Biological, Serological, and Molecular Variabilities of Clover Yellow Vein Virus

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ABSTRACT

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A comparative study was made on the host reactions, serological properties, and nucleotide sequences of the coat protein (CP) gene of 10 clover yellow vein virus (CIYVV) isolates and one bean yellow mosaic virus (BYMV) isolate collected from different host plant species and locations in Japan. Two strains of CIYVV isolates, grouped on the basis of host reactions on *Chenopodium amaranticolor*, *C. quinoa*, *Nicotiana clevelandii*, *N. benthamiana*, *Vicia faba*, and *Trifolium repens*, corresponded to two serotypes determined by double-antibody sandwich- and triple-antibody sandwich-enzyme-linked immunosorbent assay using three polyclonal and nine monoclonal antibodies. These results were also con-

firmed by nucleotide sequence analysis of the CP gene. The CP gene of CIYVV isolates of strain 1, including the Australian isolate CIYVV-B, had 93 to 98% nucleotide identities and 97 to 99.6% amino acid identities. The CP of CIYVV isolates of strain 2, including the New Zealand isolate CIYVV-NZ, had 92 to 98% nucleotide identities and 95 to 98% amino acid identities. The nucleotide identities and the amino acid identities between the two CIYVV strains were 82 to 84%, and 90 to 94%, respectively. When compared with the CP sequences of 12 CIYVV isolates, the CP sequence of the BYMV isolate had 71 to 73% nucleotide identity and 73 to 77% amino acid identity. Amino acid sequence differences among CIYVV isolates from strains 1 and 2 were located mostly at the N-terminal regions of the CP. Our results indicated that the CIYVV isolates studied could be separated into two strains on the basis of host reactions, serology, and the nucleotide sequence of the CP gene.

Clover yellow vein virus (CIYVV), first isolated from white clover by Hollings and Nariani (16) in England, is distributed worldwide (3,10,17,20,21) and causes severe diseases in many kinds of leguminous and ornamental plants (9,10,19,27). CIYVV is a member of the bean yellow mosaic subgroup of the genus *Potyvirus*, which includes bean yellow mosaic virus (BYMV), pea mosaic virus, sweetpea mosaic virus, and white lupin mosaic virus (3, 13,24). The taxonomy of bean yellow mosaic subgroup viruses has been controversial, because their properties are very similar to one another. Some researchers considered CIYVV a strain of BYMV because (i) they have overlapping host ranges; (ii) they produce morphologically similar pinwheel cytoplasmic inclusions; and (iii) antisera against CIYVV strongly interact with BYMV and vice versa (3,4,21). The confusion regarding their taxonomic relations was resolved by comparing structural information of the coat protein (CP) gene and the 3'-noncoding region (3'-NCR) of the viral RNA. Nucleotide sequences of the CP gene of several CIYVV and BYMV isolates have been published (6,7,12,28,34,35), and the sequences fell into distinct clusters, indicating that CIYVV and BYMV are distinct *Potyvirus* species (35).

In Japan, a virus from leguminous plants originally described as a necrotic strain of BYMV (BYMV-N) (20) is now considered to be CIYVV (9), and CIYVV was also described from *Calanthe* sp. (19). BYMV-N is, however, different from other CIYVV isolates in host reactions and serological properties (20,27). These variabilities among CIYVV isolates and their relatedness to BYMV

have made it difficult to develop reliable diagnostic techniques for CIYVV. The determination of variability within CIYVV and the understanding of mechanisms and factors affecting this variability are also of considerable agronomic significance, particularly for developing resistance gene deployment strategies.

In this study, 10 CIYVV isolates and one BYMV isolate, collected from different hosts and locations in Japan, were compared by host reactions, serological properties, and the sequences of the CP gene in order to shed light on the variability of CIYVV. Our results clearly showed that the Japanese CIYVV isolates were classified into two strains by host reactions, serological properties, and the sequences of the CP gene.

