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Further Characterization of Cymbidium Mosaic Virus from *Vanda* Orchid

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A virus causing necrotic spots and necrotic flecks on the leaves of *Vanda* orchids in Japan was identified as cymbidium mosaic virus (CyMV) on the basis of host range, stability in crude sap, particle morphology, serological test and physico-chemical properties. The virus was transmitted by sap inoculation to 12 of 57 species in 6 of 12 families tested, but not by aphid *Mizus persicae* or through seeds. Systemic infection occurred in all Orchidaceae plants tested and only one in non-orchidaceae (*Sesamum indicum*). In *Tetragonia expansa* sap, the virus was infective at a dilution of 10^{-5} but not at 10^{-6}, after heating at 65°C for 10 min, and was still active after 1 month aging *in vitro*. Flexuous rod particles, c. 475 × 13 nm, were observed. In ultrathin sections of leaf tissues from diseased plants, virus particles were found to aggregate in the cytoplasm. The molecular weight of the protein subunit and RNA determined by gel electrophoresis, was 27.8×10^3 and 2.2×10^6, respectively. Double-stranded RNAs with estimated molecular weights of 5.4×10^6, 4.0×10^6, 3.6×10^6 and 3.0×10^6 were isolated from infected plants.

Key words : *Vanda* orchid, Cymbidium mosaic virus, Potexvirus

INTRODUCTION

Cymbidium mosaic virus (CyMV) was reported for the first time by Jensen (1950, 1951) to induce mosaic or black streak in *Cymbidium* orchid. Since this decade, CyMV has been recognized to be an infectious disease to many orchid genera (Hu *et al.* 1993, Inouye and Leu 1983, Zettler *et al.* 1990). Likewise, the virus reduces the plant vigor and flower quality which affect the economic value (Corbett 1960, Pearson and Cole 1991).

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CyMV is a very common disease of orchids in Japan causing chlorotic areas and necrotic streaks in the leaves of *Cymbidium* and leaf necrosis in *Cattleya* (Inouye 1968). Moreover, ashes white ringspots in the leaves of *Vanda* orchids caused by CyMV was reported by Inouye and Mitsuhata (1981). In the present study, we identified and characterized the causal agent of a virus disease of *Vanda* orchids showing necrotic spots and necrotic flecks in their leaves, on the basis of host range, stability in crude sap, transmission, electron microscopy and physico-chemical properties of the virus.

MATERIALS AND METHODS

1. *Virus isolation and host range*

The virus used in this study was isolated from *Vanda* orchids showing necrotic spots and necrotic flecks collected from commercial orchid nurseries in Japan (Fig. 1-A). The diseased *Vanda* orchid plants were maintained in a greenhouse for the inoculum sources. *Tetragonia expansa* produced obvious symptoms after sap inoculation and was suitable as a host for virus propagation. After three successive single lesion tranfers on *T. expansa*, the isolate was established in *T. expansa* for further tests.

In host range studies, several differential hosts (Table 1) were used. To confirm the virus infection, samples of inoculated leaves and other leaves (systemic infections) were assayed by back-inoculation to *C. amaranticolor* and by electron microscopy.

2. *Stability in crude sap*

Virus stability in crude sap was tested using the extract of the diseased *T. expansa* prepared in 0.01 M phosphate buffer (PB), pH 7.0. These tests included thermal inactivation point, dilution end point and longevity *in vitro*.

3. *Transmission*

Aphid transmission tests were performed in a non-persistent manner in a greenhouse using *Mizus persicae* Sulzer. The aphids were starved for 1 hr before an acquisition period on infected *C. amaranticolor* leaves and transferred to test plants.

For seed transmission tests, seeds were collected from *Sesamum indicum* showing severe symptoms after mechanical inoculation in the greenhouse. The seeds harvested from these plants were planted in pasteurized soil to observe the appearance of symptoms. The presence or absence of the virus was determined by back inoculation to *C. amaranticolor* and by electron microscopy.

