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Author(s)	MATSUMOTO, Tsutomu; KODAMA, Tadashi; MURAYAMA, Daiki
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ON THE ALFALFA MOSAIC VIRUS STRAINS OCCURRING ON FORAGE CROPS IN HOKKAIDO

Tsutomu MATSUMOTO*, Tadashi KODAMA**
and Daiki MURAYAMA*

(* Laboratory of Plant Pathology, Faculty of Agriculture
Hokkaido University, Sapporo, JAPAN
** Institute for Plant Virus Research, Ministry of
Agriculture and Forestry, Chiba, JAPAN)

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INTRODUCTION

Alfalfa mosaic virus (AMV) is very widespread and occurs on a relatively wide range of hosts including economic crops such as annual and perennial legumes, potato and tobacco. AMV is rapidly disseminated by aphids (21, 31) and overwinters in the various perennial legumes from which aphids transmit the virus to annual crops in the spring (14). Thus AMV will be a potent pathogen of various legumes and potato which are of particular economic importance for agriculture in Hokkaido. However, no records are available upto the present concerning the occurrence of AMV in this area.

Since its first discovery on alfalfa by WEIMER (39), AMV has been known to occur in various countries in the world (6, 7, 16, 20, 28, 32). In the main land of Japan, the occurrence of AMV was first reported by SYODA *et al.* (35) in 1953 and its properties were described in detail by ASUYAMA *et al.* (1). Thereafter, strains of AMV were reported to occur in crops such as tobacco (9, 31), Ladino clover (12), white clover (37) and soybean (17). Recently a new strain of AMV was isolated by KOMURO *et al.* (18, 19) from potato (cv. Wheeler) which showed the characteristic symptoms similar to that of potato calico disease as described by BLACK and PRICE (5).

The present study deals with the isolation and identification of AMV strains from alfalfa, red clover and Ladino clover in Hokkaido. The preliminary results were reported in the annual meeting of the Phytopathological Society of Japan in 1965 (26).

MATERIALS AND METHODS

A. Source and Maintenance of Previously Described AMV Isolates

The following four isolates which have been described previously were

used for comparative purposes in this study.

Strain AA-1 : furnished by Dr. IIDA and Mr. IIZUKA, Tohoku Agricultural Experiment Station.
Strain AL-9 : ditto
Strain Tobacco B : furnished by Dr. TOMARU, Hatano Tobacco Experiment Station.
Strain ATCC-106 : furnished by Dr. BANCROFT, Purdue University, Indiana, U.S.A.

The subisolate of each strain was made by serial local lesion transfers on bean (cv. Kairyo Otebo) or cowpea (cv. Kurodane Sanjyaku). The subisolates were maintained in desiccated tobacco tissues (cv. Ky-57) at 4°C throughout this study.

B. Isolation and Maintenance of the New Virus Isolates

Isolation of AMV strains was attempted from leguminous forage crops in Hokkaido. Date and place of collection and symptoms on source plants were listed in Table 1. The infected leaves from each source plant were cut into small pieces, desiccated *in vacuo* at 4°C and kept as the virus reservoir.

TABLE 1. The source plant, date and place of collection, and symptom on the source plant.

Source Plant	Date	Place	Symptom	Name of Virus Isolate
alfalfa	June 6, 1964	Tsukisappu, Sapporo	mild mosaic	HN-1
Ladino clover	July 24, 1964	Kotoni, Sapporo	yellow mosaic	HN-2
alfalfa	July 28, 1964	University Farm, Hokkaido University, Sapporo	yellow mosaic	HN-3
alfalfa	do.	do.	mild mosaic	HN-4
red clover	do.	do.	yellow mosaic	HN-5
red clover	do.	do.	mild mosaic	HN-6

For isolation of the virus through serial local lesion transfers, the desiccated tissues were macerated with small amount of 0.01 M phosphate buffer (pH 6.8) and inoculated on the carborundum-dusted primary leaves of bean (cv. Kairyo Otebo). The subisolate derived from each virus source was obtained after serial local lesion transfers on cowpea (cv. Kurodane Sanjyaku). The sub-

isolates were allowed to grow in tobacco (cv. Ky-57) and maintained in a form of desiccated tissue at 4°C throughout this study.

