# Characterization of Two Viruses Isolated from Patchouli in Japan

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**ABSTRACT**

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Two sap-transmissible viruses were isolated from patchouli (*Pogostemon patchouli*) in Japan. The virus source plants showed faint mosaic, mottling, or no symptoms. In electron microscopic examinations, however, elongated (760 nm in length) or spherical (27 nm in diameter) viruslike particles were found. The spherical virus, designated patchouli mild mosaic virus (PaMMV), infected plants in seven families and was found to be serologically related to, but different from, broad bean wilt virus. The elongated virus, named patchouli mottle virus (PaMoV), had a narrower host range and was identified as a member of the Potyviridae on the basis of particle morphology, formation of cytoplasmic inclusions, and a distant serological relationship with turnip mosaic potyvirus.

Patchouli (*Pogostemon patchouli* Pellet. = *P. cablin* (Blanco) Benth.), a member of Labiatae, is vegetatively propagated in Indonesia, the Philippines, and other Southeast Asian countries for aromatic oils used in soaps and other toiletries. Many propagants have viruslike symptoms and reductions in yields within several years after first planting. Viruses reported in patchouli include patchouli mosaic virus (PaMV) (2), tobacco necrosis virus, and a rhabdovirus (3,4), but few studies have been conducted for viruses in patchouli in Asia.

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Patchouli plants from Southeast Asia are being grown in several botanical gardens and experimental farms in Japan, and many of the plantings have high incidences of viruslike symptoms. The identification and characterization of two viruses isolated from the plants and designated patchouli mild mosaic virus (PaMMV) and patchouli mottle virus (PaMoV) are reported here. A preliminary report was published (7).

## MATERIALS AND METHODS

**Sources of virus isolates.** Patchouli plants originating from Southeast Asia were established in several locations in Japan. Many plants showed symptoms of mild mosaic or mottle while others appeared healthy. Plants used in this study were collected from two botanical gardens and maintained in glasshouses. On the basis of host reactions of some differential indicator plants to inocula derived from the patchouli collections and on electron microscopy of the patchouli and indicator plants, two virus isolates, PaMMV and PaMoV, were selected for further study.

**Host range.** All indicator plants were grown from seeds and maintained in aphid-free greenhouses or air-conditioned glass chambers at 20–25 C with a day length of about 12 hr. Inoculations were made by rubbing tissue extracts ground in 0.1 M phosphate buffer (pH 7.0) on indicator plants dusted with 600- or 800-mesh Carborundum. In some instances, a small amount of a Celite-bentonite mixture (1:1) was added directly to the extract. Indicator plants were observed for up to 2 mo for symptom development. Latent infections were detected by inoculation to *Chenopodium amaranticolor* Coste & Reyn. or *C. quinoa* Willd. or by electron microscopy.

**Electron microscopy.** Extracts from infected plants of both PaMMV and PaMoV were negatively stained with 2% phosphotungstic acid (pH 6.0) and observed with a transmission electron microscope (JEM-100CX or 100SX). Ultrastructural studies were done with the infected leaf tissues fixed in 2% glutaraldehyde, followed by 2% osmium tetroxide, and embedded in EPON 812. Immunoserological electron microscopy

was done with carbon-fronted 400-mesh grids floated for 15 min on a drop of purified virus or infected leaf sap, mixed with diluted antiserum to virus for 5 min, stained with 2% uranyl acetate, and examined in the electron microscope.

**Stability in vitro.** Virus sources were extracts of systemically infected leaves of *C. quinoa*, which were assayed onto *C. amaranticolor* to determine virus viability. Thermal inactivation point between 50 and 70 C, longevity in vitro at 20 C, and dilution end point were determined for both viruses.

**Aphid transmission.** Aphid transmission trials were conducted with only PaMMV and *Myzus persicae* (Sulzer). Aphids were starved for 2–3 hr, allowed an acquisition period of 5 min on infected plants, placed on healthy plants to feed for 24 hr, and then removed.