4. *Electron microscopy*

Leaf dip and purified virus preparations were stained with 2% uranyl

acetate or with 2% phosphotungstic acid, pH 7.0. Immunoelectron microscopical decoration tests were performed according to Milne and Luisoni (1977).

For ultrastructural studies, small pieces of diseased *Cymbidium* leaves were prefixed in 6% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were cut with a ultramicrotome model 2088 Ultotome V, stained with 2% uranyl acetate and lead citrate. A Hitachi model H-7100 electron microscope was used for observation.

5. *Virus purification*

The virus was extracted and purified by the method of Frowd and Tremaine (1977) with slight modifications. Infected *T. expansa* leaves were homogenized in 0.5 M citrate buffer, pH 6.5 containing 0.005 M ethylenediaminetetraacetate (EDTA) and 0.2% thioglycolic acid. Homogenates were filtered through double layers of cheesecloth and centrifuged at 6,100 $\times g$ for 15 min. The aqueous phases were treated with 2% Triton X-100 (stirred for 15 min) and after addition of 6% polyethylene glycol (PEG) 6,000 and 0.25% sodium chloride stirred for 1 hr. The mixture was centrifuged at 6,100 $\times g$ for 20 min. The pellet was resuspended in 0.005 M borate-EDTA buffer, pH 9.0. After low speed centrifugation (6,100 $\times g$ for 15 min), 1% Triton X-100 was added to the supernatant, which was then stirred for 15 min. The mixture was layered onto 30% sucrose cushion and centrifuged at 121,000 $\times g$ for 1.5 hr. After repetition of sucrose-cushion and low speed centrifugation, the supernatant was centrifuged at 135,000 $\times g$ for 3 hr in sucrose-cesium chloride density-gradient columns prepared in 0.005 M borate-EDTA buffer. The visible virus band was dialyzed overnight against the same buffer. The virus preparation was centrifuged at 121,000 $\times g$ for 1.5 hr and the pellet was resuspended in 0.01 M PB, pH 7.0 and then centrifuged at 6,100 $\times g$ for 15 min. All procedures were carried out at 4°C, except sucrose-cesium chloride density-gradient centrifugation at 15°C. This purified virus was stocked at 4°C for further studies.

6. *Determination of capsid protein molecular weight*

The molecular weight of viral coat protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) consisting of a 4% stacking and 15% resolving gel (Laemmli 1970). After electrophoresis, the gel was stained in Coomassie Brilliant Blue. For estimation of the molecular weight, the following markers were used: phosphorylase b. (Mr 94×10^3), albumin (Mr 67×10^3), ovalbumin (Mr 43×10^3), carbonic anhydrase (Mr 30×10^3), trypsin inhibitor (Mr 20.1×10^3) and α-lactalbumin (Mr 14.4×10^3).

7. *Purification and electrophoresis of ssRNA and dsRNA*

Single-stranded RNA (ssRNA) from the purified virus was extracted according to Steinhart and Oshiro (1990). Purified virus was suspended in an extraction buffer (10 mM Tris-HCl, pH 7.5 ; 200 mM sodium acetate ; 1 mM EDTA ; 0.5% SDS and 100 mM proteinase K). The solution was then deproteinated by phenol : chloroform (1 : 1 by volume) extractions, and the viral ssRNA was concentrated by ethanol precipitation. The molecular weight of the viral ssRNA was determined by agarose gel electrophoresis after denaturing by formaldehyde for 10 min at 65°C.

Double-stranded RNA (dsRNA), extracted from the infected leaves of *T. expansa*, was subjected to two cycles of cellulose chromatography on Whatman CF-11 (Valverde *et al.* 1990). The final dsRNA suspension was concentrated by the addition of 0.1 vol. of 3.0 M sodium acetate and 2.5 vol. of cold 95% ethanol. The dsRNA was electrophoresed in 6% polyacrylamide gel and stained with silver as described by Blum *et al.* (1987). Molecular weight standards for size estimation were cucumber mosaic virus (Takanami *et al.* 1977) and arabis mosaic virus (Inouye *et al.* 1992).

