SOYBEAN CRINKLE LEAF AND COWPEA MILD MOTTLE VIRUSES

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ABSTRACT

The occurrence of two whitefly-borne viruses, soybean crinkle leaf (SCLV) and cowpea mild mottle viruses (CMMV), on soybean in Thailand was recognized for the first time in 1983 and 1982, respectively. CMMV occurred also on soybean and peanut in Malaysia and Indonesia. SCLV, a new virus on soybean, was transmitted by the whitefly, *Bemisia tabaci*, in a persistent manner and by grafting but not by aphids, sap inoculation, or through seeds of soybean. Minimum acquisition and inoculation access periods in whitefly transmission ranged from 30 to 60 min and from about 10 to 30 min, respectively. The latent period in the whitefly vector ranged between 8 and 10 hr, and the retention period in the vector lasted 9 days. SCLV affected 11 plant species in three families (Compositae, Leguminosae, and Solanaceae). Partially purified preparations contained geminate virus-like particles (about 18 × 30nm). CMMV, consisting of rod-shaped particles about 680nm in length containing single-stranded RNA, was transmitted by the whitefly in a semi-persistent manner, by sap inoculation and through soybean seeds, but not by aphids. Among 26 plant species tested by sap inoculation, the virus systemically infected mainly plants of the family Leguminosae, and it produced poorly defined local lesions on inoculated leaves of *Chenopodium amaranticolor*.

Introduction

During surveys of virus diseases of soybean (*Glycine max* (L) Merr.) in Thailand, many plants showing various types of symptoms were observed. Three whitefly-borne viruses were isolated from these plants.

The first one, soybean crinkle leaf virus (SCLV) which is a whitefly-borne virus transmitted in a persistent manner but not sap-transmissible, was isolated by whitefly transmission from soybean plants showing crinkle leaf and vein enation symptoms.

The second one, cowpea mild mottle virus (CMMV) which is a whitefly-borne virus transmitted in a semi-persistent manner and sap-transmissible, was isolated by mechanical inoculation from soybean plants showing mild mosaic, mosaic, rugose mosaic symptoms, etc.

The third one, mungbean yellow mosaic virus, a whitefly-borne virus transmitted in a persistent manner and sap-transmissible, was isolated by whitefly transmission from soybean plants showing yellow mosaic symptoms.

This paper describes the characteristics of whitefly transmission and some properties of SCLV and CMMV.

Soybean crinkle leaf virus

The virus was isolated by whitefly transmission from naturally infected soybean plants collected at Phitsanulok, North Thailand, and subsequently maintained in soybean plants either by grafting or by whitefly transmission.

1 Symptoms on soybean and occurrence

Infected soybean plants in the fields showed twisting or curling of leaves with veinal enations on the undersurfaces of the leaves (Fig. 1,2). In addition, foliage of infected plants was

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Fig. 1 Leaf curl symptoms on soyben plant infected with soybean crinkle leaf virus.

Fig. 2 Vein enations on undersurface of soybean leaf infected with soybean crinkle leaf virus.

dark green which enabled infected plants to be distinguished from nearby uninfected plants.

In the greenhouse, infected soybean plants showed yellow netting of veins at 10–14 days after inoculation. Thereafter, the symptoms included veinal enations on the undersurfaces of the leaves and cupping or twisting of leaves.

These diseased soybean plants were observed in the fields of five provinces in North and North East Thailand.

2 Transmission

(1) Sap transmission Inoculum for sap inoculation was prepared by grinding diseased leaves in 0.05 M phosphate buffer, pH 7.0 or 8.0, containing 10 mM sodium diethyldithiocarbamate (DIECA) and 1 mM L-cysteine or 20 mM sodium sulfite. Sap inoculation was performed by rubbing the Carborundum-dusted leaves with a cotton swab dipped in inoculum.

None of the soybean (54), Top Crop bean (88), tomato (48), *Cassia tora* (4), tobacco (6), *Nicotiana glutinosa* (6), and petunia (4) plants inoculated with sap prepared from infected soybean, Top Crop bean, *C. tora,* tomato and *Datura stramonium* plants became infected.

(2) Aphid transmission Aphid transmission tests were carried out using non viruliferous aphids, *Aphis craccivora, A. glycines, Myzus persicae,* and by first starving aphids for 2 hr in a glass beaker before allowing an acquisition access period of 15 min on the diseased plants. After acquisition access, 10 aphids were transferred to each healthy soybean seedling (10–14 days old) for an inoculation access period of 1 day, which was terminated by spraying with insecticides. In other tests, aphid transmission was carried out without starvation before acquisition access, and a 1 day acquisition access period was allowed before inoculation access as before.