C. Symptomatology and Host Range

Symptomatology and host range of 10 virus isolates were examined on 18 species (9 families) of plants grown in a green-house from June to October. Observation of the symptoms was continued until 30 days after inoculation and if no symptoms appeared, the possible latent infection was judged from the result of back-inoculation to bean. Symptomatology of 10 isolates was compared on each species of test plants at the same time.

D. Properties *in vitro* of the Virus Isolates

1. Tolerance to dilution.

The virus isolates were separately grown in tobacco (cv. Ky-57) for 12 days and the infectious crude extracts were prepared by grinding one g of each infected tissue with 9 ml of 0.01 M phosphate buffer, pH 7.4 (a dilution of 1 : 10). The saps were extracted through two layers of gauze and then subjected to serial dilutions with the same buffer. The infectivity of each diluted extract was assayed by rubbing the extract with a gauze pad on young primary leaves of bean (cv. Kairyo Otebo). The inocula were applied in an incomplete block design using no less than eight replications.

2. Thermal inactivation.

The virus isolates were separately grown in tobacco (cv. Ky-57) for 10 days. The infectious extracts prepared as described above were extracted through two layers of gauze and heated them in thin glass tubes at thermostatically controlled, desired temperatures for 10 minutes. After rapid cooling in ice water, the surviving infectivity of each inoculum was assayed as described above.

3. Aging *in vitro*.

Three virus isolates (HN-1, HN-3 and HN-6) were separately grown in tobacco (cv. Ky-57) for 13 days and the crude extracts were prepared as described above. The extracts were kept at 22°C for various periods of time and the surviving infectivity of each inoculum was assayed as described above.

E. Cross Protection Test

Physalis floridana and tobacco (cv. Ky-57) were used for cross protection tests among the virus isolates. In the case of *P. floridana*, ATCC-106 was used as the primary virus (established virus) which causes mild mosaic and

other isolates as the secondary virus (challenge virus) which causes distinct yellow mosaic along the leaf veines. Reinoculations were made 15 days after the first inoculation. In the case of tobacco (Ky-57), HN-4 showing symptomless infection was used as the primary virus and other virus isolates as the secondary virus which cause distinct mosaic or necrosis. Reinoculations were made 10 days after the first inoculation.

F. Serological Procedures

1. Preparation of the AMV-antiserum.

As previously demonstrated by KODAMA *et al.* (15), a virus isolate AA-1 was specifically reactive with the AMV-antiserum kindly furnished by Dr. BANCROFT which had been prepared by immunizing a rabbit with the purified ATCC-106 preparation (3). AA-1 was purified by the combined method of the chloroform-n-butanol treatment and differential centrifugation essentially as described by BANCROFT and KAESBERG (2). The purified AA-1 preparation was emulsified with an equal volume of "complete" Freund adjuvant. A rabbit received 3 intramuscular injections at one week intervals and then 2 intravenous injections at 3 day intervals. The antiserum obtained showed a titer of 1 : 512 against 0.1 mg/ml of the purified AA-1 preparation in a precipitin ring test (3).

2. Preparation of antigens.

For the precipitin ring test as described by BANCROFT *et al.* (3), the crude extracts were prepared by grinding the tobacco tissues infected with each of the virus isolates for 10 days in the presence of 9 volumes of 0.01 M phosphate buffer (pH 7.4). The saps were extracted through gauze and then centrifuged at 10,000 rpm for 10 minutes. The resultant supernatants were used as the antigens. For the agar-gel diffusion test (29), the crude extracts from tobacco infected for 13 to 28 days were prepared in the presence of a half volume physiological saline, extracted through gauze and used as the antigens.

RESULTS AND DISCUSSION

A. Comparative Host Ranges and Symptomatological Characteristics of the Virus Isolates

The results from studies on comparative host range and symptomatology of 10 virus isolates are summarized in Table 2. Of 18 species tested, the following species were infected with all the virus isolates tested: bean (cv. (Kairyo Otebo; Masterpiece), cowpea (cv. Kurodane Sanjyaku), broad bean

TABLE 2. Comparative host range and symptomatology of the virus isolates.