RESULTS

1. *Host range and symptomatology*

The host range study involved 57 species in 12 families in which the reactions of test plants varied (Table 1). The virus systemically infected Orchidaceae : *Cattleya*, *Cymbidium*, *Dendrobium* and *Phalaenopsis* and only one non-orchidaceae, *Sesamum indicum*. This virus caused local lesions on *T. expansa*, *C. amaranticolor*, *C. murale* : first, green spots were formed on the inoculated leaves, later turning to yellowish-green. While inoculated leaves were lost prematurely, the upper inoculated leaves showed chlorotic spots. *C. quinoa* : both chlorotic and necrotic spots were formed on the inoculated leaves. *Cassia occidentalis* : small brown necrotic spots appeared on the inoculated bifoliate leaves (Fig. 1-B) . *Datura stramonium* : green spots developed on the inoculated leaves which turned to lesions with a yellowish margin, while on inoculated young leaves, both chlorotic spots and necrotic spots were formed (Fig. 1-C). *Nicotiana benthamiana* : local lesions with green spots and necrotic spots appeared on the inoculated leaves. *Gomphrena globosa*, *Beta vulgaris*, *Cucumis sativus*, and *Cucurbita maxima* were infected locally by the virus without symptoms. Thus, the host range of the virus responsible for necrotic spots and necrotic flecks of *Vanda* orchids was similar to that reported for CyMV (Inouye 1968).

Table 1. Host range of cymbidium mosaic virus (CyMV) from *Vanda* orchid

Systemically-susceptible plants	
Orchidaceae	*Cattleya* sp., *Cymbidium* sp., *Dendrobium* sp., *Phalaenopsis* sp.
Pedaliceae	*Sesamum indicum* (cvs. Kurogoma, Shirogoma)
Locally-susceptible plants	
Aizoaceae	*Tetragonia expansa*
Amaranthaceae	*Gomphrena globosa* *
Chenopodiaceae	*Beta vulgaris* *, *Chenopodium amaranticolor*, *C. quinoa*, *C. murale*
Cucurbitaceae	*Cucumis sativus* (cvs. Shougoin, Tsubasa)*, *Cucurbita maxima* *
Leguminosae	*Cassia occidentalis*
Solanaceae	*Datura stramonium*, *Nicotiana benthamiana*
Non-susceptible plants	
Amaranthaceae	*Celosia argentea*
Caryophyllaceae	*Dianthus barbatus*, *D. caryophillus*
Chenopodiaceae	*Spinacia oleracea*
Compositae	*Callistephus chinensis*, *Crysanthemum coronarium*, *Helianthus annuus*, *Lactuca sativa*, *Zinnia elegans*
Cruciferae	*Brassica campestris*, *B. rapa*, *B. sativus*, *Raphanus sativa*
Cucurbitaceae	*Citrulus lanatus*, *Cucumis melo*, *C. moschata*, *C. pepo*, *Lagenaria siceraria*, *Pharbitis nil*
Gramineae	*Zea mays*
Leguminosae	*Astragalus sinicus*, *Glycine max*, *Medicago sativa*, *Phaseolus vulgaris* (cvs. Hatumidori, Kintoki, Top Crop, Yamasiro Kurosando), *Pisum sativum*, *Trifolium incarnatum*, *T. pratense*, *T. repense*, *Vigna radiata*, *V. unguiculata* (cvs. Akadanesanjaku, Kurodanesanjaku), *Vicia faba*
Solanaceae	*Capsicum annuum*, *Hyoscyamus niger*, *Lycopersicon esculentum*, *Nicotiana clevelandii*, *N. glutinosa*, *N. rustica*, *N. tabacum* (cvs. Samsun, White Burley), *Petunia hybrida*, *Physalis floridana*, *Solanum melongena*

