Biological and Genetic Diversity of *Wheat yellow mosaic virus* (Genus *Bymovirus*)

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Accepted for publication 25 September 2013.

ABSTRACT

Ohki, T., Netsu, O., Kojima, H., Sakai, J., Onuki, M., Maoka, T., Shirako, Y., and Sasaya, T. 2014. Biological and genetic diversity of *Wheat yellow mosaic virus* (genus *Bymovirus*). *Phytopathology* 104:313-319.

The biological and genetic diversity of *Wheat yellow mosaic virus* (WYMV) isolates in Japan was characterized. On the basis of wheat cultivar reactions, 14 WYMV isolates from various places were classified into pathotypes I, II, or III. These were distributed in central, northern, and southern areas of Japan, respectively. WYMV isolates comprised three genotypes (A, A' and B) based on amino acid differences in RNA1 and two genotypes (a and b) based on amino acid differences in RNA2. A

correlation was found between the WYMV RNA1-based genotype and pathotype, suggesting that factors associated with pathogenicity map to RNA1. Genotype Aa and A'a were distributed mainly in the central to southern areas of Japan, and genotype Bb was found in northern areas of Japan, as shown by reverse-transcription polymerase chain reaction restriction fragment length polymorphism analysis. Chinese isolates YA and YZ were closely related to genotypes Bb and Aa, respectively. Wheat was introduced from China to Japan in the 4th and 5th centuries, and the two genotypes of WYMV might also have been introduced with the crop from China and later adapted to local wheat cultivars in Japan.

Wheat yellow mosaic virus (WYMV) causes yellow mosaic and dwarf symptoms on wheat (*Triticum aestivum* L.) and causes yield losses of >30% in wheat in Japan. WYMV was first identified in Japan and has been reported in China and Japan (6,8). A similar yellow mosaic disease on wheat is caused by *Wheat spindle streak mosaic virus* (WSSMV) in North America, Europe, and Africa (10,24,26,27,33,35). These viruses belong to the genus *Bymovirus* in the family *Potyviridae*. The virions are flexuous filaments with two modal lengths (≈550 and 275 nm) and a diameter of 13 nm. They contain two linear, positive-sense, single-strand RNAs (31,32). The complete sequence of WYMV has been determined for Japanese isolate JA (17) and two Chinese isolates, YA and YZ (3). RNA1 is ≈7.7 kb long and encodes a polyprotein that contains the coat protein and seven putative nonstructural proteins: P3, 7K, cylindrical-shaped inclusion protein (CI), 14K, genome-linked viral protein (VPg), protease (Pro), and RNA-dependent RNA polymerase (NIb). RNA2 is ≈3.7 kb long and encodes a polyprotein that contains a 28-kDa protein (P1) and a 73-kDa protein (P2). WYMV and WSSMV are transmitted by the soil-inhabiting *Polymyxa graminis* and have similar host ranges that are restricted to *Hordeum* spp. However, because of the low amino acid sequence identity of their coat proteins, WYMV and WSSMV are classified as distinct *Bymovirus* spp. (4,17,28,36).

Control measures for the yellow mosaic disease caused by WYMV have included cultural practices, chemical treatments,

and introduction of resistant cultivars. However, resting spores of *P. graminis*, which can carry and transmit the virus, can remain viable in the soil for a long period, and recommended cultural practices such as crop rotations and late fall plantings of wheat seed do not adequately control the disease (2). Chemical fumigation of the soil can be effective but is not a realistic option because the fungicide is too expensive for wheat production.

Thus, introducing resistant cultivars is considered the most effective method to control the disease. The development of WYMV-resistant cultivars requires ongoing analysis for resistance genes in wheat. Thus far, the dominant resistance genes *YmYF*, *Ymlb*, and *YmMD* have been mapped on the long arm of chromosome 2D of Chinese wheat 'Yangfu 9311', European wheat 'Ibis', and American wheat 'Madsen', respectively (14,20,30). In an analysis of microsatellite (simple-sequence repeat) markers these resistance genes were found to be closely related but no evidence was provided as to whether any of these three genes are the same. The quantitative trait locus associated with resistance to WYMV, *QYm.njau-5A.1*, was mapped on the long arm of chromosome 5A of 'Xifeng' wheat, and DNA markers that closely link with *QYm.njau-5A.1* were found (38). In addition to these dominant resistance genes, three or four recessive genes also seem to be involved in WYMV resistance (7). With the use of these DNA markers linked to WYMV-resistance genes, the development of the new WYMV-resistance cultivars should be accelerated.

