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Some Properties of Bean Yellow Mosaic Virus Isolated from *Calanthe* sp. (Orchidaceae) in Japan

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Bean yellow mosaic virus (BYMV) was isolated from *Calanthe* sp. showing mild chlorosis on the leaves, collected in Yamaguchi Prefecture in Japan. The virus was transmitted by the aphid *Myzus persicae* in a non-persistent manner, and by sap-inoculation to 29 out of 46 plant species from 9 out of 12 families tested. Stability in crude sap, morphology of virus particles, shape of cylindrical inclusions and the presence of cytoplasmic crystalline inclusions in the infected cells were similar to those of BYMV isolates previously reported. The virus contained a single protein species with a molecular weight of 35,000. In a microprecipitin test and double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), the virus showed a close serological relationship to isolates of BYMV from both crocus and gladiolus, and showed a distant relationship to clover yellow vein virus. Three BYMV isolates used in this study were found to be serologically related to each other, but the virus was more closely related to the BYMV crocus isolate than to gladiolus isolate.

Key words : *Calanthe* sp., bean yellow mosaic virus, clover yellow vein virus, serological relationship.

INTRODUCTION

A potyvirus occurring in *Calanthe* (Orchidaceae), three viruses calanthe mild mosaic (CalMMV) (Gara *et al.* 1998), clover yellow vein (ClYVV) (Inouye *et al.* 1988) and turnip mosaic (TuMV) (Matsumoto *et al.* 1993) viruses have been reported in Japan. During a survey of viruses occurring in *Calanthe* in Japan, bean yellow mosaic virus (BYMV) was isolated from a plant [(*C. sieboldii* × *C. masuca*) × *C. izu-insularis*] showing mild chlorosis

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on the leaves, collected in an orchid nursery in Yamaguchi Prefecture in Japan. This paper mainly deals with the characterization of the virus with host range, symptomatology, vector transmission, stability in crude sap, viral protein, morphology of virus particles, ultrastructure of the infected cells and serological relationship between the virus and other isolates of BYMV and ClYVV isolated from ornamental plants.

MATERIALS AND METHODS

1. *Virus source and maintenance.*

The virus designated Cal.90-11 was isolated from *Calanthe* sp., showing mild chlorosis on the leaves, collected in Yamaguchi Prefecture in Japan in 1990 (Plate I-1). After two successive single lesion transfers on *Chenopodium quinoa*, the virus was maintained in *Vicia faba*. Two isolates of BYMV from crocus (Cro-4) (Kaneshige *et al.* 1991) and from gladiolus (BYMV-G) and one isolate of ClYVV from *Calanthe* (Cal-35) (Inouye *et al.* 1988) were also used in this study for comparison with the present virus.

2. *Host range.*

Plants were inoculated with sap from infected leaves of *V. faba* showing mosaic symptoms. The inoculated plants were maintained in a greenhouse at 15 to 25°C and the symptoms were observed up to 3 wk after inoculation. The infection was confirmed by back inoculation to *Chenopodium amaranticolor* or *C. quinoa*, and by electron microscopy.

3. *Virus purification.*

Viruses were purified by a modification of the method of Uyeda *et al.* (1975) from infected leaves of *V. faba*. The infected leaves were homogenized with a mortar and pestle in two volumes of 0.1 M Tris-HCl buffer, pH 7.0, containing 0.01 M EDTA and 0.5% Na_2SO_3. The homogenate was pressed through two layers of cheesecloth and centrifuged at 3,300 $\times g$ for 10 min. The supernatant was added with 2% Triton X-100, 4% polyethylene glycol (mol. wt 6,000) and 0.1 M NaCl, and stirred for 70 min at 4°C. After centrifugation at 7,000 $\times g$ for 20 min, the precipitate was resuspended in 0.01 M phosphate buffer (PB), pH 7.4, containing 0.5 M urea and 1% Triton X-100, and then the mixture was stirred for 10 min before centrifugation at 7,000 $\times g$ for 20 min. The supernatant was layered on a 20% sucrose cushion and centrifuged at 75,000 $\times g$ for 15 min. The resulting pellet was resuspended in 0.01 M PB, pH 7.4, containing 0.5 M urea. After low-speed centrifugation the supernatant was layered on a CsCl-sucrose density gradient and