*Latent infection

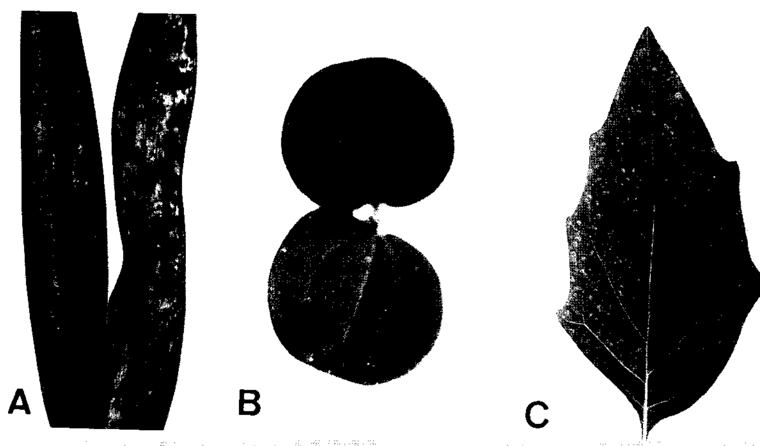

Fig. 1. Symptoms of naturally infected *Vanda* orchid and test plants inoculated with cymbidium mosaic virus (CyMV) from *Vanda* orchid. (A) Field infected *Vanda* orchid, exhibiting necrotic spots and necrotic fleks. (B) Local lesions on an inoculated leaf of *Cassia occidentalis*, 5 days after inoculation. (C) Local lesions on an inoculated leaf of *Datura stramonium*, 10 days after inoculation.

2 . *Stability in crude sap*

Sap from *T. expansa* showing local lesions was infective for 10 min after heating at 65°C but not at 70°C, and after dilution to 10^{-5} but not to 10^{-6}. In aging tests, the virus remained infective in juice after storage for one month at 20°C.

3 . *Transmission*

In the aphid transmission test, *Mizus persicae* failed to transmit the virus from *C. amaranticolor* to *C. amaranticolor* in a non-persistent manner.

Seedlings produced from seeds of severely infected *S. indicum* showed no symptoms. The virus was not detected in a *S. indicum* by either return inoculation or serological tests.

4 . *Electron microscopy*

The particle length of the virus was measured on 300 particles. The particle lengths ranged from about 75 to 950 nm and the modal length was found to be 475 nm, with a width of approximately 13 nm (Fig. 2). Sinuous particles were observed in leaf dip preparation from diseased *Vanda* orchid and from artificially infected plants (Fig. 3-A) or in purified preparations (Fig. 3-B).

