in Tsurunashi Kintoki bean.

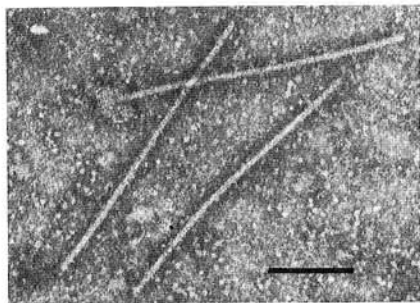


Fig. 2. Electron micrograph of a negatively stained dip preparation from diseased soybean. Bar = 200 nm.

determined in tube precipitin tests.

**Transmission and virus-vector relationships.** Virus-free whiteflies, *Bemisia tabaci* Genn., were reared on healthy hibiscus (*Hibiscus* sp.) or tobacco (*Nicotiana tabacum* L.); virus-free aphids, *Aphis craccivora* Koch and *A. glycines* Matsumura, were reared on healthy broad bean and soybean, respectively.

For aphid transmission tests, *A. craccivora* and *A. glycines* were starved for 1 hr and given acquisition access periods of 15 min to 24 hr on diseased soybean, inoculation access periods of 1–24 hr on healthy soybean or bean plants, and successive access periods of 24–48 hr in serial transfers to healthy soybeans or beans. Ten to 15 aphids were used per test plant.

Transmission efficiency using single adult whiteflies was determined by first allowing an acquisition access period of 1 day on infected soybean plants. After acquisition, each whitefly was transferred to a healthy soybean plant at the primary leaf stage and allowed an inoculation access period of 1 day.

For tests of the minimum acquisition

access period, groups of adult whiteflies were allowed an acquisition access period of 10 or 30 min or 1, 3, 6, or 24 hr on infected soybean plants. After acquisition access, 10 or 40 whiteflies were allowed an inoculation access of 1 day on each test plant. To determine the minimum inoculation access period, groups of whiteflies were allowed an acquisition access of 1 day on infected soybean plants. Immediately after acquisition, 10 whiteflies were allowed an inoculation access period of 10 or 30 min or 1, 3, 6, or 24 hr on healthy soybean plants.

Virus retention was determined by first allowing whiteflies an acquisition access period of 1 day on infected soybean plants; 10 whiteflies were then transferred daily for 18 days to a healthy soybean test plant. In another test, each group of 10 whiteflies that had acquisition access on infected soybean for 1 day was allowed an inoculation access period of 10 or 30 min or 1, 3, 6, 12, or 24 hr on a healthy soybean test plant. Immediately after each inoculation access period, these whiteflies were transferred to additional healthy test plants for an inoculation access of 1 day. The number of whiteflies per plant decreased with increased transmission times because some insects died during the transfers.

To test for a latent period of the virus in whitefly, groups of 15 whiteflies were allowed an acquisition access period of 5–10 min on infected soybean plants. Immediately after acquisition access, each of the 15 whiteflies was allowed an inoculation access of 5, 10, or 30 min or 1, 3, 6, or 24 hr on healthy soybean plants.

Whitefly transmission of purified virus was determined by the membrane feeding method (12) using Parafilm. Sucrose (20%, w/v) was added to the purified virus preparation, and groups of 10

whiteflies were allowed 1–2 days each for acquisition and inoculation access periods.

Test plants in whitefly transmission tests were observed for symptoms for about 2 wk after inoculation and then checked for the presence of particles by the leaf dip technique.

**Partial characterization of nucleic acid.** Nucleic acid was extracted from partially purified virus by the SDS-phenol method (13). Isopycnic ultracentrifugation was performed according to the method of Szybalski (15) by mixing the nucleic acid (final concentration;  $A_{260}$  0.1/ml) with cesium sulfate (final density; 1.618 g/cm<sup>3</sup> or 1.640 g/cm<sup>3</sup>) in 0.01 M tris-HCl buffer (pH 8.0) and centrifuging for 42 hr at 38,000 g at 25 C with MSE Centriscan 75 analytical ultracentrifuge using ultraviolet optics. Reaction of the nucleic acid with formaldehyde was analyzed as described by Miura et al (11).