In contrast to advances in resistance against WYMV, analysis of the viral factors and the mechanism involved in virulence of WYMV have not progressed as much. Pathological differences among WYMV isolates have been reported in Japan and China (3,12,22). To help elucidate the viral factors involved in the virulence of WYMV isolates, Chen et al. (3) compared genomic sequences among WYMV isolates that induced different reactions

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*The e-Xtra logo stands for "electronic extra" and indicates that the online version contain three supplemental tables.

on various wheat cultivars but viral factors remain to be identified. For effective progress in breeding resistant cultivars, it is very important to clarify the interaction between wheat resistance genes and viral factors.

In this study, we examined the biological and genetic diversity of WYMV in Japan to advance our understanding of population structure and obtain useful information for wheat breeding programs.

MATERIALS AND METHODS

Sampling and assay for WYMV pathotype. To investigate host reactions and genomic sequences of WYMV, we collected leaves of one wheat tiller with yellow mosaic symptoms from 14 locations among 10 prefectures in Japan during 2009 and 2010 (Table 1; Fig. 1) and stored them at -80°C until use. WYMV has no local lesion host plants, and a single lesion isolation experiment could not be conducted. To confirm whether more than one WYMV was present in the wheat samples, we preliminarily sequenced the partial coat protein region of WYMV from three to five wheat samples from each field of the 14 locations in Japan. Because mixed-wave peaks were barely observable by direct sequencing, we assumed that the wheat samples did not include more than one WYMV and considered them to be WYMV isolates.

The pathotypes of 14 isolates were determined based on the reactions of ‘Nambukomugi’, ‘Fukuhokomugi’, and ‘Hokkai 240’ wheat (Table 1) after mechanical inoculation of leaves dusted with carborundum and inoculum prepared by grinding leaves in 50 mM phosphate buffer (pH 7.0). After plants were held in the dark for 5 to 7 days at 5°C, plants were maintained in a screen-house for 1 to 2 months in the winter. Infection with WYMV was confirmed by symptom expression and double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) (18).

Total RNA extraction, cDNA synthesis, sequencing, and phylogenetic analysis. Leaf samples were ground in 2-ml microfuge tubes with a sterile 4-mm stainless steel grinding ball and powdered by adding liquid nitrogen and shaking at 15,000 rpm for 5 min with a Multi-Beads Shocker (Yasui Kikai Corp., Osaka, Japan). Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized using SuperScript III (Invitrogen, Carlsbad, CA) and a random 6-mer primer according to the manufacturer’s recommendations. In all, ≈0.2 µg of total RNA and 100 ng of primer were denatured at 65°C for 5 min and incubated on ice for 2 min. After adding reagents, the reactions were incubated at room temperature for 5 min, 50°C for 1 h, and 70°C for 10 min, then incubated at 37°C

for 20 min with 40 U of RNase H (Takara Bio, Shiga, Japan). The mixtures were diluted 1:2.5 water for experimental use. The coding regions of the 14 isolates were amplified by KOD plus polymerase (Toyobo, Osaka, Japan). The primers for amplification and sequencing were designed based on nucleotide sequences of the JA isolate (accessions D86634 and D86635). The 5’ and 3’ untranslated regions (UTRs) of four isolates (Chitose, Morioka, Tsukuba, and Yanagawa) were determined. The 5’ UTR was amplified by rapid amplification of cDNA ends methods according to Weng and Xiong (34). The 3’ UTR was amplified using the natural poly(A) tail. The polymerase chain reaction (PCR) products were treated with 2.5 U of exonuclease I (Takara Bio) and 0.25 U of shrimp alkaline phosphatase (Takara Bio) at 37°C for 20 min and 80°C for 30 min, then cleaned with a QIAquick PCR Purification Kit (Qiagen). The PCR products were directly sequenced using a BigDye Terminator v1.1 Cycle-Sequencing Kit (Applied Biosystems, Foster City, CA) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The nucleotide sequences of full-length or coding regions from the 14 WYMV isolates were registered in the DDBJ database (RNA1 accessions: AB627806 to AB627819 and RNA2: AB627821 to AB627834, respectively). The nucleotide and amino acid sequences of isolates JA, YA (AJ239039 and AJ242490), and YZ (AJ131981 and

Fig. 1. Distribution in Japan of pathotypes of Wheat yellow mosaic virus used in this study. Pathotypes I, II, and III are distributed in central, northern, and southern areas of Japan, respectively.

