Mechanical Transmission, Purification, and Some Properties of Whitefly-Borne Mung Bean Yellow Mosaic Virus in Thailand

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ABSTRACT

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The whitefly-borne mung bean yellow mosaic virus (MYMV) in Thailand was transmitted by mechanical inoculation. Among the several buffers used in attempted transmissions, 0.1 M potassium or sodium phosphate, pH 7.8, gave the highest transmission rates. The optimal incubation temperatures for symptom expression ranged from 25 to 30 C in the growth chamber or 30 C in the daytime and 20 C at night in the greenhouse. Host range of MYMV was limited to seven plant species in the family Leguminosae. Determinations of the stability of the virus in plant sap gave the following results: thermal inactivation point of 40–50 C for 10 min, dilution end point of 10^{-2}–10^{-3}, and longevity in vitro of 1–2 days at 20 C. Purified virus preparations had an ultraviolet light absorption spectrum typical of that of nucleoprotein with a A_{260}/A_{280} value of about 1.3–1.4. Purified preparations and leaf-dip samples contained geminate particles of about 18 × 30 nm. Infectivity was associated with the presence of purified virus particles.

Additional key word: geminivirus

In 1977, an outbreak of mung bean yellow mosaic disease (MYMD) was reported in northern Thailand (11). The disease caused almost total yield loss in infected mung bean (*Vigna radiata* (L.) Wilcz.) plants in the field. Laboratory tests indicated that the causal agent of the disease was transmitted by the tobacco whitefly, *Bemisia tabaci* Genn. (11). Prior to that report, a similar whitefly-borne disease had been observed in India (6). The disease in India was very severe in black gram (*V. mungo* (L.) Hepp.) and caused as much as 100% yield loss when plants were infected at the seedling stage (7). Despite the severity of the MYMD in both Thailand and India, very little was known previously about the nature of the causal agent except that it was whitefly-borne and was not transmissible by mechanical inoculation (7). Isolation and characterization of the causal agent of MYMD have not been reported but ultrastructural changes induced in infected mung bean plants (10) were characteristic of those caused by whitefly-transmitted geminiviruses (1,3–5,9).

In this paper, we report the mechanical transmission and purification of mung bean yellow mosaic virus (MYMV) in Thailand and describe some of the properties indicating that MYMV is a member of the geminivirus group.

MATERIALS AND METHODS

Virus source, maintenance, and mechanical inoculation. Mung bean plants showing yellow mosaic symptoms were collected from fields in northern Thailand. The virus was maintained in mung bean plants by whitefly and graft transmission in a greenhouse at Tsukuba, Japan, or in an insect-proof house at Bangkhen, Thailand. Seedlings of mung bean used for mechanical inoculation tests were 5–7 days old. All inocula were prepared by grinding systemically infected young mung bean leaves in buffers (about 4 ml/g tissue) with a chilled mortar and pestle. Inoculations were made by rubbing Carborundum-dusted primary leaves of the test plants with cotton wool soaked in the homogenate. Various buffers (potassium phosphate, sodium phosphate, borate, and Tris-HCl) with molarities of 0.05, 0.1, and 0.2, as well as 0.1 M potassium phosphate buffer with pH values of 4.5, 5.0, 6.0, 6.5, 7.0, 7.5, 7.8, 8.0, 8.5, 9.0, and 9.5 were used in the transmission studies.

The effect of the incubation temperature on symptom expression of inoculated plants was also tested at 15, 20, 25, 30, and 35 C in a growth chamber or at 30 C in the daytime and 20 C at night in a greenhouse. On this occasion, the inoculum was ground in 0.1 M potassium phosphate, pH 7.8, containing 0.001 M KCN and inoculated to 30 test plants. The combination that gave maximum transmission rates was used in subsequent experiments.

Host range and stability in sap. Host range of MYMV was determined by mechanical inoculation of 26 plant species belonging to six families. Young seedlings were used in these trials and were inoculated under optimal conditions for MYMV transmission. Inoculated plants were assayed by back-inoculation to mung bean seedlings 24 days after inoculation. In sap extracted from infected mung bean leaves, the thermal inactivation point (TIP), dilution end point (DEP), and longevity in vitro of the virus were determined using mung bean seedlings as test plants. The sap for TIP and LIV tests was diluted fourfold in 0.1 M sodium phosphate buffer, pH 7.8.