## RESULTS

**Symptomatology, host range, and properties.** Symptoms on soybean varied with the cultivar. Cultivars SJ4, Shirotsurunoko, and Okuharawase showed slight veinclearing and leaf malformation, either downward curling or upward cupping (Fig. 1A). Cultivar Toyosuzu showed distinct mosaic, vein necrosis, and top necrosis (Fig. 1B).

Tsurunashi Kintoki bean plants showed leaf malformation, mild mottle, and stunting (Fig. 1C). Peanut developed veinclearing and mild mottle, which later became less distinct. Inoculated leaves of *Chenopodium amaranticolor* showed poorly defined local lesions 10–14 days after inoculation.

*Gomphrena globosa*, *Vicia faba*, and *Cucumis sativus* did not develop symptoms, but back-inoculation tests indicated that inoculated leaves of these plants contained virus. Back-inoculation tests also indicated that *Nicotiana clevelandii*, *Phaseolus angularis*, *Pisum sativum*, *Vigna mungo*, *V. radiata*, *V. sesquipedalis*, and *V. unguiculata* were systemically infected with the virus without visible symptoms.

The virus did not infect *Brassica rapa*, *Chenopodium quinoa*, *Datura stramonium*, *Lycopersicon esculentum*, *Nicotiana glutinosa*, *N. tabacum*, *Petunia hybrida*, *Sesamum indicum*, *Spinacia oleracea*, *Trifolium pratense*, *T. repens*, and *Zinnia elegans*.

DEP, TIP, and LIV of the virus were between  $10^{-5}$  and  $10^{-6}$ , 70 and 75 C for 10 min, and 21 and 28 days at 20 C, respectively.

**Electron microscopy and purification.** Negatively stained preparations from infected soybean leaves showed slightly flexuous, rod-shaped particles (Fig. 2), most of which were 10–15 nm wide and 650–700 nm in length (Fig. 3). Particles observed in sap from plants infected by whitefly transmission and mechanical

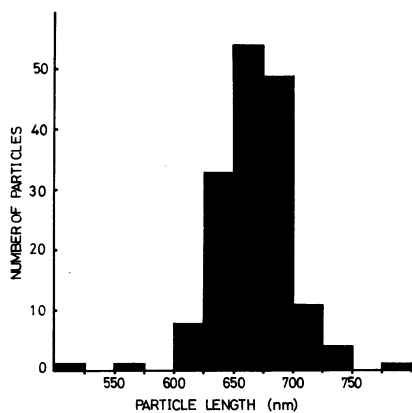


Fig. 3. Histogram of particle length distributions of cowpea mild mottle virus.

inoculation were similar in size.

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  $A_{260}/A_{280}$  ratio was 1.37 and  $A_{max}(260)/A_{min}(244)$  was 1.16. Soybean plants inoculated with the purified virus preparations showed symptoms similar to those described above. In membrane feeding tests, whiteflies transmitted the purified virus to 10 of 21 soybean plants.

**Serology.** The purified virus reacted with antisera to cowpea mild mottle virus (homologous titre, 1:4,096) diluted up to 1:4,096; to carnation latent virus (1:640) diluted up to 1:64; and to chrysanthemum virus B (1:10,000) diluted up to 1:64. However, it did not react with antisera to potato virus S (1:512) or citrus tatter leaf virus (1:128) diluted 1:8 to 1:4,096.

**Transmission and virus-vector relationships.** The virus was easily transmitted by sap inoculation. Inoculated soybean plants showed symptoms similar to those described above about 7 days after inoculation. The virus was also transmitted at rates up to 100% by *B. tabaci* when groups of about 40 whiteflies were allowed 1-day acquisition and inoculation access periods. The virus was transmitted through seed of soybean (1/110), but not by the aphids *A. craccivora* and *A. glycines*.