TABLE 1. Pathotypes of Japanese isolates of Wheat yellow mosaic virus (WYMV) isolates determined using three wheat cultivars in this study

Isolate name	Prefecture of origin	Cultivar (number of infected/total inoculated)			Pathotype ^a
		Nambukomugi	Fukuhokomugi	Hokkai240	
Kitami	Hokkaido	8/21	0/25	0/24	II
Chitose	Hokkaido	26/47	0/42	0/44	II
Date	Hokkaido	5/23	0/32	1/16 ^b	II
Kyowa	Hokkaido	24/44	0/41	2/35 ^b	II
Morioka	Iwate	31/43	0/42	0/41	II
Tome	Miyagi	13/20	0/22	0/23	II
Utsunomiya	Tochigi	30/44	13/43	0/41	I
Tsukuba	Ibaraki	27/40	18/43	0/42	I
Mutsuzawa	Chiba	18/44	27/44	0/44	I
Matsumoto	Nagano	8/23	13/23	0/16	I
Suzuka	Mie	3/23	10/22	0/20	I
Yamaguchi	Yamaguchi	10/45	32/44	0/41	I
Yanagawa	Fukuoka	9/29	7/18	10/20	III
Yamato	Fukuoka	6/25	6/20	4/11	III

^a I = systemic infection on Nambukomugi and Fukuhokomugi but not on Hokkai 240, II = systemic infection only on Nambukomugi, and III = systemic infection on all three cultivars.

^b WYMV infected but did not cause symptoms.

AJ131982) were obtained from the database. The identities among WYMV isolates were calculated with GENETYX software (Genetyx Corp., Tokyo). For phylogenetic analysis, the coding regions were aligned with ClustalX software ver. 2.0 (13) then a phylogenetic tree was constructed using the same software and the neighbor-joining method (bootstrap = 1,000 replicates).

Restriction fragment length polymorphism. For reverse-transcription (RT)-PCR restriction fragment length polymorphism (RFLP), three primer pairs were designed based on the alignment of 14 isolates of WYMV (Supplemental Table 1). On the basis of amino acid sequences specific to genotypes of WYMV, restriction enzymes *NheI*, *Aor51HI*, and *TaqI* (Takara Bio) were selected. Digestion of PCR products with WR-F1/-R1, WR-F2/-R2, and WR-F3/-R3 indicated genotype A or A' for RNA1, genotype A' for RNA1, and genotype a for RNA2, respectively. The cDNA was synthesized in the same way as for the sequencing analysis, and PCR was performed using KOD dash polymerase (Toyobo). The 25 µl of mixture was prepared with 1× KOD dash buffer, 0.2 mM dNTPs, 0.2 µM each primer, 0.625 U of KOD dash, and 0.5 µl of the cDNA reactions. The amplification conditions were 30 cycles of 94°C for 20 s, 55°C for 15 s, and at 74°C for 20 s. For digestion by the restriction enzymes, 10 µl of mixture was prepared with 2.5 µl of PCR reaction, 1× enzyme buffer, 0.01% bovine serum albumen, and 2 U of a restriction enzyme. The mixtures were incubated at 37°C for 2 h with *NheI* and *Aor51HI* or at 65°C for 2 h with *TaqI*, and the products were separated on a 2% agarose gel in 1× Tris-acetate-EDTA buffer at 100 V for 30 min and visualized with ethidium bromide. Some PCR products that were not digested with proper restriction enzymes were cloned by a TOPO TA Cloning kit (Invitrogen), and the sequence confirmed as mentioned already.