centrifuged at 125,000 $\times g$ for 3 hr (Maeda and Inouye 1993). The virus zone was collected and dialyzed against 0.01M PB, pH 7.0. In the purification of BYMV-G, 0.5 M potassium phosphate buffer, pH 8.4, containing 0.5% Na_2SO_3 and 0.5 M borate-KCl buffer, pH 8.0 were used for extraction and resuspension of the virus, respectively.

4. *SDS-polyacrylamide gel electrophoresis (SDS-PAGE).*

SDS-PAGE in a 2 mm thick polyacrylamide slab gel (4% stacking gel and 12.5% separation gel) was done by the method of Laemmli (Kaneshige *et al.* 1991).

For molecular weight estimation, SDS-degraded virus proteins were co-electrophoresed with the Pharmacia marker proteins.

5. *Serology.*

Antiserum to Cal.90-11 was produced in a rabbit by five intramuscular injections at 2-wk intervals of purified virus emulsified with Freund's complete adjuvant for the first injection and with Freund's incomplete adjuvant for subsequent injections. The rabbit was bled 2 wk after the final injection. The antiserum was absorbed with the extract from healthy *V. faba* leaves before use. The titer of the antiserum was determined by a microprecipitin test. The double antibody sandwich direct enzyme-linked immunosorbent assay (DAS-ELISA) was done by the method of Clark and Adams (1977). Antisera to the isolates of BYMV (Cro-4 and BYMV-G) (Kaneshige *et al.* 1991) and ClYVV (Cal-35) (Inouye *et al.* 1988) were also used for serological tests.

6. *Electron microscopy.*

The crude extract from the infected leaves was stained with either 2% phosphotungstic acid (PTA) (pH 6.5) or 1% ammonium molybdate (pH 7.0) and examined with a Hitachi-7100 electron microscope. For ultramicrotomy, leaf tissue was embedded in Epoxy resin and ultrathin sections were stained with uranyl acetate and lead citrate.

RESULTS

1. *Host range.*

Cal.90-11 isolate infected by mechanical inoculation 29 out of 46 plant species from 9 out of 12 families tested. The virus induced mild chlorosis in *Calanthe* sp. Species infected systemically with symptoms were *Vicia faba* (Plate I-2), *Pisum sativum* (cvs. Sanjuunichi-kinusaya and France), *Phaseolus*

vulgaris cvs. Kintoki (Plate I-3), Top Crop and Hatsumidori, *Astragalus sinicus*, *Cassia occidentalis*, *Medicago sativa* and *Trifolium incarnatum*. On the other hand *Sesamum indicum* cvs. Shiro and Kuro, *Chenopodium amaranticolor*, *Nicotiana benthamiana* and *N. clevelandii* were systemically infected without symptoms.

C. quinoa, *C. murale*, *P. vulgaris* cv. Kurosando and *Tetragonia expansa* developed only local lesions in the inoculated leaves.

Beta vulgaris, *Chrysanthemum coronarium*, *Cucurbita maxima*, *C. moschata*, *C. pepo*, *Glycine max*, *Gomphrena globosa*, *Hyoscyamus niger*, *N. glutinosa*, *N. rustica*, *N. tabacum* (cvs. White Burley, Samsun and Samsun NN), *Spinacea oleracea*, *Vigna radiata* and *Zinnia elegans* were infected locally but showed no symptoms.

The virus failed to infect 16 plant species, *Cucumis sativus*, *C. melo* var. *conomon*, *Citrullus lanatus*, *Petunia hybrida*, *Solanum melongena*, *Lycopersicon esculentum*, *Dature stramonium*, *Brassica campestris* subsp. *rapa*, *Raphanus sativus*, *Trifolium repens*, *T. pratense*, *Vigna angularis*, *V. sinensis* cvs. Kurodane-sanjaku and Akadane-sanjaku, *Dianthus superbus* and *Zea mays*.