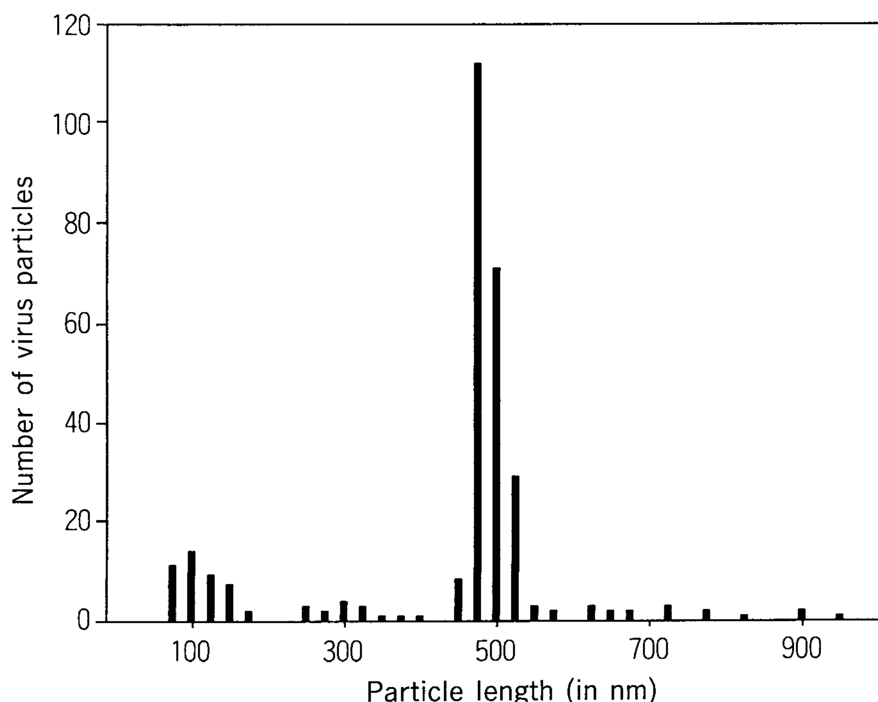

Fig. 2. Particle length distribution of CyMV from *Vanda* orchid.

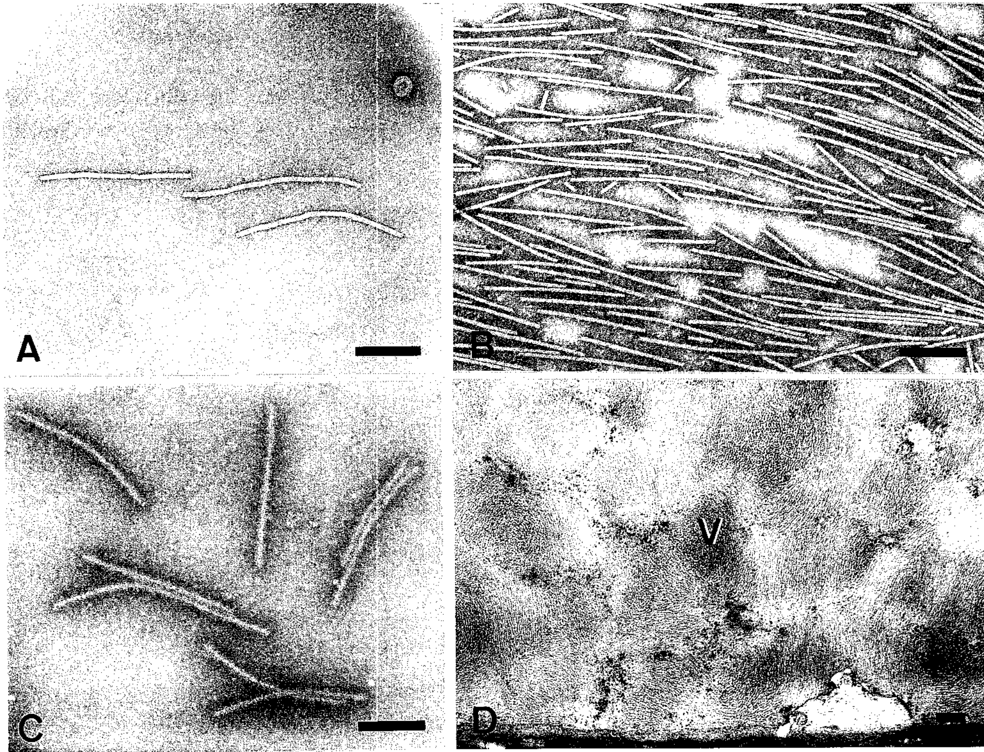

Fig. 3. Electron micrographs of CyMV from *Vanda* orchid. (A) Virus particles negatively stained with 2% uranyl acetate in a dip preparation from an infected leaf of *T. expansa*. (B) Purified virus particles stained with 2% PTA, pH 7.0. (C) Immunosorbent electron microscopy using CyMV antiserum at a 1/200 dilution. (D) Ultrathin section of mesophyll cells of infected *Cymbidium*, with viral inclusions (V) in the cytoplasm which resemble "finger print". Scale bars 200 nm.