MATERIALS AND METHODS

Virus isolates and host reaction. Isolates of the viruses studied and their original hosts are shown in Table 1. Each virus isolate was sap-inoculated mechanically to at least 12 plants of each species tested in the greenhouse at 22 to 25°C. All plants not showing symptoms were tested 3 to 4 weeks after inoculation by back-inoculation to *Chenopodium amaranticolor*.

Production of monoclonal antibodies (MAbs). Three 5-week-old male BALB/c mice were immunized by intraperitoneal injection of 100 µg of purified virions of CIYVV-90-1 or CIYVV-92-3 emulsified in Freund's complete adjuvant and were boosted 27 days later by an intravenous injection of the same amount of the purified virions in saline. At day 30, the spleen was removed and 0.9×10^8 cells were fused with the myeloma partner PAI using polyethylene glycol 4000. Selected hybridoma cells were cloned and injected into the peritoneal cavity of BALB/c mice for bulk production of MAbs (14).

Enzyme-linked immunosorbent assay (ELISA). Double-antibody sandwich (DAS)-ELISA tests with polyclonal antisera to CIYVV-90-1, CIYVV-92-3, and BYMV-90-2 (26,27), and triple-

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DDJB (DNA Data Base of Japan) accession numbers for CIYVV-MB3, -92-3, -N, -189-1, -90-1, -94-6, -P180, and BYMV-90-2 are from D89538 to D89545.

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antibody sandwich (TAS)-ELISA tests with monoclonal antisera to CIYVV-90-1 and CIYVV-92-3 were used in this experiment. Experiments were conducted with crude extracts of virus-infected *Nicotiana benthamiana* or *Vicia faba* plants ground 1/100 (wt/vol) in phosphate-buffered saline-Tween (PBS-T) or with purified virus preparations at a final concentration of 1.0 µg/ml in the same buffer. A negative control (extract from noninfected *N. benthamiana* plants) was added on each plate. The MABs (tissue culture supernatant diluted 1:10² or ascitic fluids diluted 1:10⁴) and anti-mouse immunoglobulin G conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis) diluted 1:5 × 10³ were incubated for 1 h at 25°C. Substrate was incubated for 1 h at 25°C, and an absorbance value at 405 nm of the reaction was measured.

Sequencing of CIYVV-90-1, CIYVV-92-3, and BYMV-90-2. CIYVV-90-1, CIYVV-92-3, and BYMV-90-2 were purified from infected broad bean leaves, and the viral RNAs were extracted as described by Uyeda et al. (35). Viral cDNAs were synthesized and unidirectionally ligated to Uni-ZAP XR lambda phage vector (Stratagene Inc., La Jolla, CA) at the *EcoRI/XhoI* site using a ZAP-cDNA synthesis kit (Stratagene Inc.) according to the manufacturer's instructions. Recombinant phage DNAs were encapsidated by a Gigapack Gold packaging extract (Stratagene Inc.) and amplified in *Escherichia coli* strain XL1-Blue MRF' according to the instructions provided by Stratagene Inc. Insert-bearing clones were identified by immunoscreening (18) using the antiserum to CIYVV-90-1 or BYMV-90-2. The selected lambda recombinants were converted to phagemids by in vivo excision according to the manufacturer's instructions (Stratagene Inc.). The clone CY9013 for CIYVV-90-1, the clone CY9233 for CIYVV-92-3, and the

clone BY9021 for BYMV-90-2 were used for nucleotide sequencing. The plasmids were unidirectionally deleted by *ExoIII*/mungbean-nuclease treatment (Promega Corp., Madison, WI). The nucleotide sequence was performed in both directions by the dideoxynucleotide chain termination procedure of Sanger et al. (25) using an Applied Biosystems 373A DNA sequencer (Perkin-Elmer Cetus, Foster City, CA). Multiple alignments of sequences were carried out using the program GENETYX-MAC (Software Development, Tokyo).