2. *Aphid transmission.*

An aphid transmission test was conducted using *Myzus persicae* reared on virus-free *Brassica campestris*. Cal.90-11 was readily transmitted by the aphids in a non-persistent manner from infected *V. faba* to healthy *V. faba*.

3. *Stability of the virus in crude sap.*

In the crude sap of infected *V. faba* leaves, the virus was infective at a dilution of 10^{-4} but not at 10^{-5}. The virus lost infectivity after heating for 10 min at 55°C, and after 16 days at 20°C.

4. *Electron microscopy.*

In leaf-dip preparations from the infected leaves, flexuous filamentous virus particles ca. 750 nm long (Plate I-4) and fragments of cylindrical inclusions were observed. In ultrathin sections of the infected leaves of *V. faba*, cylindrical inclusions with pinwheels and laminated aggregates, and crystalline aggregates (dense body) were observed in the cytoplasm of the infected cells (Plate I-6). The inclusions were very similar in appearance to those induced by BYMV including Cro-4 (Kaneshige *et al.* 1991) an ClYVV (Cal-35) (Kaneshige *et al.* 1991).

5. *Coat protein.*

Coat proteins prepared from purified Cal.90-11 particles migrated as a single band with a molecular weight of 35,000 in SDS-PAGE. The molecular weight of coat protein was similar to those of BYMV (Cro-4, BYMV-G) and ClYVV (Cal-35).

6. *Serology.*

Reciprocal serological reaction tests were conducted among the isolates of Cal.90-11, Cro-4, BYMV-G and Cal-35 using respective antiserum. In a microprecipitin test, Cal.90-11 antigen reacted with antisera to Cal.90-11, Cro-4, BYMV-G and Cal-35 up to a dilution of 1/512, 1/128, 1/32 and 1/8, respectively (Table 1). In an immunoelectron microscopy, all virus particles of Cal.90-11 were positively decorated with antibodies to Cal.90-11 (Plate I -5) and Cro-4 of BYMV, but did not with antibodies to Cal-35 of ClYVV. In DAS-ELISA, similar results were obtained as in microprecipitin tests. In DAS-ELISA using respective antiserum, Cal.90-11 was related more closely to Cro-4 than to BYMV-G, and was distantly related to Cal-35.

Table 1. Homologous and heterologous titers of antisera to Cal.90-11, Cro-4 and BYMV-G isolates of bean yellow mosaic virus, and Cal-35 isolate of clover yellow vein virus in microprecipitin tests

Antigen	Antiserum			
	Cal.90-1	Cro-4	BYMV-G	ClYVV
Cal.90-11	**512**	128	32	9
Cro-4	256	**512**	32	16
BYMV-G	128	64	**512**	32
ClYVV	8	8	8	**512**

DISCUSSION

A potyvirus isolated from *Calanthe* sp. in Japan was distinguished from the three potyviruses, CalMMV (Gara *et al.* 1998), ClYVV (Inouye *et al.* 1988) and TuMV (Matsumoto *et al.* 1993), that have been reported to occur in *Calanthe*. The virus was identified as bean yellow mosaic virus (BYMV) on the basis on host range, symptomatology, particle morphology and serology. BYMV on *Calanthe* had been reported from USA (Hammond and Lawson 1988) and Korea (Chang *et al.* 1991), but the detailed properties of the viruses isolated from *Calanthe* have not been reported. The present virus, Cal.90-11, developed systemic mosaic on *Vicia faba*, *Pisum sativum* and *Phaseolus vulgaris*, but did not produce necrosis on these plants and did not infect *Brassica* or *Raphanus* plants. The host range and symptoms of the virus on some selected plants were similar to those of the ordinary strain of BYMV