RESULTS

Distribution of WYMV pathotypes in Japan. On the basis of the reaction of Nambukomugi, Fukuhokomugi, and Hokkai 240 wheat, the 14 isolates of WYMV from various places in Japan were grouped into pathotypes I, II, or III (Table 1). Isolates from Utsunomiya, Tsukuba, Mutsuzawa, Matsumoto, Suzuka, and Yamaguchi prefectures were classified as pathotype I, which causes a systemic infection on Nambukomugi and Fukuhokomugi but not on Hokkai 240. The Kitami, Chitose, Date, Kyowa,

Morioka, and Tome isolates were classified as pathotype II, which causes a systemic infection only on Nambukomugi. Two isolates, Yanagawa and Yamato, were classified as pathotype III, which caused infection on all three wheat cultivars. Data showed a geographical component to the distribution of the three pathotypes (Fig. 1). WYMV isolates of pathotype II were found in northern areas of Japan, pathotype I in the central areas of Japan, and pathotype III in southern areas of Japan.

The JA isolate of WYMV was previously found to be pathotype I (22). Although Chinese isolates YA and YZ were reported to differ in their host reactions (3), we could not presume the pathotypes of the two Chinese isolates because the wheat cultivars used for determination of the pathotypes by Chen et al. (3) differed from ours.

Comparison of genomic sequence. The nucleotide sequence of the entire genome of 4 isolates of WYMV (Chitose, Morioka, Tsukuba, and Yanagawa) and the coding regions of the 10 other isolates were determined and compared with those of JA, YA, and YZ isolates previously reported. The coding regions on RNA1 of 17 isolates, including JA, YA, and YZ, consisted of 7,215 nucleotides (nt) and encoded a polyprotein of 2,404 amino acids. The coding region of 14 isolates of WYMV on RNA2 comprised 2,712 nt and 903 amino acids, 1 amino acid shorter than those of isolates JA, YA, and YZ. The coding region for RNA1 and RNA2 among 17 WYMV isolates shared high amino acid identities (>96.2 and 93.1% respectively) (Supplemental Table 2). The 5' UTR of four isolates was 162 nt long in RNA1 and 172 nt in RNA2. The 3' UTR was 258 nt long in RNA1 and 767 nt long in RNA2, except for the polyA tail. The 5' and 3' UTR shared identities of >90.1 and 97.3% in RNA1 and 89.9 and 98.7% in RNA2, respectively (Supplemental Table 3).

Comparison of amino acids among isolates. On the basis of amino acid differences in the coding region of RNA1 and RNA2, WYMV isolates were divided into several genetic variants (Tables 2 and 3). WYMV grouped into genotypes A, A', and B based on RNA1 amino acid composition (Table 2). Genotypes A and B differed in 13 characteristic amino acids, mainly located within the CI, VPg, Pro, and Nib regions. The sequence of genotype A' was similar to that of genotype A, with only four amino acid differences (Table 2); A' is likely to have derived from A. The phylogenetic analysis using nucleotide sequences of the coding regions of WYMV RNA1 also confirmed a close relationship

TABLE 2. Characteristic amino acids at various positions correlated with the genotype of *Wheat yellow mosaic virus* in RNA1^a

			Position of amino acid															
			P3	CI									14K	VPg	Pro		NIb	
Isolate	Pathotype ^b	Genotype ^c	228 ^d	397	418	421	443	655	679 ^d	820	1017	1040	1095	1318	1468	1540	1587	1828
Kitami	II	B	H	N	D	A	L	R	N	V	V	T	L	K	I	S	I	K
Chitose	II	B	H	N	D	A	L	R	N	V	V	T	L	K	I	S	I	K
Date	II	B	H	N	D	A	L	R	N	V	V	T	L	K	I	S	I	K
Kyowa	II	B	H	N	D	A	L	R	N	V	V	T	L	K	I	S	I	K
Morioka	II	B	H	N	D	A	L	R	N	V	V	T	L	K	I	S	I	K
Tome	II	B	H	N	D	A	L	R	N	V	V	T	L	K	I	S	I	K
Utsunomiya	I	A	H	N	E	T	I	K	S	I	V	I	F	T	L	T	T	R
Tsukuba	I	A	H	N	E	T	I	K	S	I	V	I	F	T	L	T	T	R
Mutsuzawa	I	A	H	N	E	T	I	K	S	I	V	I	F	T	L	T	T	R
Matsumoto	I	A	H	N	E	T	I	K	S	I	V	I	F	T	L	T	T	R
Suzuka	I	A	H	N	E	T	I	K	S	I	V	I	F	T	L	T	T	R
Yamaguchi	I	A	H	N	E	T	I	K	S	I	V	I	F	T	L	T	T	R
Yanagawa	III	A'	R	G	E	T	I	K	S	I	L	I	F	K	L	T	T	R
Yamato	III	A'	R	G	E	T	I	K	S	I	L	I	F	K	L	T	T	R
JA	I	A	H	N	E	T	I	K	S	I	V	I	F	T	L	T	T	R
YA	n.d.	B	H	H	D	A	L	R	N	V	V	T	L	T	L	S	I	K
YZ	n.d.	A	H	N	E	T	I	K	S	I	V	I	L	T	L	T	T	K