None of the test plants exposed to aphids that had been previously allowed acquisition access on diseased soybean plants became infected, regardless of whether non persistent or persistent types of transmission test were performed.

(3) Whitefly transmission Whitefly, *Bemisia tabaci* Genn., transmitted SCLV in preliminary tests from soybean to soybean.

In transmission efficiency tests by whiteflies that had been allowed an acquisition access period of 2 days on infected soybean plants, single whiteflies were unable to transmit SCLV, but groups of 5, 10, 20, and 40 whiteflies transmitted it at a rate of 20, 26, 40, and 73%, and about 40 insects were required to achieve high transmission rates (Table 1).

Table 1 Effect of insect number on the transmission of soybean crinkle leaf disease by *Bemisia tabaci*

Number of insects per test plant	No. of infected/ inoculated plants
1	0 / 14
5	3 / 15
10	4 / 15
20	6 / 15
40	11 / 15

Disease source plant and test plant: Shirotsurunoko soybean.
Acquisition and inoculation access periods: each 2 days.

The minimum acquisition and inoculation access periods by groups of 40 whiteflies ranged between one-half to 1 hr and one-sixth to one-half hr, respectively, and transmission rates increased with the increase in the duration of the access periods for acquisition or inoculation (Tables 2, 3).

Whiteflies required a period of 8–10 hr after acquisition access before they could transmit SCLV, and retained their transmission ability for at least 9 days after acquisition.

Table 2 Effect of acquisition access period on the transmission of soybean crinkle leaf disease by *Bemisia tabaci*

Test	Acquisition access period						
	10 min	30 min	1 hr	3 hr	6 hr	24 hr	48 hr
I	0/15[a)]	0/15	1/15	1/12	7/15	8/15	11/15
II	0/9	1/11	0/15	4/15	9/15	13/15	12/15

Test plant: Yuzuru soybean, Inoculation access period: 2 days.
a): No. of infected plants/inoculated plants.

Table 3 Effect of inoculation access period on the transmission of soybean crinkle leaf disease by *Bemisia tabaci*

Test	Inoculation access period						
	10 min	30 min	1 hr	3 hr	6 hr	24 hr	48 hr
I	3/15[a)]	3/15	7/14	14/15	4/12	13/14	11/11
II	0/15	4/15	0/15	3/15	3/15	4/9	4/8

Test plant: Yuzuru soybean, Acquisition access period: 2 days.
a): Number of infected plants/inoculated plants.

(4) Seed transmission In seed transmission tests, none of the 172 seedlings from seeds harvested from diseased soybean plants grown in the greenhouse showed crinkle leaf symptoms.

3 Host range

Thirty six plant species in 10 families were inoculated using viruliferous whiteflies (40–50 insects per plant). Infection in each plant was indexed by back inoculation to soybean by whitefly transmission about 4 hr after inoculation.

The following 11 plant species and cultivar were infected with SCLV and showed vein-clearing symptoms at about 10–14 days after inoculation by whiteflies and later showed leaf curl or crinkle leaf symptoms. Those plants were *Cassia tora, Datura stramonium, Glycine max, Lycopersicon esculentum, Nicotiana clevelandii, N. debneyi, N. glutinosa, N. tabacum, Petunia hybrida, Phaseolus vulgaris* cv. Top Crop, and *Zinnia elegans.*

Other 26 plant species and cultivars were not susceptible to SCLV.

4 Purification and electron microscopy

Purification of the causal virus from systemically infected soybean and tomato plants was performed following the methods developed by Osaki *et al.* (1973) for the purification of tobacco leaf curl virus.

Partially purified preparations of SCLV included many virus-like geminate particles (Fig. 3),

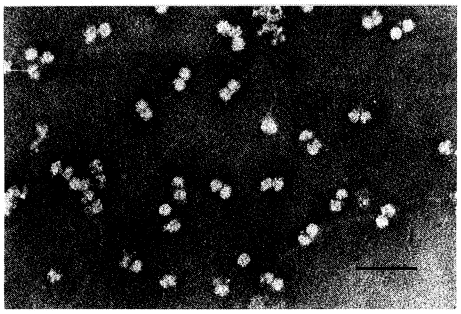

Fig. 3 Virus-like particles in partially purified samples of soybean crinkle leaf virus. Bar = 100 nm.

but these preparations did not show the infectivity by whitefly transmission in membrane feeding methods.