Virus purification. Systemically infected leaves of French bean (*Phaseolus vulgaris* L. 'Top Crop') were harvested about 2 wk after mechanical inoculation. Healthy leaves of Top Crop bean were used as controls. Frozen leaves were homogenized with a Waring Blendor in 0.1 M potassium phosphate buffer, pH 7.8, containing 0.1% thioglycolic acid, 0.01 M sodium diethyldithiocarbamate, and 0.001 M sodium ethylenediaminetetraacetate (2 ml/g tissue). The extract was

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Table 1. Effect of extraction buffer on the infectivity of mung bean yellow mosaic virus

Buffers (pH 7.8)	Infectivity at buffer molarities of		
	0.05 M	0.1 M	0.2 M
Potassium phosphate	9[a]	9[a] (15)[b]	0[a]
Sodium phosphate	3	9 (15)	0
Borate	5	8 (9)	2
Tris-HCl	5	0 (2)	5

[a] Indicates the number of plants infected per 10 mung bean seedlings inoculated.
[b] Figures in parentheses are the number of plants infected per 15 mung bean seedlings inoculated (each buffer containing 0.001 M KCN).

clarified by adding one-half volume of chloroform and stirring at 4 C for 30 min. The emulsion was broken by centrifugation at 5,000 *g* for 10 min, and the aqueous phase was recovered.

Polyethylene glycol (PEG: mol wt 6,000) and sodium chloride were added to the aqueous phase to give a final concentration of 6% and 0.2 M, respectively. After stirring at 4 C for 2 hr, the mixture was centrifuged at 15,000 *g* for 30 min and the precipitates were dissolved in 0.1 M potassium phosphate buffer, pH 7.8, and clarified by centrifugation (9,000 *g* for 10 min) before being subjected to ultracentrifugation at 125,000 *g* for 90 min. The pellets were resuspended in potassium phosphate containing 6% PEG and 0.2 M NaCl. The resuspended pellets were layered onto PEG discontinuous reverse solubility gradients prepared by layering 12.5 ml of 40% sucrose and then another 12.5 ml of 10% sucrose containing 4% PEG and 0.2 M NaCl into each tube. About 2 ml of the sample was layered onto each gradient and centrifuged in a Hitachi RPS 25 swinging rotor at 12,000 rpm for 30 min.

The opaque band located at the interface between sucrose layers containing 4 and 0% PEG was recovered and concentrated by ultracentrifugation as before. Resuspended pellets were subjected to sucrose density gradient centrifugation in a Hitachi RPS 27-2 swinging rotor at 26,000 rpm for 3 hr using 10–40% linear sucrose gradients. After centrifugation, gradients were analyzed and fractionated by an ISCO Model 640 density gradient fractionator coupled with an ISCO Model UA-5 absorbance monitor. Ultraviolet light (254 nm) absorbing fractions were collected, pooled, and concentrated by ultracentrifugation as before and used for electron microscopy and absorbance spectrum analysis. The corresponding fractions from a sister gradient were also collected similarly and used for infectivity assays by mechanical inoculation to mung bean seedlings.

Electron microscopy. Samples for electron microscopy were mounted on collodion-carbon-coated grids and stained with 2% sodium phosphotungstate (PTA), pH 3.5, or 2% uranyl acetate. Observations were made with a Hitachi Model H300 or H500 electron microscope.