^a CI, cylindrical-shaped inclusion body protein; VPg, viral protein linked to the genome; Pro, protease; Nib, RNA-dependent RNA polymerase. Entries in bold have characteristic amino acids correlated with genotype B and those in italics have A'.

^b Abbreviation: n.d. = not determined.

^c Sequences from Tsukuba, Morioka and Yanagawa isolates are named genotype A, B, and A', respectively.

^d Amino acid targeted in restriction fragment length polymorphism analysis.

between genotypes A and A' (Fig. 2). Fourteen WYMV isolates were divided into two clades; all six isolates in genotype B belonged to one clade, and six isolates in genotype A and two isolates in genotype A' belonged to the other clade. With regard to a correlation between genotype and pathotype, genotype A, B, and A' on RNA1 corresponded to pathotype I, II, and III, respectively.

WYMV isolates grouped into genotypes a and b based on RNA2 amino acid composition (Table 3). Twelve amino acids, mainly located on P2, were different between both genotypes. Genotype a and b on RNA2 generally linked to pathotype I and II, respectively. However, the RNA2 of the Matsumoto isolate indicated that pathotype I belongs to genotype b. In addition, no amino acid substitutions related to pathotype III were found on

RNA2. Therefore, these results strongly suggested that WYMV RNA1 rather than RNA2 contains the viral factors associated with the pathotype.

Of 14 isolates, 7 were either genotype Aa or A'a, 6 were Bb, and only 1 was the reassortant genotype Ab. Interestingly, the Chinese isolates YA and YZ of WYMV also agreed well with the amino acid specificity for genotypes (Tables 2 and 3); isolate YA isolate was close to genotype Bb and YZ was close to genotype Aa.

Analysis of genotype by RT-PCR-RFLP. To examine the distribution of each genotype in Japan, we designed an RT-PCR-RFLP to characterize each genotype. Genotypes A, A', and B on RNA1 were assessed by the digestion pattern of PCR products obtained with primer pair WR-F1/-R1 and *Nhe*I and primers WR-

Fig. 2. Unrooted phylogenetic analysis based on nucleotide sequences of RNA1 of 14 isolates of *Wheat yellow mosaic virus* in Japan. Unrooted phylogenetic tree was constructed using the neighbor-joining (NJ) method (bootstrap = 1,000 replicates). Only bootstrap values >700 are shown. Bar indicates phylogenetic distance.

Fig. 3. Determination of genotype using reverse-transcription polymerase chain reaction restriction fragment length polymorphism. Bands **A**, amplified with primer WR-F1/-R1 and digested with *Nhe*I; **B**, amplified with WR-F2/-R2 and digested with *Aor*51HI; and **C**, amplified with WR-F3/-R3 and digested with *Taq*I. Lane M, 100-bp DNA ladder (Toyobo); and wheat samples from lane 1, Kitami; lane 2, Date; lane 3, Utsunomiya; lane 4, Matsumoto; lane 5, Yanagawa; and lane 6, Yamato.