Decoration of the purified virus particles was done with the antiserum raised against CyMV (common strain) (Fig. 3-C). No decoration was obtained with the antisera to white clover mosaic virus or potato virus X.

In ultrathin sections of mesophyll cells of diseased *Cymbidium*, banded body inclusions consisted of large aggregate resembling "finger print" were scattered in the cytoplasm (Fig. 3-D).

5 . *Physico-chemical properties*

As shown in Fig. 4, the electrophoresis of coat protein resulted in a major protein band at M*r* of c. 27.8×10^3, slightly larger than previously published values for CyMV (Frowd and Tremaine 1977, Steinhart and Oshiro 1990).

A single species was observed when the RNA extracted from purified virus particles electrophoresed in denaturing formaldehyde and stained with

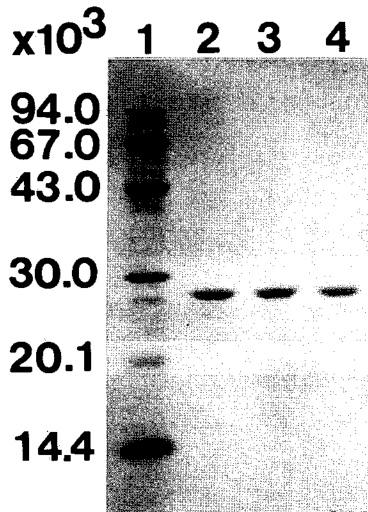

Fig. 4. SDS-PAGE of CyMV coat proteins. Lane 1: molecular weight marker proteins, lane 2: CyMV-*Vanda*, lane 3: CyMV-*Cymbidium*, and lane 4: CyMV-*Calanthe*.

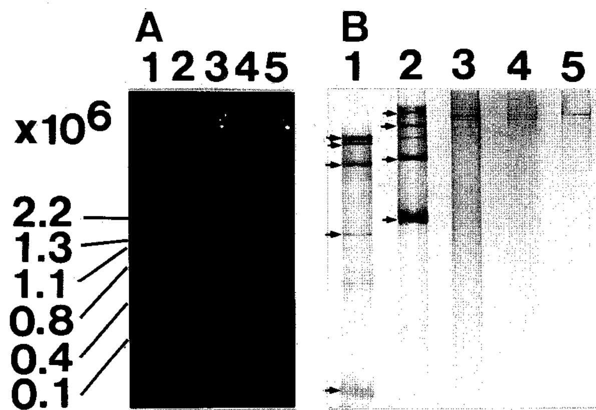

Fig. 5. (A) Electrophoresis of virus RNA denaturated with formaldehyde in 1% agarose gel. Cucumber mosaic virus (lane 1) and tabacco mosaic virus (lane 2) were used as markers, lane 3: CyMV-*Vanda*, lane 4: CyMV-*Cymbidium*, and lane 5: CyMV-*Calanthe*. (B) Double-stranded RNA pattern of CyMV in 6% polyacrylamide gel. Cucumber mosaic virus (lane 1) and arabis mosaic virus (lane 2) were used as markers, lane 3: CyMV-*Vanda*, lane 4: CyMV-*Cymbidium*, and lane 5: CyMV-*Calanthe*. Molecular weights of bands (arrows) in lane B1 are (top to bottom) 2.46, 2.26, 1.66, 0.66, and 0.14×10^6; and in lane B2 are 5.02, 3.10, 1.74, and 0.76×10^6.

ethidium bromide. The molecular size of the virus ssRNA was estimated to be 2.2×10^6 (Fig. 5-A).

Two species of the most heavily stained dsRNA were found with an estimated Mr of 4.0×10^6 and 3.6×10^6. Moreover, minor dsRNA (Mr 5.4×10^6) of a slower mobility than the most heavily stained dsRNA, and one with a faster mobility (Mr 3.0×10^6) were also detected (Fig. 5-B).

DISCUSSION

A virus causing necrotic spots and necrotic flecks on *Vanda* orchids in Japan was identified as cymbidium mosaic virus (CyMV) on the basis of host range, stability in crude sap, electron microscopy and physico-chemical properties of the virus in this study.