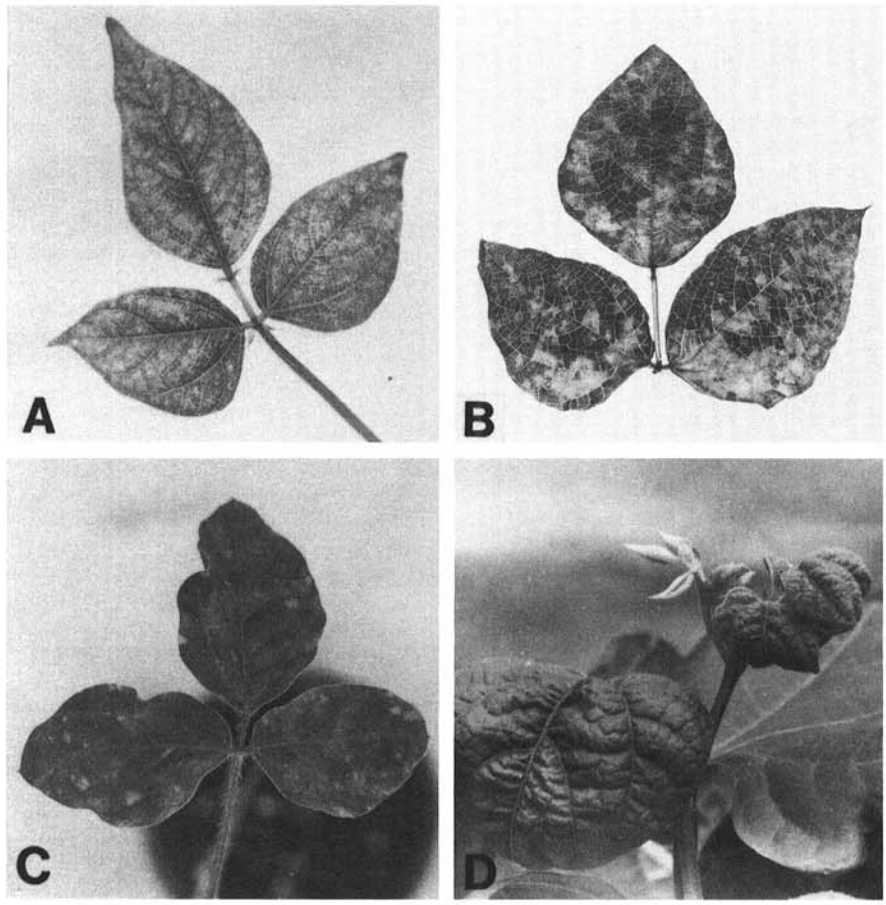

Fig. 1. Symptoms of infection with mung bean yellow mosaic virus: **(A)** vein yellowing in mung bean, **(B)** yellow mosaic in mung bean, **(C)** yellow mosaic in soybean, and **(D)** severe downward curling in Top Crop bean.

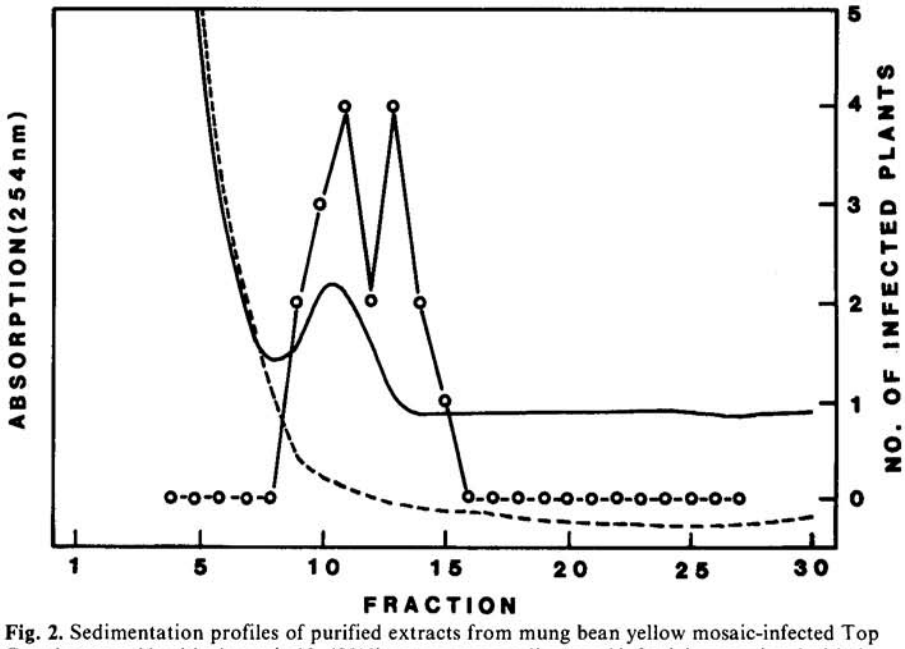

Fig. 2. Sedimentation profiles of purified extracts from mung bean yellow mosaic-infected Top Crop beans and healthy beans in 10–40% linear sucrose gradients and infectivity associated with the fractions collected. Sedimentation from the left. --- = Healthy leaf extract absorbance, —— = infected leaf extract absorbance, and o——o = infectivity (each fraction collected was inoculated to five mung bean seedlings).

RESULTS

Mechanical inoculation. In preliminary experiments, MYMV could not be transmitted by mechanical inoculation. In later trials, however, the virus could be transmitted using conventional inoculation techniques. Among the several buffers used in attempted transmissions, 0.1 M potassium or sodium phosphate, pH 7.8, gave transmission rates as high as 90–100% (Table 1). In potassium phosphate buffer at pH 4.5, 5.0, 6.0, 6.5, 7.0, 7.5, 7.8, 8.0, 8.5, 9.0, and 9.5, the percentages of mung bean seedlings with yellow mosaic symptoms were 25, 15, 50, 80, 100, 95, 100, 90, 90, 85, and 70%, respectively. The optimum pH values of phosphate buffer for transmission ranged between 7.0 and 7.8. At 15, 20, 25, 30, and 35 C in the growth chamber, the percentages of transmission were 0, 80, 87, 93, and 80%, respectively, and 97% in the greenhouse, where the temperature varied from 20 C minimum at night to 30