TABLE 3. Characteristic amino acids at various positions correlated with the genotype of *Wheat yellow mosaic virus* in RNA2^a

Isolate	Pathotype ^b	Genotype ^c	Position of amino acid											
			P1	P2										
			51	261 ^d	267	274	305	315	358	359	364	369	474	509
Kitami	II	b	G	Q	F	T	Y	N	H	S	S	I	N	F
Chitose	II	b	G	Q	F	T	Y	N	H	S	S	I	N	F
Date	II	b	G	Q	F	T	Y	N	H	S	S	I	N	F
Kyowa	II	b	G	Q	F	T	Y	N	H	S	S	I	N	F
Morioka	II	b	G	Q	F	T	Y	N	H	S	S	I	N	F
Tome	II	b	G	Q	F	T	Y	N	H	S	S	I	N	F
Utsunomiya	I	a	S	R	S	A	R	G	Y	N	P	V	S	I
Tsukuba	I	a	S	R	S	A	R	G	Y	N	P	V	S	I
Mutsuzawa	I	a	S	R	S	A	R	G	Y	N	P	V	S	I
Matsumoto	I	b	G	Q	F	T	Y	N	H	S	S	I	N	F
Suzuka	I	a	S	R	S	A	R	G	Y	N	P	V	S	I
Yamaguchi	I	a	S	R	S	A	R	G	Y	N	P	V	S	I
Yanagawa	III	a	S	R	S	A	R	G	Y	N	P	V	S	I
Yamato	III	a	S	R	S	A	R	G	Y	N	P	V	S	I
JA	I	a	S	R	S	A	R	G	Y	N	P	V	S	I
YA	n.d.	b	G	Q	F	T	Y	N	N	S	S	I	N	F
YZ	n.d.	a	S	R	S	A	R	G	Y	N	P	V	S	F

^a Entries in bold have characteristic amino acids correlated with genotype b.

^b Abbreviation: n.d. = not determined.

^c Sequences from Tsukuba and Morioka are named genotype a and b.

^d Amino acid targeted in restriction fragment length polymorphism analysis.

F2/-R2 and *Aor51HI*; and genotypes a and b on RNA2 using primers WR-F3/-R3 and *TaqI*. Representative RFLP patterns of WYMV isolates from six places are shown in Figure 3. Wheat samples from Kitami, Date, Utsunomiya, and Matsumoto indicated genotypes Bb, Bb, Aa, and Ab, respectively. Wheat samples from Yanagawa and Yamato correspond to a mixed infection of genotypes Aa and A'a on RNA1 (Fig. 3, lanes 5 and 6). Isolates from Yanagawa and Yamato had mixed infections of genotypes A and A' but they were classified into pathotype III because wheat samples dually infected with genotypes Aa and A'a, would yield results for pathotype III.

More WYMV isolates from various areas in Japan were characterized by RT-PCR-RFLP (Table 4). Samples from northern areas of Japan (Kitami, Chitose, Date, Kyowa, and Morioka) were all genotype Bb. In samples from Tome, genotype Bb was generally detected as well, although a few samples had Ab genotypes. In the central areas of Japan, except for Matsumoto, almost all samples were genotype Aa. The virus with reassortant genotype Ab seemed to be widely distributed in Matsumoto and less so in Suzuka. In the southern areas (Yamaguchi, Yanagawa, and

Yamato), genotypes Aa and A'a were detected in the same fields. Genotype Ba was not found in the present study. In summary, WYMV genotypes Aa/A'a and Bb were the primary genotypes in Japan, and WYMV of the reassortant genotype was mainly observed in Matsumoto.

DISCUSSION

Here, we characterized the genetic diversity and pathogenicity of WYMV isolates in Japan. Cultivar inoculation tests showed three WYMV pathotypes. This result is consistent with those of Ohto et al. (22). When genomic sequences of WYMV were grouped into genotypes based on their characteristic amino acids (Tables 2 and 3) and compared with the pathotypes, the genotype based on RNA1 coincided with each pathotype. Therefore, differences of pathogenicity map to amino acids encoded by WYMV RNA1.