Virus Isolate / Test Plant	HN-1		HN-2		HN-3		HN-4		HN-5		HN-6		ATCC-106		Tobacco B		AA-1		AL-9	
	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S
Bean (Kairyo Otebo)	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—
Bean (Masterpiece)	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—
Cowpea (Kurodane Sanjyaku)	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—
Broad bean (Wase Soramame)	LL	—	LL	—	LL	Mo N	LL	Mo N	LL	—	LL	Mo N	LL	—	LL	Mo N	LL	Mo N	LL	—
Pea (Alaska)	LL	Lat	LL	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Lat	LL	Lat
Soybean (Shiro Tsurunoko)	VN	Mo VN	VN	Mo VN	VN	Mo VN	VN	Mo VN	VN	Mo VN	VN	Mo VN	Lat	Mo VN	VN	Mo VN	VN	Mo VN	VN	Mo VN
Azuki bean (Wase Dainagon)	Lat	Lat	Lat	Mo	Lat	Mo	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Lat	Mo	Lat	Lat
Tobacco (Ky-57)	N	Mo	N	Mo	Lat	Mo	Lat	Lat	N	Mo N	N	Mo N	N	Mo N	N	Mo N	N	Mo N	N	Mo N
Potato (Norin No. I)	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—
Tomato (Marglobe)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Physalis floridana	Lat	Mo Ma	Lat	Mo Ma	Lat	Mo Ma	Lat	Mo Ma	Lat	Mo Ma	Lat	Mo Ma	Lat	Lat Chl	Lat	Mo Ma	Lat	Mo Ma	Lat	Mo Ma
Cucumber (Kariwa Fushinari)	LL	—	LL	Mo	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	Mo	LL	—
Pumpkin (Houko Kabocha)	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—
Vinca rosea	Lat	Lat	Lat	Lat	Lat	Mo Ma	Lat	Lat	Lat	Mo Ma	Lat	Mo Ma	Lat	Lat	Lat	Mo Ma	Lat	Lat	Lat	Mo Ma
Gomphrena globosa	LL	—	LL	—	LL	—	RS	—	LL	—	LL	—	LL	—	LL	—	LL	—	LL	—
Carrot (Gosun Ninjin)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Aster (Ariake Ponpon)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Radish (Tokinashi)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Corn (Golden Cross Bantum)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

The following abbreviations are used: L=local infection, S=Systemic infection, LL=local lesions, Ma=malformation, Mo=mosaic or mottle, N=necrosis, RS=ringspots, VN=vein necrosis, Chl=chlorosis, Lat=latent infection, (—)=no infection as judged by the back-inoculation to bean.

(cv. Wase Soramame), soybean (cv. Shiro Tsurunoko), pea (cv. Alaska), azuki-bean (cv. Wase Dainagon), tobacco (cv. Ky-57), potato (cv. Norin No. 1), *Physalis floridana*, cucumber (cv. Kariwa Fushinari), pumpkin (cv. Houko Kabocha), *Vinca rosea*, and *Gomphrena globosa*.

Tomato (cv. Marglobe), carrot (cv. Gosun Ninjin), radish (cv. Tokinashi), aster (cv. Ariake Ponpon) and corn (cv. Golden Cross Bantum) are insusceptible to all the virus isolates tested. It has been reported that tomato is susceptible to potato calico strain of AMV (5, 18, 19), to an AMV strain from tobacco (9) and to AMV-425 (8). However, tomato was shown to be insusceptible to all the virus isolates used in this study. Our observation is consistent with those of the reaction of tomato to various AMV strains as previously reported by many workers (4, 27, 34, 36, 38, 41, 42).

The present results indicated that the virus isolates have the host range and symptomatological characteristics similar to those of many of the AMV strains described previously.

B. Properties *in vitro* of the Virus Isolates in Crude Extracts

1. Tolerance to dilution

The tolerance to dilution of 8 virus isolates was examined using bean half-leaves as the local lesion assay system. HN-1, HN-2 and HN-4 withstood a dilution of $1:10^{4.5}$ but not $1:10^{5}$, whereas HN-3, HN-5 and HN-6 withstood a dilution of $1:10^{5}$ but not $1:10^{5.5}$ (Table 3). The dilution experiment with ATCC-106 and Tobacco B also suggests that their dilution end

TABLE 3. Tolerance to dilution of the virus isolates in crude extracts.