**PaMMV purification.** Infected *C. quinoa* leaves were harvested about 5–10 days after inoculation. The tissues were homogenized in 0.2 M citrate buffer (pH 6.5) containing 0.1% of 2-mercaptoethanol (v/v). The homogenate was filtered through cheesecloth, and then Triton X-100 was added to 1% (v/v). The mixture was stirred for 30 min at 37 C and for 30 min at 4 C, then was centrifuged at 8,000 *g* for 10 min. The supernatant was layered onto 6 ml of a 30% sucrose cushion and centrifuged at 250,000 *g* for 90 min. The pellets were resuspended in 0.1 M citrate buffer (pH 7.5) containing 1 mM dithiothreitol. After two cycles of differential centrifugation, the resuspended pellets were layered onto 10–40% sucrose density gradients and centrifuged at 110,000 *g* for 150 min. The fractions containing each of the separate nucleoprotein components of the virus were pooled, diluted, and concentrated by centrifugation at 250,000 *g* for 90 min.

**Serology.** Antisera against PaMMV were prepared in rabbits by three to six intramuscular injections, each containing 1 mg of purified virus emulsified with an equal volume of Freund's complete adjuvant, at intervals of 2 wk. The rabbits were bled 1 wk after an intravenous injection (1 mg) following the series of intramuscular injections. The serum was tested by immunodiffusion in agar against a diluted preparation of 1.0 mg/ml of homologous virus. The gel diffusion tests were done in petri dishes containing 0.75% agar in 0.01 M citrate buffer (pH 7.6) with 0.02% sodium azide. Two broad bean wilt virus (BBWV) antisera and lamium mild mosaic virus (LMMV) antiserum were also used.

**Determination of molecular weight of the PaMMV coat protein.** Molecular weights of the coat proteins were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (5). The gels were then stained with Coomassie brilliant blue.

**Extraction of the PaMMV nucleic acids.** Double-stranded RNA from infected *C. quinoa* leaf was extracted by a modified procedure described by Dodds and Bar-Joseph (1).

## RESULTS

**PaMMV.** The virus was first recovered from a patchouli plant with very mild mosaic. It was maintained in *C. quinoa* and transmitted easily by mechanical inoculation to 13 species in seven families (Table 1), including *Nicotiana tabacum* L., *Vicia faba* L., *Gomphrena globosa* L., *C. amaranticolor, C. quinoa, Spinacia oleracea* L., and *Tetragonia expansa* Thunb. ex J.A. Murray. *C. amaranticolor* and *C. quinoa* showed developing chlorotic local lesions and mild systemic distortion, and *T. expansa* showed chlorotic spots both in inoculated and in upper leaves. *Antirrhinum majus* L., *N. glutinosa* L., three cultivars (Samsun, Xanthi-nc, and Xanthi) of *N. tabacum*, and *Petunia* × *hybrida* Hort. Vilm.-Andr. were only locally infected in a latent manner. Patchouli showed very mild systemic mottling.

Electron microscopic observation of purified preparations and leaf dips of each plant revealed isometric particles with a diameter of approximately 27 nm (100 particles counted). Numerous virus particles also were observed in the cytoplasm of infected leaves in ultrathin sections.

In crude extracts, PaMMV was infective at a dilution of $1 \times 10^3$ to $10^4$ at 50–55 C for 10 min and lost infectivity after 6 days at 20 C. The sap in phosphate buffer had lower infectivity than that in citrate buffer.

None of healthy *C. quinoa* seedlings exposed to *M. persicae* that had fed on diseased plants developed symptoms of PaMMV.

The purification procedure using citrate buffer yielded three virus bands in the final sucrose density gradient column. By electron microscopy, the top component consisted mostly of empty particles, whereas the middle and bottom components comprised mostly intact particles (Fig. 1).

By immunoserological electron microscopy, the antiserum with a dilution end point of 1/1,024 reacted with purified preparations. The reaction was strong with antiserum to BBWV serotype II but weak with antiserum to BBWV serotype I. The reaction with antiserum to LMMV was very weak, and no serological relationship was observed with antisera to cucumber mosaic virus and arabis mosaic virus. PaMMV reacted and spurred with antiserum to BBWV serotype I and crossed with antiserum to BBWV serotype II in gel immunodiffusion tests (Fig. 2).