In symptomatology, the virus caused local lesions without systemic infection on *Chenopodium amaranticolor*, *Cassia occidentalis* and *Datura stramonium*. The lesions in these plants were similar to those reported for CyMV (Corbett 1959, Inouye 1968, Jensen and Gold 1955). *C. amaranticolor*, *C. occidentalis* and *D. stramonium* have been reliable test plants for identification of CyMV (Francki 1970). In addition, we found that *Tetragonia expansa* was also a good test plant for this virus and suitable as a source plant for virus purification.

The shape and size of the virus particles were the same as those reported for CyMV (Francki 1970, Gold and Jensen 1951, Inouye 1968, Kado and Jensen 1964, Thornberry and Phillippe 1964). These characteristics were readily distinguishable from odontoglossum ringspot virus and vanda mosaic virus, which also have occurred in *Vanda* orchids, and were reported by Zettler *et al*. (1990) and Murakishi (1952). In ultrathin sections of mesophyll cells of diseased *Cymbidium*, virus particles were dispersed throughout the cytoplasm resembling "finger print". These morphological characteristics were taken to be the same as that found for CyMV (Hamilton and Valentine 1984, Hanchey *et al*. 1975, Ko *et al*. 1985, Pisi *et al*. 1982).

The capsid subunit protein of the virus was found to be a single-polypeptide species with a Mr of 27.8×10^3 and was similar to the value obtained for CyMV by Frowd and Tremaine (1977), but slightly greater than that reported by Steinhart and Oshiro (1990). This discrepancy also occurred with different strains of the virus (Frowd and Tremaine 1977) and potato virus X in different conditions (Tung and Knight 1972). However, we also examined the coat protein subunit of other CyMV-isolates such as *Cymbidium* and *Calanthe* isolates, which had the molecular weight similar to that of CyMV isolated from *Vanda*.

M*r* of ssRNA determined by gel electrophoresis was 2.2×10^6 for all CyMV-isolates which was estimated by comparing its mobility with those of RNA from tobacco mosaic virus (TMV-OM) and cucumber mosaic virus (CMV-Y). The ssRNA of CyMV isolated from *Vanda* was within the range of values reported for potexviruses (Matthews 1991). Thus it is evident that the virus isolated from *Vanda* orchids is cymbidium mosaic virus.

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バンダから分離されたCymbidium Mosaic Virusの諸性質

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日本において葉にえそ斑やえそ輪紋を示すバンダから分離されたウイルスは，宿主範囲，病葉汁液中での安定性，粒子長，物理化学的性質，外被タンパク質ならびにウイルスRNAの分子量等からcymbidium mosaic virus (CyMV) と同定された．本ウイルスを12科57種の植物に汁液接種したところ，数種のラン科植物とゴマに全身感染した． また，本ウイルスは汁液接種で容易に伝搬されたが，アブラムシ (*Mizus persicae*) では伝搬されず，種子伝染も認められなかった．ツルナの病葉汁液中での安定性は不活化温度が65～70℃(10分)，希釈限度が10^{-5}～10^{-6}，保存限度が20℃で1～2ヶ月であった．DN法による電顕観察では屈曲性のある長さ約475×13 nmのひも状粒子が多数認められ，感染葉 (*Cymbidium*) の超薄切片にはウイルス粒子がfingerprint状として細胞質内に存在することが確かめられた．ゲル電気泳動により外被タンパク質とウイルスRNAの分子量の解析を行ったところ，それぞれ27.8×10^3，2.2×10^6であった．また，本ウイルスに罹病したツルナ葉からは，主に4種の感染に特異的な二本鎖RNA (分子量約5.4×10^6，4.0×10^6，3.6×10^6 and 3.0×10^6) が検出された．

キーワード：バンダ，シンビジウムモザイクウイルス，ポティックスウイルス