Virus Isolate	Dilution of the crude extracts							
	10^{-1}	10^{-2}	10^{-3}	$10^{-3.5}$	10^{-4}	$10^{-4.5}$	10^{-5}	$10^{-5.5}$
HN-2	N	N	1159	402	57	4	0	0
HN-1	N	N	1921	783	84	22	0	0
HN-3	N	N	N	1697	305	101	3	0
HN-5	N	N	1977	1033	541	291	38	0
HN-6	N	N	2169	1460	726	293	25	0
Tobacco B	N	N	N	353	71	—	—	—
HN-4	N	N	609	135	13	12	0	0
ATCC-106	N	N	N	815	317	—	—	—

The number represents the local lesions on 8 half-leaves of bean.
The N designates numerous lesions and the—no tests.

points may be within a range between $1:10^{4.5}$ and $1:10^{5.5}$ (Table 3). Thus all the isolates tested are demonstrated to possess a similar dilution end point value and the value lies almost similar dilution end point of AMV strains reported by Tomaru and Udagawa (38).

2. Thermal inactivation point

The thermal inactivation points of 8 virus isolates were tested using bean half-leaves as the local lesion assay system (Table 4). Isolates HN-2 and HN-4 were comparatively susceptible to heating at 50°C for 10 minutes, and their thermal inactivation threshold was between 55 and 60°C for 10 minutes. HN-3 showed thermal inactivation point within the similar range. The thermal inactivation points of other isolates (HN-1, HN-5, HN-6, Tobacco B and ATCC-106) were within a range between 60 and 65°C. The results indicate that HN-2 and HN-4 are relatively heat-labile as compared with other isolates used in this study.

TABLE 4. Thermal inactivation of the virus isolates.

Virus Isolate	Temperature °C					
	45	50	55	60	65	Control
HN-2	N	391	7	0	0	N
HN-1	N	N	N	15	0	N
HN-3	N	N	422	0	0	N
HN-5	N	N	1386	4	0	N
HN-6	N	N	2058	2	0	N
Tobacco B	N	N	1485	6	0	N
HN-4	N	438	35	0	0	N
ATCC-106	N	N	1820	11	0	N

The number represents total lesion number on 8 half-leaves of bean.
The N designates numerous lesions.
The control: The saps were kept at 4°C.

3. Aging *in vitro*

The aging *in vitro* of HN-1, HN-3 and HN-6 were tested at 22°C (Table 5). HN-1 and other two (HN-3 and HN-6) were noninfectious in the crude extracts after 7 day storage and 6 day storage at 22°C, respectively. Among the various AMV strains, the shortest longevity *in vitro* has been reported for alfalfa yellow spot mosaic strain (12 to 24 hours) (43) and the longest for alfalfa mosaic 2 (9 days) (30), respectively. The longevities of the virus isolates obtained in the present work seem to be slightly longer than the reported range for many AMV strains (3 to 5 days).

TABLE 5. Aging *in vitro* of the virus isolates in crude extracts.

Virus Isolate	Length of aging (days)							
	0	1	2	3	4	5	6	7
HN-1	N	N	2575	711	75	17	2	0
HN-3	N	N	740	513	187	13	0	0
HN-6	N	N	N	3887	112	2	0	0

The number represents total lesion number on 8 half-leaves of bean.
The N designates numerous lesions.

The properties of viruses in the infectious crude extracts such as tolerance to dilution, thermal inactivation point and aging *in vitro* have been considered to be of practical importance for description and classification of plant viruses (33). The results of such tests will largely depend upon the concentration of the infectious virus in the crude sap, environmental conditions and also the sensitivity of the test plants. The standardization of these tests are difficult with AMV because its concentration in source plants greately varies with increasing infection ages (23). AMV is one of the plant viruses whose concentrations in their systemic host plants decreased very rapidly with decreasing specific infectivity (infectivity per unit weight of virus) after they have reached the maximum concentration early in infection ages (23). Thus the dilution end point of AMV will be directly influenced by the infection ages of the source plant and the strict standardization for testing this property is often difficult for this reason. Further, MILBRATH (24) examined the thermal stabilities of 44 isolates of AMV in the crude extracts and reached the conclusion that the difference in the thermal stability among the AMV isolates was the direct reflection of the difference in virus concentration in infectious crude extracts. Our present results also indicated that the AMV isolates showing relatively low virus concentration in the crude sap possessed relatively low thermal inactivation points. Moreover, the aging *in vitro* as tested in the present and previous works is actually dependent upon the initial concentration and inactivation rate of virus in the crude sap (40). Thus special caution was taken to use the virus-infected tissues with the maximum virus content, which is attained about 10 to 14 days after inoculation, in this study. Considering these factors influencing the results of tests on properties *in vitro* of AMV, the AMV isolates tested in the present work were considered to possess properties *in vitro* within the reported ranges for many of the previously described AMV isolates.