The coat protein of PaMMV contained two polypeptides with $M_r$ 43,000 and 26,000 (Fig. 3). The double-stranded

**Table 1.** Host range and symptomatology of patchouli mild mosaic virus (PaMMV)

| Host species[a] | Symptoms[b] | |
|---|---|---|
| | Local | Systemic |
| *Antirrhinum majus* | L | NV |
| *Chenopodium amaranticolor* | CS, NS | D |
| *C. quinoa* | CS | CS, D |
| *Spinacia oleracea* | NS | D, NS |
| *Gomphrena globosa* | NS | L, M |
| *Tetragonia expansa* | CS | CS |
| *Phaseolus radiatus* | NS | NV |
| *Vicia faba* | NS | L |
| *Vigna sesquipedalis* | NS | NV |
| *Nicotiana glutinosa* | L | NV |
| *N. rustica* | L | L |
| *N. tabacum* | | |
| White Burley | L | L |
| Burley 21 | L | L |
| Samsun | L | NV |
| Xanthi-nc | L | NV |
| *Petunia* × *hybrida* | L | NV |
| *Pogostemon patchouli* | L | M |

[a]Not infected by PaMMV: *Lactuca sativa, Zinnia elegans, Sesamum indicum, Pisum sativum, Perilla frutescens, Cucumis sativus, Cucurbita pepo*, and *Salvia splendens*.
[b]CS = chlorotic spot, D = distortion, L = latent infection, M = mosaic, N = necrosis, NS = necrotic spot, NV = no virus recovered by back-inoculation.

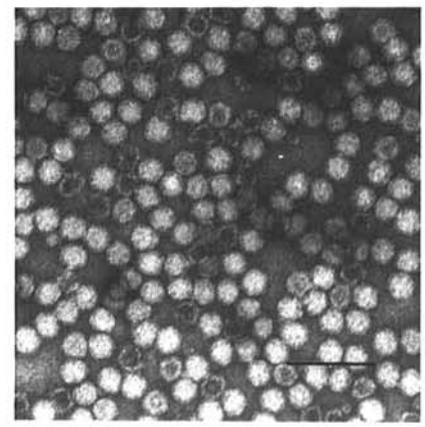

**Fig. 1.** Purified patchouli mild mosaic virus particles. Scale bar = 100 nm.

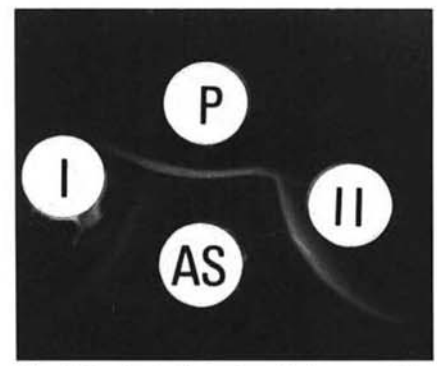


**Fig. 2.** Serological reactions in immunodiffusion test. Wells contain patchouli mild mosaic virus (PaMMV) antiserum (AS) and virus preparations of broad bean wilt virus serotypes I (I) and II (II) and PaMMV (P).

RNA replicative forms of both RNAs were detected in infected *C. quinoa* but not in healthy *C. quinoa*. Their estimated molecular weights were 4.2 and 2.7 × $10^6$ (Fig. 4).

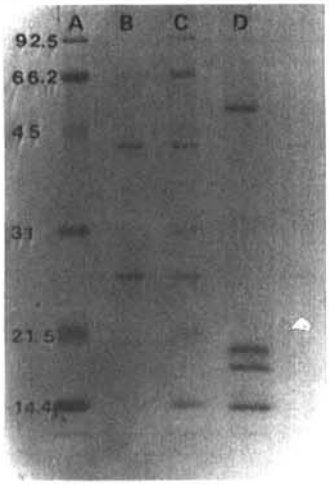


**Fig. 3.** Migration pattern of patchouli mild mosaic virus (PaMMV) capsid proteins in 10% sodium dodecyl sulfate-polyacrylamide gel. Lane A = protein markers, lane B = purified PaMMV, lane C = protein markers and purified PaMMV, and lane D = partially purified healthy *Chenopodium quinoa* extract.