In transmission tests using one whitefly per plant, the virus was transmitted to 10 of the 58 Shiroturunoko soybean plants tested. The minimum period for virus acquisition and inoculation by whiteflies was not experimentally determined because whiteflies transmitted the virus in the shortest time tested (10 min) for each access period. Percentage of transmission increased with the increase in acquisition and inoculation access periods (Table 1).

In serial transfer tests, whiteflies retained the virus only for 1 day. In another serial transfer test, whiteflies transmitted the virus to the second test plants when allowed an inoculation access period of 1 hr or less on the first

Table 1. Effect of acquisition and inoculation access periods on transmission of cowpea mild mottle virus by *Bemisia tabaci*

| Test                     | Plants infected/inoculated (no.) after access period <sup>a</sup> |        |       |       |       |       |
|--------------------------|---|--------|-------|-------|-------|-------|
|                          | 10 min  | 30 min | 1 hr  | 3 hr  | 6 hr  | 24 hr |
| Acquisition <sup>b</sup> |   |        |       |       |       |       |
| 1 <sup>c</sup>           | 12/12   | 9/9    | 8/8   | 13/13 | 2/2   | 21/21 |
| 2 <sup>d</sup>           | 9/30  | 19/29  | 15/29 | 15/17 | 30/30 | 29/29 |
| Inoculation <sup>e</sup> |   |        |       |       |       |       |
| 1 <sup>d</sup>           | 17/27   | 14/25  | 21/25 | 22/27 | 23/26 | 19/22 |
| 2 <sup>d</sup>           | 16/30   | 19/29  | 25/30 | 23/30 | 28/28 | 23/27 |

<sup>a</sup> Shiroturunoko soybean plants.

<sup>b</sup> Followed by a 1-day inoculation access period.

<sup>c</sup> 40 insects per plant.

<sup>d</sup> 10 insects per plant.

<sup>e</sup> Following a 1-day acquisition access period.

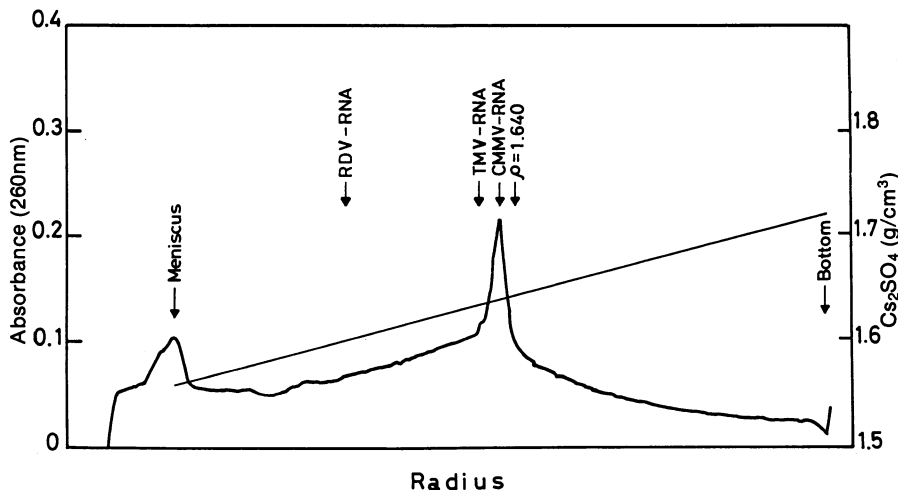


Fig. 4. Isopycnic ultracentrifugation of cowpea mild mottle virus ribonucleic acid (RNA) in cesium sulfate ( $\text{Cs}_2\text{SO}_4$ ). RNA was mixed with  $\text{Cs}_2\text{SO}_4$  in 0.01 M tris-HCl buffer (pH 8.0) to produce a solution of density 1.640  $\text{g}/\text{cm}^3$ . The sample was centrifuged at 88,000 g for 42 hr at 25 C with MSE Centrican 75. Heavy line = absorbance, light line = density. CMMV = cowpea mild mottle virus, RDV = rice dwarf virus, TMV = tobacco mosaic virus.

test plant, but not of 3 hr or longer. These results indicate that virus retention was about 1 hr.