C. Interference among the Virus Isolates

The results obtained in the cross protection tests among the AMV isolates were summarized in Table 6. Using *Physalis floridana* as the test plant and ATCC-106 as the primary virus, it was demonstrated that ATCC-106 interfered with AA-1, HN-2, HN-6, and HN-4. Using tobacco (cv. Ky-57) as the test plant and HN-4 as the primary virus, it was observed that HN-4 interfered with AA-1, HN-2, HN-6 and ATCC-106. The results clearly show that ATCC-106 and HN-4 are distinct AMV strains readily distinguished from other isolates used in the present work.

TABLE 6. Interference of the virus isolates

Test Plant	Primary Virus	Secondary Virus	Degree of Interference	Remarks
Physalis floridana	ATCC-106	AA-1	Almost complete	Chlorotic mottle on some plants
	ATCC-106	HN-2	do.	do.
	ATCC-106	HN-6	do.	do.
	ATCC-106	HN-4	do.	do.
Tobacco (Ky-57)	HN-4	AA-1	do.	Mild mosaic on some plants
	HN-4	HN-2	do.	do.
	HN-4	HN-6	do.	Mild chlorosis or mosaic on some plants
	HN-4	ATCC-106	do.	Mild chlorosis on some plants

In a number of previous works on AMV, the bean primary leaves have been used for the cross protection tests in which the AMV strains causing systemic infection on bean are used as the primary virus and the results from reinoculation with the AMV strains producing local lesion on bean are usually quantitative. Tobacco was used as the test plant for cross protection of AMV strain only in a few case (4, 5). *Physalis floridana* was used for the first time as the test plant for cross protection of AMV isolates in the present work, but the results were not quantitative in the present test systems. The recently reported AMV isolates (38) producing systemic infection on bean and cowpea will make it possible to perform quantitative cross protection test with AMV isolates used in the present work.

D. Serological Relation among the Virus Isolates

The results of the precipitin ring tests on the virus isolates were shown

TABLE 7. Serological reaction of the virus isolates with the alfalfa mosaic virus-antiserum (precipitin ring test).

Dilution of Antiserum	Virus Isolate										Extract of Healthy Plant
	HN -1	HN -2	HN -3	HN -4	HN -5	HN -6	ATCC -106	Tobacco B	AA -1	AL -9	
1 : 4	+	+	+	+	+	+	+	+	+	+	−
1 : 8	+	+	+	+	+	+	+	+	+	+	−
1 : 16	+	+	+	+	+	+	+	+	+	+	−
1 : 32	+	+	+	+	+	+	+	+	+	+	−
1 : 64	±	−	+	+	±	−	±	±	+	+	−
1 : 128	−	−	+	−	−	−	−	−	−	±	−
1 : 256	−	−	−	−	−	−	−	−	−	−	−
1 : 512	−	−	−	−	−	−	−	−	−	−	−
1 : 1024	−	−	−	−	−	−	−	−	−	−	−

in Table 7. The positive reactions were obtained at a dilution of the AMV-antiserum of 1 : 32 against HN–1, HN–2, HN–5, HN–6, Tobacco B and ATCC–106. HN–4, AA–1 and AL–9 gave positive reactions at a dilution of the antiserum of 1 : 64 and HN–3 at a dilution of 1 : 128, respectively. The crude extract from healthy plant (sap control) and the buffer alone gave no positive reaction against all the dilutions of antiserum. The agar-gel double diffusion tests showed that only a single precipitin line was formed within 3 to 5 days between the AMV-antiserum and each of the crude extract of tobacco infected with each of 10 virus isolates. No positive precipitin line was formed between the antiserum and the healthy crude extract. The serological tests clearly show that the newly isolated 6 virus isolates are serologically related to AMV.

From the present studies on comparative host range and symptomatology, properties *in vitro*, interference and serological relationship, it is concluded that all the 6 virus isolates from alfalfa, red clover and Ladino clover are strains of AMV. Furthermore, in electron microscopy and sedimentation analysis, the purified preparations of some of these virus isolates revealed the characteristic particles and multicomponent schlieren patterns typical of AMV. (unpublished results).

E. Classification of AMV Isolates on the Basis of Their Symptom Expression on Some Test Plants

The strains of AMV have been classified into two types on the basis of their symptom expression on bean (7, 10, 11) or on cowpea (25). Using both

bean and cowpea as the indicator plants, BANCROFT *et al.* (3) proposed a convenient system for classifying the AMV strains into 4 types. Also IIZUKA and IIDA (13) reported a system for classifying them into 3 types in which bean, cowpea and soybean were used as the indicator plants.