(Inouye 1964, Inouye 1968). BYMV induces the cylindrical inclusions consist of pinwheels and laminated aggregates in the cytoplasm of infected cells. According to the classification of Edwardson (Edwardson 1974), the ultrastructure of the inclusions was assigned to be subdivision II of potyvirus. Dense bodies (crystalline cytoplasmic inclusions) similar to those induced by BYMV isolates (Edwardson 1974, Inouye 1973), were also observed in the cytoplasm of infected cells. In a microprecipitin test and DAS-ELISA, Cal. 90-11 reacted with antiserum to the homologous virus and with antisera to Cro-4 and BYMV-G isolates of BYMV, but reacted weakly with antiserum to ClYVV as reported previously (Hollings and Stone 1974, Inouye *et al.* 1988, Kaneshige *et al.* 1991, Leammli 1970). In the serological relationship among the strains of BYMV, Cal.90-11 was closer to Cro-4 than to BYMV-G. Lawson *et al.* (1970) reported that BYMV gladiolus isolate showed a distinct serological relationship to BYMV red clover isolate in a SDS-immunodiffusion test and in DAS-ELISA. Kaneshige *et al.* (1991) reported that BYMV Cro-4 was more closely related to BYMV pea isolate (classified as BYMV-O strain) than to the other BYMV isolates in a microprecipitin test and in DAS-ELISA. In this study, we revealed that the serological relationship between Cro-4 and BYMV-G isolate from Iridaceae was less close, while that between the Cro-4 isolate from Iridaceae and Cal.90-11 isolate from Orchidaceae were more close. Incidence of BYMV on Orchidaceae has been reported on *Calanthe*, *Masdevallia* (Hammond and Lawson 1988) and *Spiranthes* (Chang *et al.* 1991), but in Japan it has been found only on *Calanthe*. Comparison among Cal.90-11 and some isolates of BYMV reported in Orchidaceae, revealed that the host range and ultrastructure of the infected cells induced by Cal.90-11 were similar to those of the *Masdevallia* isolate, but the *Masdevallia* isolate infected neither *P. sativum* nor *Medicago sativa* (Lesemann and Koenig 1985). BYMV isolates from *Calanthe* and *Spiranthes* (Chang *et al.* 1991) in Korea were identified based on host range, particle morphology and immunoelectron microscopy.

As no differences were found among the isolates of BYMV from Orchidaceae (Bos 1970, Inouye 1964, 1968, Kaneshige *et al.* 1991), the source of BYMV occurring in *Calanthe* plants may be BYMV in leguminous plants (Inouye 1964, 1968) or ornamental plants (Bos 1970), and BYMV seems to occur widely in *Calanthe* in Japan.