For electron microscopy, pieces of infected soybean leaves were fixed with 4% glutaraldehyde at 5°C for 1.5 hr, and post-fixed with 25 osmium tetroxide in 0.1 M phosphate buffer, pH 7.5, at 5°C for 5 hr. After washing and dehydration, they were embedded in a mixture of low-viscosity

epoxy resin. Thin sections were cut with glass knives on a Porter Blum Model MT 2B microtome, and stained with uranyl acetate and lead citrate. They were examined under a Hitachi H 500 or H 300 electron microscope.

The aggregations of virus-like particles were observed in the nuclei of infected cells.

Cowpea mild mottle virus

The virus was isolated from naturally infected soybean plants collected at Phitsanulok, North Thailand in 1979 and maintained in soybean plants by mechanical inoculation.

1 Symptoms on soybean and occurrence

Symptoms on soybean varied with the cultivars. Cultivars SJ4, Shirotsurunoko and Okuharawase showed slight vein-clearing and leaf malformation, either downward curling or upward cupping. Cultivar Toyosuzu showed distinct mosaic, vein necrosis and top necrosis.

In the inoculation tests of collected samples to differential host plants CMMV was detected from plants showing a wide range of symptoms, including mild mosaic, mosaic, rugose mosaic (Fig. 4), etc. The reasons for the diversity of the symptoms observed could not be clarified.

Fig. 4 Rugose mosaic symptoms on soybean infected with cowpea mild mottle virus.

2 Host range

The virus infected 14 plant species in 5 families among 26 plant species in nine families inoculated with sap from soybean plants infected with whiteflies.

The virus infected systemically peanut, soybean and Kintoki bean, inducing visible symptoms.

Infected peanut plants showed vein-clearing and mild mottle which later became less distinct.

Kintoki bean plants showed leaf malformations, mild mottle and stunting.

Bean (Top Crop, Yamashiro Kurosando), azuki bean, pea, blackgram, mungbean, asparagus bean, cowpea, *Nicotiana clevelandii* were infected systemically without showing any symptoms.

Chenopodium amaranticolor exhibited poorly defined local lesions on the inoculated leaves.

Gomphrena globosa, broadbean, cucumber were infected with the virus in inoculated leaves without exhibiting symptoms.

Other 11 plant species were not infected with the virus.

3 Transmission and virus-vector relationships

The virus was transmitted easily by sap inoculation.

(1) Aphid transmission Aphids, *Aphis craccivora* and *A. glycines,* failed to transmit the virus from bean or soybean to bean (0/6) or soybean (0/33) in a non persistent and persistent manner.

(2) Whitefly transmission The virus was transmitted effectively by whitefly, *Bemisia tabaci.*

In transmission tests using one whitefly per plant, the virus was transmitted to 10 of the 58 Shirotsurunoko 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. Percentage of transmission increased with the increase in the duration of the acquisition and inoculation access periods (Tables 4, 5).

Table 4 Effects of acquisition access period on transmission of cowpea mild mottle virus by *Bemisia tabaci*

Test	Acquisition access period[a]					
	10 min	30 min	1 hr	3 hr	6 hr	24 hr
I[b]	12/12[d]	9/9	8/8	13/13	2/2	21/21
II[c]	9/30	19/29	15/29	15/17	30/30	29/29

Test plant: *Glycine max* cv. Shirotsurunoko.
a): Followed a 1 day inoculation access period.
b): Fourty insects were transferred to each test plant.
c): Ten insects were transferred to each test plant.
d): No. of infected plants/no. of inoculated plants.

Table 5 Effects of inoculation access period on transmission of cowpea mild mottle virus by *Bemisia tabaci*

Test	Inoculation access periods[a]					
	10 min	30 min	1 hr	3 hr	6 hr	24 hr
I[b]	17/27[c]	14/25	21/25	22/27	23/26	19/22
II[b]	16/30	19/29	25/30	23/30	28/28	23/27

Test plant: *Glycine max* cv. Shirotsurunoko.
a): Following a 1 day acquisition access period.
b): Ten insects were transferred to each test plant.
c): Number of infected plants/number of inoculated plants.

In serial daily transfer tests, whiteflies retained the virus only for one 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 test plant, but not 3 hr or longer. These results indicate that the virus was retained in the vector for about 1 hr.

In the tests of latent period in vector, 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 was presumably no latent period for the virus in the vector.

4 Stability in crude sap

Stability of the virus in crude sap of diseased Kintoki bean leaves was as follows. Dilution end point ranged between 10^{-5} and 10^{-6}, thermal inactivation point between 70 and 75°C (10 min) and longevity in vitro between 21 and 28 days at 20°C.