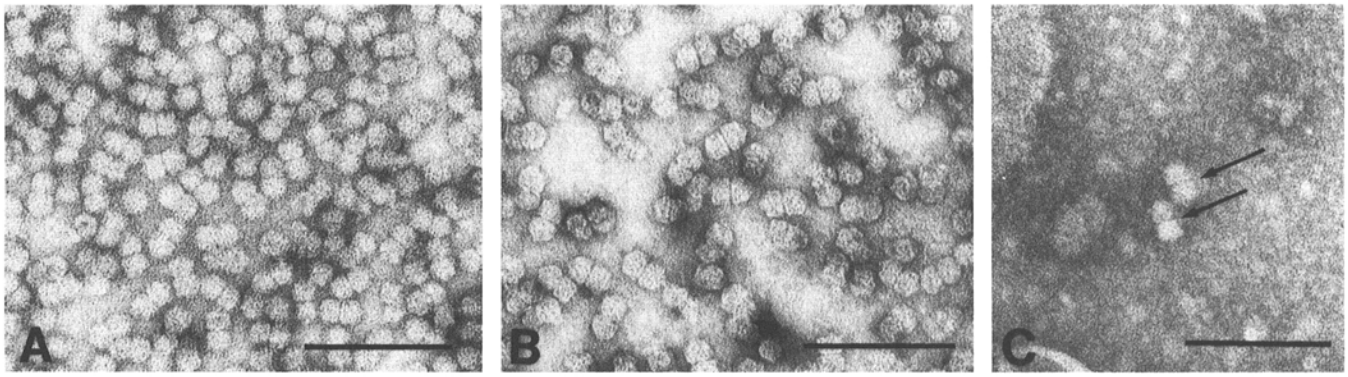

Fig. 3. Electron micrographs of mung bean yellow mosaic virus particles. **(A and B)** Purified virus preparations negatively stained with 2% sodium phosphotungstate (PTA), pH 3.5, and 2% uranyl acetate, respectively. **(C)** Infected mung bean leaf-dip samples negatively stained with 2% PTA. Arrows indicate geminate particles. Bar = 100 nm.

C maximum in the daytime. Although no symptoms appeared at 15 C, 70% of the test plants showed symptoms when maintained at 15 C for 23 days and then kept in the greenhouse for 22 days.

Host range, symptomatology, and stability in sap. Among the 26 plant species belonging to six families used in the mechanical inoculation tests, only seven species of the family Leguminosae were infected with MYMV. Symptoms consisted mainly of yellow mosaic or leaf curl. No symptoms appeared on inoculated primary leaves. In systemically infected leaves of azuki bean (*Phaseolus angularis*), black gram, mung bean (Fig. 1A,B), and soybean (*Glycine max*) (Fig. 1C), vein yellowing along the veinlets appeared at the early stage, then developed into yellow mosaic symptoms. First emerging trifoliolate leaves of mung bean showed severe downward curling.

In infected French bean (*P. vulgaris*), jack bean (*Canavalia ensiformis*), and lima bean (*P. lunatus*), vein yellowing developed in leaflets, followed by mild or severe downward curling (Fig. 1D). These plants did not show yellow mosaic symptoms in the advanced stages of infection. Back-inoculation to mung bean seedlings from infected plants resulted in yellow mosaic symptoms in test plants. MYMV did not infect *Tetragonia expansa* (Aizoaceae), *Gomphrena globosa* (Amaranthaceae), *Chenopodium amaranticolor, C. quinoa* (Chenopodiaceae), *Cucumis sativus* (Cucurbitaceae), *Arachis hypogaea, Cassia occidentalis, Cassia tora, Centrosema pubescens, Dolichos lablab, Pisum sativum, Vicia faba, V. sesquipedalis, V. unguiculata* (Leguminosae), and *Datura stramonium, Lycopersicon esculentum, Nicotiana glutinosa, N. tabacum,* and *Petunia hybrida* (Solanaceae).

In sap extracted from infected mung bean leaves, the virus showed a TIP of 40–50 C for 10 min, DEP between 10^{-2} and 10^{-3}, and LIV of 1 or 2 days at 20 C.

Virus purification and electron microscopy. After centrifugation in PEG reverse concentration gradients, two major bands were observed, one located slightly below the meniscus and the other, which was opaque, located at the interface between the two layers of sucrose containing 4 and 0% PEG. Sucrose density gradient centrifugation and electron microscopy revealed that the first band contained predominantly phytoferritins, whereas the second band contained geminate particles. The geminate particles sedimented in 10–40% linear sucrose gradient as a single band located at fractions 9–15 (Fig. 2). When employed for mechanical inoculation, these fractions proved infective in five mung bean seedlings (Fig. 2).