In the tests of latent period, 15 whiteflies that were allowed 5–10 min of acquisition access transmitted the virus within 5–10 min of inoculation access. These results indicate that there is probably no latent period for the virus in the whitefly vector.

**Some properties of nucleic acid.** Nucleic acid preparations from the virus had an ultraviolet spectrum with  $A_{260}/A_{233}$  and  $A_{260}/A_{280}$  ratios of about 1.93 and 1.96, respectively.

Buoyant density of the nucleic acid in cesium sulfate was 1.636  $\text{g}/\text{cm}^3$  (Fig. 4), which corresponded to the values of the other viral, single-stranded ribonucleic acid (RNA) molecules (15). Single-stranded RNA of tobacco mosaic virus and double-stranded RNA of rice dwarf virus banded at a buoyant density of 1.632  $\text{g}/\text{cm}^3$  and 1.599  $\text{g}/\text{cm}^3$ , respectively, under the same conditions. After 22 hr of incubation with formaldehyde, the nucleic acid exhibited a hyperchromicity of 22% and a shift of 2–3 nm of longer wavelength in the ultraviolet absorption spectrum (Fig. 5).

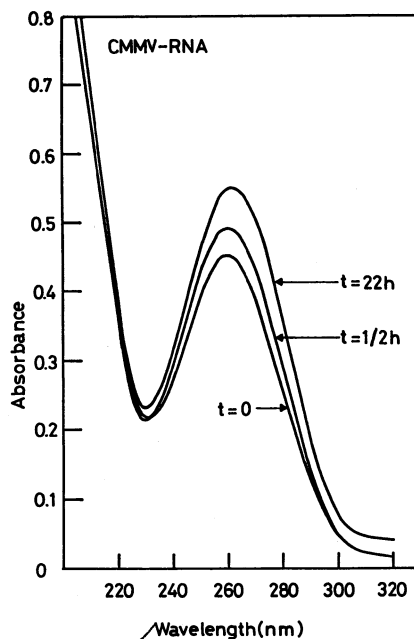


Fig. 5. Reaction of cowpea mild mottle virus (CMMV) ribonucleic acid (RNA) with formaldehyde. The ultraviolet spectra of RNA in 0.1 M sodium chloride were examined after 0, 0.5, and 22 hr of incubation at 37 C in the presence of 1.8% formaldehyde.

## DISCUSSION

On the basis of host range, symptomatology, particle morphology, and serology, the virus appeared to be a strain of CMMV; however, whitefly transmission of CMMV had not been reported previously.

The causal agents of whitefly-borne virus diseases have paired or geminate particles (1,5), which are known as geminivirus (7), or filamentous particles of 740–800 nm (cucumber vein yellowing virus) (6,14), 950 nm (sweet potato mild mottle virus) (8), and 1,000 nm (cucumber yellows virus) (16) in length. CMMV, with a particle length of 650–700 nm, can be considered as morphologically different from these viruses.

The CMMV described in this paper had certain carlaviruses characteristics; however, most carlaviruses are transmitted by aphids in a nonpersistent manner at various rates. Some, like CMMV from Africa and citrus tatter leaf virus, were not transmissible by aphids (2,9,10). Our results raise the possibility that some members of the carlavirus group that are not aphid-transmissible may be transmitted by whiteflies. Recently, we have found that the type strain (2) and a typical isolate (4) of CMMV (supplied by A. A. Brunt) are transmissible by the whitefly *B. tabaci* (Iwaki et al, unpublished).

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