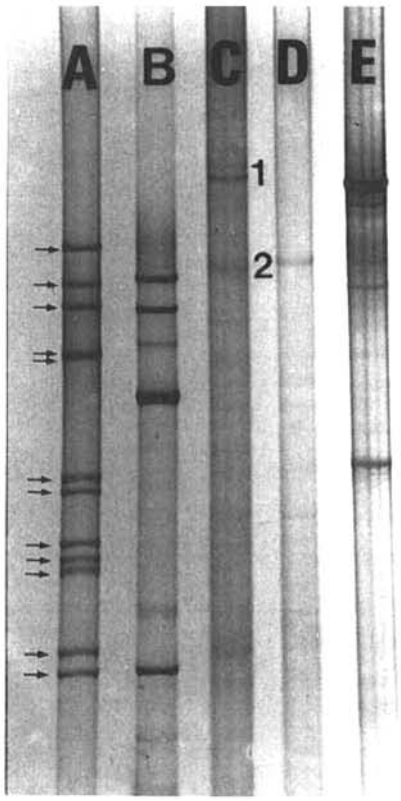


**Fig. 4.** Double-stranded RNA pattern of patchouli mild mosaic virus (PaMMV) in 5% polyacrylamide gel stained with silver. DsRNA preparation of leaves infected with lane A = rice dwarf virus, lane B = cucumber mosaic virus, lane C = PaMMV, and lane E = tobacco mosaic virus; lane D = dsRNA preparation of healthy *Chenopodium quinoa*. Molecular weights of bands (arrows) in lane A are (top to bottom) 3.1, 2.5, 2.25, 1.9, 1.8, 1.15, 1.13, 0.86, 0.78, 0.75, 0.52, and 0.52 × $10^6$.

**PaMoV.** Symptoms in patchouli plants naturally infected with PaMoV ranged from almost none to only mild mottling (Fig. 5). Symptoms were more severe in plants infected with both PaMMV and PaMoV. PaMoV was mechanically transmitted from patchouli to *C. quinoa, T. expansa*, and *Sesamum indicum* L., causing systemic infection, and to *C. amaranticolor* and *G. globosa*, causing local symptoms (Table 2). Other plants of 18 species in seven families, including Labiatae, were immune to PaMoV.

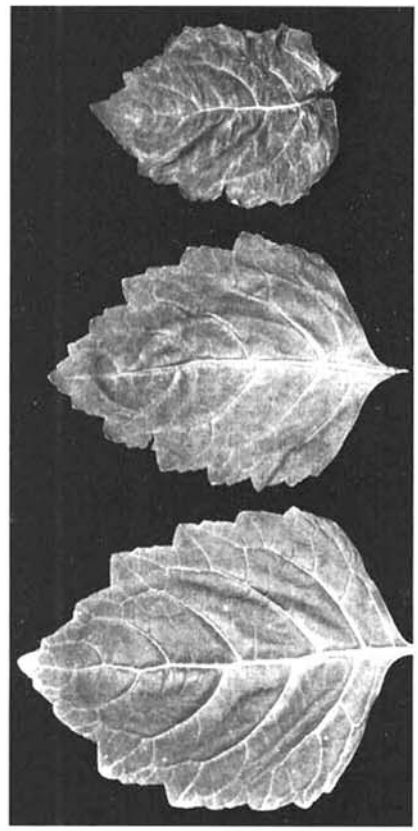

**Fig. 5.** (Bottom) Healthy patchouli leaf compared with leaves infected with (middle) patchouli mottle virus and (top) both patchouli mottle virus and patchouli mild mosaic virus.

**Table 2.** Host range and symptomatology of patchouli mottle virus (PaMoV)

| | Symptoms[b] | |
|---|---|---|
| **Host species[a]** | **Local** | **Systemic** |
| *Chenopodium amaranticolor* | CS | NV |
| *C. quinoa* | CS | CS |
| *Gomphrena globosa* | CS | NV |
| *Tetragonia expansa* | CS | CS |
| *Sesamum indicum* | | |
| Shirogoma | M | M, N |
| Kurogoma | M | M, N |

[a]Not infected by PaMoV: *Spinacia oleracea*; *Phaseolus vulgaris* cvs. Top Crop and Masterpiece; *Vigna sesquipedalis*; *Brassica campestris* cv. Komatsuna; *Cucumis sativus*; *Cucurbita pepo*; *Perilla frutescens*; *Mentha spicata*; *Salvia splendens*; *Vinca rosea*; *Lycopersicon esculentum*; *Nicotiana glutinosa*; *N. clevelandii*; *N. tabacum* cvs. Burley 21, Samsun, and Xanthi-nc; *Petunia* × *hybrida*; *Lactuca sativa*; and *Zinnia elegans*.
[b]CS = chlorotic spot, M = mosaic, N = necrosis, NV = no virus recovered by back-inoculation.

In crude extracts, PaMoV was infective at a dilution of 1 × $10^3$ to $10^4$ after 10 min at 60–65 C and lost infectivity after 3 days at 20 C.

Fifty particles were measured from leaf dip preparations of *C. quinoa* and averaged approximately 760 nm in length. Cytoplasmic laminated inclusions typical of potyviruses were seen in ultrathin sections of infected leaves (Fig. 6).