Symptoms obtained were similar to those shown by mung bean plants infected with MYMV (Fig. 1A,B). The size of the purified geminate particles was about 18 × 30 nm (Fig. 3A,B). Electron microscopy of leaf-dip samples prepared from young mung bean leaf tissue infected with MYMV also revealed particles of similar size and shape (Fig. 3C). The corresponding fractions from healthy tissue treated similarly failed to show a peak and geminate particles. The preparations with geminate particles had an ultraviolet light absorption spectrum characteristic of that of nucleoprotein with a A_{260}/A_{280} value of 1.3–1.4 (Fig. 4). Assuming the extinction coefficient ($E^{0.1\%}_{1cm}$ 260 nm) was 7.7 as for the geminate particles of bean golden mosaic virus (2), the yield of the geminate particles associated with MYMD was below 1 mg/kg tissue.

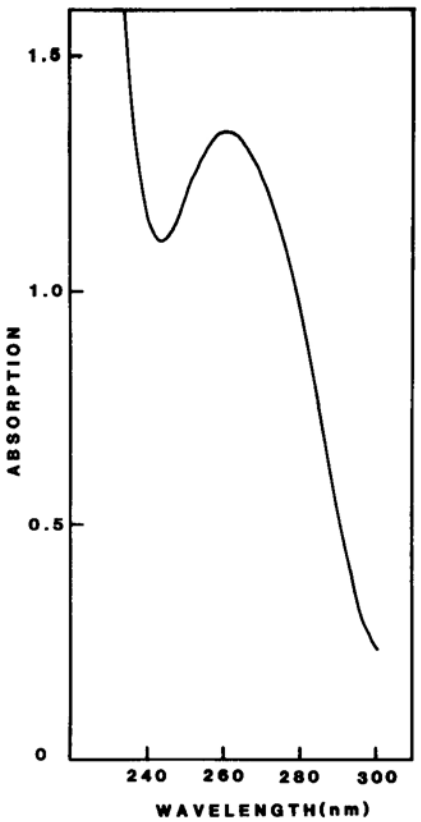

Fig. 4. Ultraviolet light absorption spectrum of purified mung bean yellow mosaic virus preparations.

DISCUSSION

The geminate particles found in the leaf-dip preparations from mung bean plants infected with MYMV together with the infectivity of the particles revealed in the purification experiments clearly indicate for the first time that the causal agent of MYMD is a geminivirus. These results confirm the original contention based on ultrastructural studies that the MYMD was caused by such a virus (10). Although early reports both from India (7) and Thailand (11) indicated that MYMV was not transmissible by mechanical inoculation, attempts in our subsequent trials were successful. MYMV can be mechanically transmitted by conventional inoculation techniques at a rate as high as 100% with or without a reducing agent in the phosphate buffers. The whitefly was able to transmit the pathogen to healthy mung bean, soybean, and Top Crop bean from mung bean infected with MYMV by mechanical inoculation (Y. Honda, *unpublished*). MYMV particles are relatively stable compared with those of many other whitefly-transmitted geminiviruses because they can be seen under the electron microscope without prior fixation with aldehydes. Geminate particles were detectable by the leaf-dip method after negative staining with PTA; hence, it is possible to use this technique for preliminary diagnosis of the disease.

The yellow mosaic disease reported from India was more severe in black gram than in mung bean (12); however, the disease in Thailand was more common in mung bean and was seldom observed in black gram under natural conditions. Our inoculation studies in the greenhouse revealed that MYMV could infect black gram but that the transmission rates in black gram were lower than in mung bean. The virus reported from India also has a wider host range including plant species such as *Brachiaria ramosa* (Gramineae) and *Eclipta alba* (Compositae) (8). The host range of MYMV in Thailand appears to be restricted to plants in family Leguminosae.

By comparing the host range of MYMV in Thailand with that of bean golden mosaic virus (BGMV) in Puerto Rico, it was found that BGMV could not be transmitted by inoculation with sap or graft to black gram, mung bean and soybean from Top Crop bean (Y. Honda, *unpublished*). In contrast, MYMV could be transmitted to them. Symptoms incited by BGMV in Top Crop bean (2) were different from those caused by MYMV (Fig. 1D). The characterization of the nucleic acid of MYMV and analysis of the serological relationships between MYMV and BGMV will be undertaken in our laboratory.

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