Since the local lesion isolation from bean and cowpea was employed in isolation of the virus, we have studied in this work only the AMV strains similar to those belonging to the type 2 according to the system of BANCROFT *et al.* (3). However, the AMV isolates used in this study were found to express distinguishable symptom from each other on the following 9 indicator plants: soybean, tobacco, *Physalis floridana*, cucumber, *Gomphrena globosa*, broad bean, pea, *Vinca rosea* and azuki bean. The detailed descriptions of symptom expression were as follows.

1. soybean (cv. Shiro Tsurunoko)

Reaction a: Showing necrosis on the inoculated leaves and mosaic or necrosis on the systemically infected leaves (HN-1, HN-2, HN-3, HN-4, HN-5, HN-6, Tobacco B, AA-1, AL-9).

Reaction b: Symptomless infection in the inoculated leaves and mosaic or necrosis on the systemically infected leaves (ATCC-106). ATCC-106 appeared to be similar to pepper strain (4).

2. tobacco (cv. Ky-57)

Reaction a: Showing necrosis on the inoculated leaves and mosaic or necrosis on the systemically infected leaves (HN-1, HN-2, HN-5, HN-6, ATCC-106, AA-1, AL-9, Tobacco B)

Reaction b: Symptomless infection in both inoculated and systemically infected leaves (HN-4).

The isolates causing the reaction a are similar to those previously reported (4, 8, 22, 27, 41, 42, 43). HN-3 is similar to vein necrosis strain (44) which causes symptomless infection in stead of necrosis in the inoculated leaves and mosaic on the systemically infected leaves. There has been no record of the strains similar to HN-4 so far.

3. *Physalis floridana*

Reaction a: Symptomless infection in the inoculated leaves, yellow mosaic in the systemically infected leaves and malformation in the systemically infected top leaf (HN-1, HN-2, HN-3, HN-4 HN-5, HN-6, Tobacco B).

Reaction b: Symptomless infection in both inoculated and systemically infected leaves, but occasionally showing faint chlorosis in the systemically infected leaves (ATCC-106).

4. cucumber (cv. Kariwa Fushinari)

Reaction a: Chlorotic spots on the inoculated leaves and yellow mosaic on the systemically infected leaves (HN-2, AA-1).
Reaction b: Chlorotic spots on the inoculated leaves and no systemic infection (HN-1. HN-3, HN-4, HN-5, HN-6, ATCC-106, Tobacco B, AL-9).

The isolates causing the reaction a on cucumber are similar to pepper strain (4) and those causing the reaction b are similar to yellow-dot virus (36). Cucumber has been reported to be not susceptible to many strains of AMV (5, 22, 34, 42, 43, 44) except for AMV 1 A (41), AMV 1 B (41), tuber necrosis virus (27) and AMV-425 (8).

5. *Gomphrena globosa*

Reaction a: Red lesions on the inoculated leaves and yellow mosaic on the systemically infected leaves (HN-1, HN-2, HN-3, HN-5, HN-6, ATCC-106, Tobacco B, AA-1, AL-9).
Reaction b: Necrotic ringspots on the inoculated leaves and yellow mosaic on the systemically infected leaves (HN-4).

6. broad bean (cv. Wase Soramame).

Reaction a: Showing necrotic lesions on the inoculated leaves and mosaic or necrosis on the systemically infected leaves (HN-3, HN-4, HN-6, Tobacco B, AA-1).
Reaction b: Showing necrotic lesions on the inoculated leaves and no systemic infection (HN-1, HN-2, HN-5, ATCC-106, AL-9).

The reaction a in broad bean was similar to the previously reported reaction obtained with various AMV strains (4, 8, 41, 42, 43, 44) and the reaction b to that with yellow-dot virus strain (36).

7. pea (cv. Alaska)

Reaction a: Showing necrotic spots on the inoculated leaves and symptomless infection in the systemically infected leaves (HN-1, HN-2, AL-9).
Reaction b: Symptomless infection in both inoculated and systemically infected leaves (HN-3, HN-4, HN-5, HN-6, ATCC-106, Tobacco B, AA-1).