5 Electron microscopy

Negatively stained preparations using 2% potassium phosphotungstate, pH 6.5, from infected soybean leaves showed slightly flexuous rod particles, most of which were 10–15 nm wide and 650–700 nm long (Fig. 5).

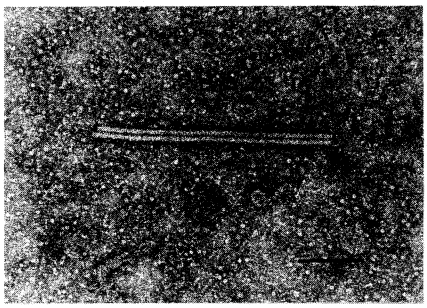

Fig. 5 Virus particles of cowpea mild mottle virus in direct negative staining preparation. Bar = 200 nm.

For electron microscopy, the samples from diseased soybean leaves were fixed with 3% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated in an acetone series, embedded in Epoxy resin, cut with glass knives, and stained with uranyl acetate and leaf citrate. These sections contained feather-like and bundle-type inclusion bodies in the cytoplasm.

6 Purification and serology

The virus was purified from infected leaves of Shirotsurunoko soybean or Kintoki bean by clarification with chloroform and carbon tetrachloride, differential centrifugation, and sucrose density gradient centrifugation.

Purified virus preparations had an ultraviolet absorption spectrum typical of nucleoprotein with maximum absorbance at 260 nm and minimum at 244 nm.

Antiserum from rabbit immunized by two series of intravenous injections with the virus and two series of intramuscular injections with the virus mixed with Freund's complete adjuvant reacted with the purified virus in tube precipitin tests at a titer of 1/2048.

The purified virus reacted with antiserum to cowpea mild mottle virus from Africa (provided by Dr. Brunt, Glasshouse Crops Research Institute, England, homologous titer, 1:4096) diluted up to 1: 4096, showing close serological relationships with cowpea mild mottle virus from Africa.

7 Partial characterization of nucleic acid

Nucleic acid preparations from the virus had a ultraviolet spectrum with A_{260}/A_{233} and A_{260}/A_{280} ratios of about 1.93 and 1.96, respectively.

Isopycnic ultracentrifugation for determining the buoyant density of nucleic acid in cesium sulfate was performed according to the method of Szybalski (1968) by mixing the nucleic acid (final concentration; A_{260} 0.1/ml) with cesium sulfate (final density 1.618 g/cm^3 or 1.640 g/cm^3) 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.

Buoyant density of nucleic acid of the virus was 1.636 g/cm^3, corresponding to single-stranded ribonucleic acid.

Reaction tests of the nucleic acid with formaldehyde (1.8%) at 37°C showed a hyperchromicity of 22% and a shift of 2-3 nm of longer wave length in the ultraviolet absorption spectrum, showing that the nucleic acid was single-stranded.

Discussion

Many whitefly-borne viruses are occurring on leguminous plants in the tropics (Bird *et al.*, 1975; Costa, 1975; Granillo *et al.*, 1975; Nene, 1972; Pierre, 1975). However, only mungbean yellow mosaic virus has been reported as a whitefly-borne virus on leguminous crops in Southeast Asia, so far (Thongmeearkom *et al.*, 1981).

In our study on virus diseases of leguminous crops in Thailand, the occurrence of a new whitefly-borne virus, soybean crinkle leaf virus (SCLV) was reported and also the whitefly transmissibility of cowpea mild mottle virus (CMMV) was confirmed.

SCLV seemed to be a new member of the geminivirus group based on its properties. SCLV affects soybean over wide areas in Thailand, and has a comparatively wider host range than the other whitefly-borne viruses, because SCLV was found to infect plants in the families Leguminosae, Solanaceae and Compositae.

In Thailand, many whitefly-borne viruses occur on tomato, tobacco, eggplant, weeds, etc. The relationships of SCLV to whitefly-borne viruses occurring on other plants require further studies.

The occurrence of CMMV was reported on cowpea and tomato in West Africa (Brunt and Kenten, 1973; Brunt and Phillips, 1981), peanut in India (Iizuka *et al.*, 1984), and beans in Brazil (Costa *et al.*, 1983). In our studies, CMMV was detected from soybean in Thailand, soybean and peanut in Malaysia and Indonesia. Thus, CMMV may be widely distributed in tropical regions.

CMMV causes usually mild symptoms on many plants, and sometimes severe symptoms, although it remains to be determined whether the diversity of the symptoms observed can be ascribed to factors relating to the plant cultivars or virus strains.

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