Immunoserological electron microscopy was conducted with antisera against zucchini yellow mosaic virus, lettuce mosaic virus, turnip mosaic virus (TuMV), watermelon mosaic virus 2, and pea seedborne mosaic virus. PaMoV reacted distantly (1/64) only with antiserum against TuMV (homologous titer 1/1,024) (Fig. 7).

## DISCUSSION

In particle morphology, molecular weight of coat protein, and serological relationship, PaMMV had similarities with BBWV (10) and LMMV (6). However, PaMMV showed differences in narrow host range, symptomatology, and spur and cross formation with two antisera to two serotypes of BBWV (11). PaMMV is easily distinguished from other fabaviruses on the basis of host ranges and symptomatology on *V. faba* and *N. tabacum* and should be considered a new fabavirus. Many viruses and isolates in the fabavirus group have been reported from many countries, but there have not been many reports from Southeast Asia. As the host range of fabaviruses is very wide, further study should determine the distribution and influence

**Fig. 6.** Cylindrical and wheel inclusions in ultrathin section of patchouli leaf infected with patchouli mottle virus. Scale bar = 500 nm.

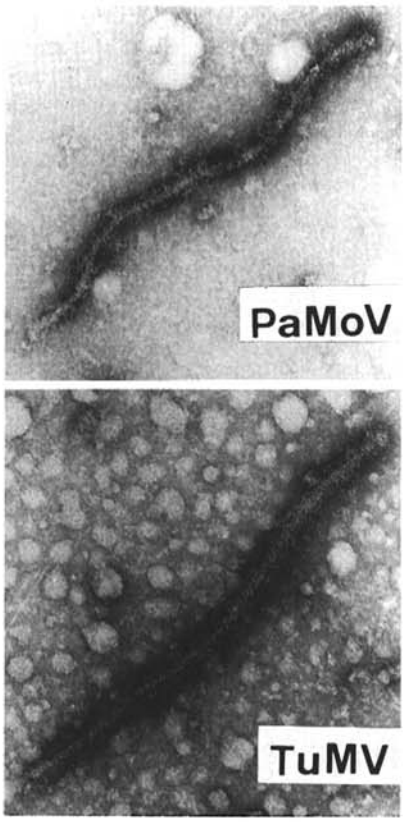


**Fig. 7.** Sap from sample infected with patchouli mottle virus (PaMoV) and turnip mosaic virus (TuMV) absorbed to grids and decorated with antiserum to TuMV diluted 1:32 (v/v) in phosphate buffer.

of fabaviruses on agricultural crop plants in Asian countries. Although *M. persicae* did not transmit PaMMV, other fabaviruses are transmitted by many aphid species (10). Thus, experiments on transmissibility by other aphid species, including aphids common on patchouli plants, should be carried out. BBWV has been classified into strains on the basis of its symptomatology and also is classified by serotypes (11). Lisa et al (6) reported that LMMV isolated from *Lamium* sp. and *Marrubium* sp. in Labiatae was a different serotype than those of BBWV. Shukla and Gough (9) also reported a strain of BBWV from *Ajuga reptans* L., a Labiatae plant, as a new serotype in Australia. From Southeast Asia, however, there are only scattered reports on the study of fabavirus serotypes, and all BBWV strains reported are either serotype I or II. Antiserum to PaMMV prepared in this study will be useful for detection and identification of PaMMV. Gama et al (4) also reported rhabdovirus-like particles and tobacco necrosis virus from patchouli. These viruses were not detected in our collections.

On the basis of the above results, the elongate virus, PaMoV, from patchouli was identified as a new member of the potyvirus group. PaMoV was distantly related serologically to TuMV, and the host range and symptomatology were completely different from other potyviruses, including TuMV. Earlier, Gama et al (2–4) reported on PaMV in Brazil, and Rao and Nagar (8) described the occurrence of PaMV in India. The host range of PaMV-Brazil is wider than that of PaMoV; it infects *Zinnia elegans* Jacq., *N. glutinosa*, and others (M. I. C. S. Gama, *personal communication*). PaMV-India may be related to PaMoV because it is serologically related to TuMV and has a narrow host range.

Crop losses of patchouli in Southeast Asia have occurred in epidemic proportions, presumably due to virus diseases. Hence, production of virus-free plants is necessary. The study of viruses on patchouli and the preparation of antisera will help to diagnose and manage these diseases and to evaluate the effectiveness of virus-free patchouli plants.

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