The present isolates causing the reaction a in pea are similar to alfalfa mosaic virus 1 (41), alfalfa mosaic virus 1 A (41), alfalfa mosaic virus 1 B (41) and alfalfa yellow mosaic virus (42). Other isolates causing the reaction b in pea are similar to Idaho alfalfa mosaic virus (43) and alfalfa yellow spot mosaic virus (43).

8. *Vinca rosea*

Reaction a: Symptomless infection in the inoculated leaves, and yellow mosaic and malformation in the systemically infected leaves (NH-3, HN-5, HN-6, Tobacco B, AL-9).

Reaction b: Symptomless infection in both inoculated and systemically infected leaves (HN-1, HN-2, HN-4, ATCC-106, AA-1).

9. azuki bean (cv. Wase Dainagon)

Reaction a: Symptomless infection in the inoculated leaves and mosaic symptom on the systemically infected leaves (HN-2, HN-3, AA-1).
Reaction b: Symptomless infection in both inoculated and systemically infected leaves (HN-1, HN-4, HN-5, HN-6, ATCC-106, Tobacco B, AL-9).

Of these 9 species, 5 species (soybean, tobacco, *P. floridana*, cucumber, *G. globosa*) were used for classifying the AMV isolates into 4 subgroups as shown in Table 8. The subgroup I includes HN-2 and AA-1, the subgroup II, HN-1, HN-3, HN-5, HN-6, Tobacco B and AL-9, the subgroup III, HN-4

TABLE 8. Classification of strains of alfalfa mosaic virus and their symptom expression.

Test Plant	Type of Infection	Subgroup I HN-2 AA-1	II HN-1, HN-3, HN-5, HN-6, AL-9, Tobacco B	III HN-4	IV ATCC-106
Soybean (Shiro Tsurunoko)	L	N	N	N	Lat
	S	Mo, N	Mo, N	Mo, N	Mo, N
Tobacco (Ky-57)	L	N	N	Lat	N
	S	Mo, N	Mo, N	Lat	Mo, N
Physalis floridana	L	Lat	Lat	Lat	Lat
	S	yellow Mo	yellow Mo	yellow Mo	Lat or Chl
Cucumber (Kariwa Fushinari)	L	CS	CS	CS	CS
	S	yellow Mo	—	—	—
Gomphrena globosa	L	red LL	red LL	RS	red LL
	S	yellow Mo	yellow Mo	yellow Mo	yellow Mo

The following abbreviations are used: L=local infection, S=systemic infection, N=necrosis, Lat=latent infection, Mo=mosaic, Chl=chlorosis, CS=chlorotic spots, LL=local lesions, RS=ringspot, (—)=no infection as judged by the back-inoculation to bean.

and the subgroup IV, ATCC-106, respectively. The subgroup I is distinguished from the subgroup II only in difference in symptom expression on cucumber. These two subgroups seem to be prevalent in Hokkaido. HN-4 has unique symptomatological characteristics and provide a useful system for cross pro-

tection test for AMV strains. We have isolated some AMV strains from red clover and alfalfa which cause systemic infection in bean or cowpea. However, these were omitted in this paper.

SUMMARY

Six virus isolates were obtained by the local lesion transfer technique as the naturally occurring pathogen of alfalfa, red clover and Ladino clover in Hokkaido. These isolates were designated HN-1, HN-2, HN-3, HN-4, HN-5 and HN-6, and were compared with the previously described alfalfa mosaic virus (AMV) strains (ATCC-106, AA-1, AL-9 and Tobacco B). Comparative host range and symptom expression of these isolates were examined on 18 species (9 families) of test plants. The isolates HN-1, HN-2 and HN-4 withstood a dilution of $1 : 10^{4.5}$, but not $1 : 10^{5}$. The isolates HN-3, HN-5 and HN-6 withstood a dilution of $1 : 10^{5}$, but not $1 : 10^{5.5}$. The thermal inactivation threshold of these virus isolates was between 55° and 65°C. The virus isolates were found to withstand aging *in vitro* at 22°C for 6 to 7 days. The cross protection tests suggested that the virus isolates were strains of AMV. The precipitin ring test and agar-gel double diffusion test showed that all the six virus isolates were serologically related to the previously described AMV strains. Consequently, the virus isolates were identified as AMV. On the basis of symptom expression on 5 different plants (soybean, tobacco, *Physalis floridana*, cucumber and *Gomphrena globosa*), the AMV strains studied in this work were classified into 4 subgroups. This is the first record of natural occurrence of alfalfa mosaic virus in Hokkaido.

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