WYMV isolates of pathotype II do not usually infect Hokkai240 wheat systemically. However, the Date and Kyowa isolates did systemically infect Hokkai240 (Table 1). There is a possibility that WYMV isolates of pathotypes II and III had co-infected the

TABLE 4. Analysis of genotype of *Wheat yellow mosaic virus* RNA1 and RNA2 by reverse-transcription polymerase chain reaction restriction fragment length polymorphism

Site	Field number	Cultivar	Number of samples	Genotype ^a					Summary
				RNA1			RNA2		
				A	B	A'	a	b	
Kitami	...	Hokushin	5	0	5	0	0	5	Bb
Chitose	...	Hokushin	4	0	4	0	0	4	Bb
Date	...	Hokushin	5	0	5	0	0	5	Bb
Kyowa	...	Hokushin	5	0	5	0	0	5	Bb
Morioka	1	Nambu ^b	3	0	3	0	0	3	Bb
	2	Nambu	4	0	4	0	0	4	Bb
Tome	1	Unknown	5	1	5	0	0	5	Ab, Bb
	2	Unknown	4	0	4	0	0	4	Bb
	3	Unknown	4	1	4	0	0	4	Ab, Bb
	4	Unknown	4	1	4	0	0	4	Ab, Bb
	5	Unknown	3	1	2	0	0	3	Ab, Bb
	6	Unknown	4	0	4	0	0	4	Bb
Tsukuba	1	Nourin61	1	1	0	0	1	0	Aa
	2	Nourin61	3	3	0	0	3	0	Aa
	3	Nourin61	3	3	0	0	3	0	Aa
	4	Nourin61	4	4	0	0	4	0	Aa
	5	Nourin61	3	3	0	0	3	0	Aa
Mutsuzawa	1	Nourin61	3	3	0	0	3	0	Aa
	2	Nourin61	3	3	0	0	3	0	Aa
	3	Nourin61	1	1	0	0	1	0	Aa
	4	Nourin61	4	4	0	0	4	0	Aa
Matsumoto	1	Shirane	4	4	0	0	0	4	Ab
	2	Shirane	4	4	0	0	0	4	Ab
	3	Shirane	4	4	0	0	1	3	Aa, Ab
	4	Shirane	4	4	0	0	0	4	Ab
	5	Shirane	4	4	0	0	0	4	Ab
	6	Shirane	4	4	0	1	0	4	Ab, A'b
Suzuka	1	Nourin61	4	4	0	0	4	0	Aa
	2	Nourin61	4	4	0	0	3	2	Aa, Ab
	3	Nourin61	4	4	0	0	4	0	Aa
	4	Nishinokaori	4	4	0	0	4	0	Aa
	5	Nourin61	4	4	0	0	4	0	Aa
	6	Tamaizumi	4	4	0	0	4	0	Aa
Yamaguchi	1	Nourin61	5	5	0	0	5	0	Aa
	2	Nourin61	3	3	0	0	3	0	Aa
	3	Nourin61	4	4	0	3	4	0	Aa, A'a
	4	Nourin61	4	4	0	4	4	0	Aa, A'a
	5	Nourin61	4	4	0	4	4	0	Aa, A'a
	6	Nourin61	3	3	0	3	3	0	Aa, A'a
Yanagawa	1	Toukai102	4	4	0	2	4	0	Aa, A'a
	2	Toukai102	3	3	0	3	3	0	Aa, A'a
	3	Toukai102	4	4	0	3	4	0	Aa, A'a
	4	Toukai102	2	2	0	2	2	0	Aa, A'a

^a Co-infection by two genotypes was detected in some samples.

^b Nambu, Nambukomugi; Shirane, Shiranekomugi.

samples but the infection rate and the absorbance values of ELISA were very low (data not shown). In addition, the isolates did not systemically infect Fukuhokomugi and did not induce any symptoms on Hokkai240, thus clearly differing from pathotype III. RFLP analysis of samples from Date and Kyowa described in this article also negated the possibility of co-infection in their samples.

Compared with other potyviruses, genetic diversity of WYMV was low. WYMV isolates collected in this experiment shared nucleotide and amino acid identities of >97.3 and 98.0% for RNA1 and 95.8 and 96.1% for RNA2, respectively. In contrast, nucleotide identities of Japanese isolates of *Bean yellow mosaic virus* (BYMV) were >75.8% (16). One of reasons for this low diversity may be the restricted host range of WYMV. The natural host of WYMV is limited to wheat but BYMV causes severe diseases in many leguminous and ornamental plants. When the nucleotide sequences of *Turnip mosaic virus* (TuMV) were compared after propagating in the host of origin and in a different host, the nucleotide diversity and the nonsynonymous/synonymous ratio of TuMV on the different host were higher than those on the original host (21). *Barley yellow mosaic virus* (BaYMV), which belongs to the same genus, *Bymovirus*, and only infects barley naturally, also showed low genetic divergence and shared an amino acid identity greater than 97.1% on RNA1 and 95.0% on RNA2 for Japanese isolates (19).