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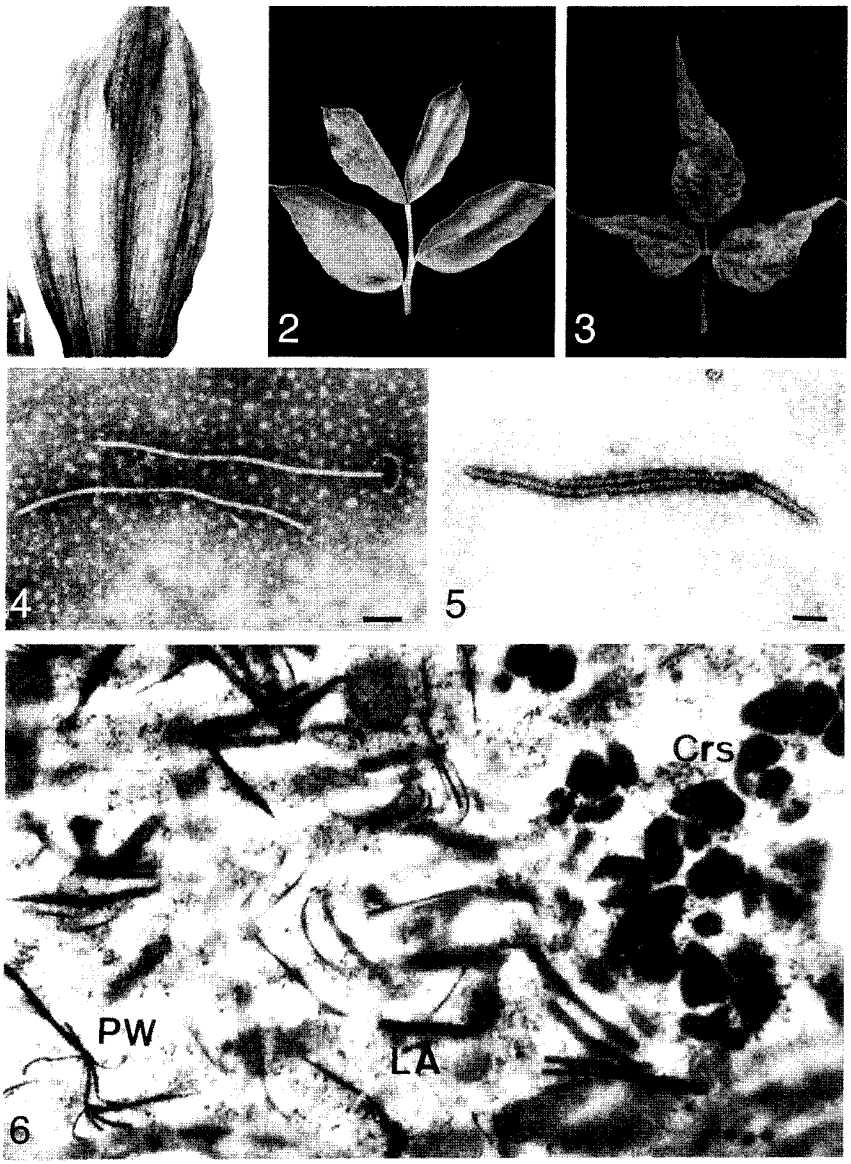
Bean yellow mosaic virus from *Calanthe*

エビネから分離されたインゲンマメ黄斑モザイクウイルスの諸性質

松本純一・前田孚憲・井上成信

山口県で採種した葉にモザイク症状を示すエビネからインゲンマメ黄斑モザイクウイルス（BYMV）が分離された。本ウイルスは汁液接種により試験した12科46種のうち9科29種に感染し，モモアカアブラムシにより非永続伝搬された。本ウイルスの粗汁液中での安定性，粒子の形態，管状封入体及び細胞質内結晶状封入体の形態は既報のBYMVのそれらと同様であった。SDS-PAGEによる外被タンパク質の分子量は35,000であった。微沈降反応及びDAS-ELISAにおいて，本ウイルスはクロッカス及びグラジョラスから分離されたBYMVと血清学的に近縁であったが，グラジョラス分離株よりもクロッカス分離株の方が本ウイルスとより近縁であった。

キーワード：エビネ，インゲンマメ黄斑モザイクウイルス，クローバー葉脈黄化ウイルス，血清学的類縁関係



Explanation of plate

1. Symptoms in *Calanthe* sp. [(*C. sieboldii* × *C. masuca*) × *C. izu-insularis*] naturally infected with BYMV (Cal.90-11) showing mild chlorosis.
2. Systemic mosaic symptoms of *Vicia faba* inoculated with BYMV (Cal.90-11).
3. Systemic mosaic symptoms of *Phaseolus vulgaris* cv. Kintoki inoculated with BYMV Cal.90-11 isolate.
4. Particles of BYMV Cal.90-11 isolate in leaf dip preparation stained with PTA. Scale bar represents 200 nm.
5. Particles of BYMV Cal.90-11 isolates decorated with homologous antiserum at a dilution of 1/200 for 30 min before negative staining. Scale bar represents 200 nm.
6. Thin section of leaf cells of *V. faba* infected with BYMV (Cal.90-11) showing pinwheel (PW) and laminated aggregate-type (LA) inclusions and dense bodies (crystalline cytoplasmic inclusion) (Crs).