Differences in RNA1 amino acid composition were mainly found in CI, VPg, Pro, and NIb (Table 2). Chen et al. (3) also reported variable parts in the CI- and NIb-coding regions of RNA1 among JA, YZ, and YA isolates of WYMV. The CI protein is a structural protein of cytoplasmic cylindrical inclusion bodies formed during infection that is assumed to possess helicase activities (17). VPg, a genome-linked viral protein that attaches to the 5' terminus of genomic RNAs, interacts with the host translation machinery (23). Pro and NIb are assumed to be a protease involved in polyprotein processing and an RNA-dependent RNA polymerase, respectively. Reverse genetic experiments identified the C terminus of gene CI as the elicitor of extreme resistance or hypersensitive reaction for *Lettuce mosaic virus*, *Soybean mosaic virus*, and TuMV (1,5,25). A single amino acid substitution in the C terminus enabled the virus to overcome resistance. In barley, barley eukaryotic translation initiation factor 4E (Hv-eIF4E) was identified as one of the recessive resistance genes to BaYMV and *Barley mild mosaic virus* (29), and the VPg was involved in virulence determination (9,11). There is no direct information on the viral factors that are involved in virulence and determination of WYMV pathotypes. However, a reverse genetic system for the genus *Bymovirus* was recently developed (37); therefore, future experiments should help determine which genomic regions of WYMV are involved in virulence and pathogenicity.

The distribution of the three pathotypes (that is, the distribution of the three WYMV RNA1 genotypes) may be partially due to the cultivars grown in local areas of Japan. Cultivars resistant to WYMV pathotype II, such as 'Nourin 61' and Fukuhokomugi, have been traditionally cultivated in central to southern areas of Japan. Therefore, pathotype II may have been excluded while pathotype I prevailed in these areas. The preferred use of certain cultivars might also explain why WYMV pathotype III would be derived from pathotype I. By cultivation of cultivars resistant to pathotype I, such as 'Shiroganekomugi', in the southern areas, a certain pathotype I virus might mutate and gain the ability to overcome cultivar resistance. In our phylogenetic analysis using nucleotide sequences of the coding region of WYMV RNA1, WYMV isolates in genotypes A (pathotype I) and A' (pathotype III) clustered in the same clade (Fig. 2). In addition, RFLP analysis showed that genotypes A and A' occurred together in the same area of Japan. The prevalence of pathotype II in the northern areas of Japan is not known for certain. However, our preliminary experiments indicate that the resistance of pathotype I can change

depending on the temperature. Nambukomugi wheat that is cultivated mainly in northern Japan was susceptible to pathotypes I and II in our experiment (Table 1). However, the cultivar showed resistance to pathotype I at a relatively high temperature (13 to 15°C) and was susceptible at a relatively low temperature. Thus, the temperature-sensitivity of the cultivar might influence the prevalence of pathotype II in the northern areas.

RT-PCR-RFLP was useful in examining the distribution of WYMV genotypes. Genotypes Aa and Bb of WYMV are mainly distributed in Japan and reassortant isolates such as genotype Ab are infrequent (Table 4). One reason may be that WYMVs with genotypes Aa or Bb were separately introduced to Japan. Wheat is thought to have originated in the Mideast and Near East ≈7,000 B.C.E. and been transported to China ≈2,000 B.C.E. It was then introduced to Japan in the 4th to 5th centuries through two routes, directly from southeast China or via the Korean Peninsula, and spread throughout Japan (15). The characteristic amino acids of each genotype were correlated with those of either isolates YA or YZ. Isolate YZ is closely related to genotype Aa, found in central Japan, and YA isolate is closely related to genotype Bb in northern Japan (Tables 2 and 3). The occurrence of WYMV is limited to Japan and China and has not spread to other areas. These results lead us to predict that two genotypes of WYMV, which originated in China, were separately introduced to Japan from China, adapted to local cultivars, and thus became established in Japan.

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