Sequencing of CIYVV-MB3, -N, -I89-1, -94-6, and -P180. Total nucleic acid extraction, reverse transcription, and polymerase chain reaction (PCR) were done as described (28). CIYVV-specific primers (5' CY-I: 5'-CGGGATCCTCAGAAGTCGCGTTGAAGAG-3' corresponding to nucleotides 1 to 20 [28]; and 3' CY-I: 5'-GCGGATCCGCTATAATAGTAAACTTCG-3' corresponding to nucleotides 976 to 996 [28], *Bam*HI site is underlined) were used to amplify the complete CP gene by PCR. The PCR was carried out using the following protocol: initial denaturation at 95°C for 3 min, and then 35 cycles of annealing (60°C for 1 min), primer extension (72°C for 1.5 min), and denaturation (94°C for 1 min). Amplified DNA fragments were separated by electrophoresis in an agarose gel and isolated from the gel using the Prep-A-Gene DNA purification system (Bio-Rad Laboratories, Richmond, CA). After digestion with *Bam*HI, the fragments were ligated to *Bam*HI-digested pBluescript II KS(-) (Stratagene Inc.) using the Takara ligation kit (Takara, Tokyo) and transformed into *E. coli* strain XL1-Blue. Recombinant plasmids were identified by restriction enzyme analysis, and nucleotide sequencing was performed by the dideoxynucleotide chain termination procedure (25).

TABLE 1. The origin and host reactions of clover yellow vein virus (CIYVV) and bean yellow mosaic virus (BYMV) isolates in Japan

Isolate	Original host	Symptoms on					
		<i>Nicotiana benthamiana</i>	<i>Nicotiana clevelandii</i>	<i>Chenopodium amaranticolor</i>	<i>Chenopodium quinoa</i>	<i>Vicia faba</i>	<i>Trifolium repens</i>
CIYVV- 90-1	<i>Vicia faba</i>	CS/Mo,N ^a	CS/Mo,NS	NS/Mo,Mal,N	NS/Mo,Mal,N	NS/M,N,LN	l/Mo
MB3	<i>Vicia faba</i>	CS,NS/Mo,N	(CS)/Mo	NS/Mo,Mal,N	NS/Mo,Mal,N	NS/M,N,LN	l/Mo,NS
No30	<i>Phaseolus vulgaris</i>	l/Mo,NS	CS/Mo	NS/Mo,Mal,N	NS/Mo,Mal,N	NS/M,N,LN	l/(Mo)
NFU	<i>Phaseolus vulgaris</i>	CS/Mo,N	(CS)/(Mo)	NS/Mo,Mal,N	NS/Mo,Mal,N	NS/M,N,LN	l/Mo
N	<i>Phaseolus vulgaris</i>	CS/CS,NS	CS/CS	NS/Mo,Mal,N	NS/Mo,Mal,N	NS/M,N,LN	l/Mo
NC	<i>Gentiana sp.</i>	CS,NS/Mo,N	CS/(Mo)	NS/Mo,Mal,N	NS/Mo,Mal,N	NS/M,N,LN	l/Mo
I89-1	<i>Impatiens sultani</i>	CS,NS/Mo,N	CS/(Mo)	NS/Mo,Mal,N	NS/Mo,Mal,N	NS/M,N,LN	l/Mo
92-3	<i>Vicia faba</i>	CS/CS	CS/-	NS/-	NS/-	NS/Mo,NS	-/-
94-6	<i>Vicia faba</i>	CS/CS	(CS)/-	NS/-	NS/-	NS/Mo,NS	-/-
P180	<i>Pisum sativum</i>	CS/CS	CS/-	NS/-	NS/-	NS/Mo,NS	-/-
BYMV- 90-2	<i>Vicia faba</i>	l/-	l/-	CS/Mo	CS/-	l/Mo	-/-

^a Symptoms on inoculated leaves are to the left of the slash and symptoms in systemically infected leaves are to the right of the slash. Symptom abbreviations are -, no infection; l, latent (symptomless) infection; CS, chlorotic spots; (CS), weak chlorotic spots; LN, lethal necrosis; Mal, malformation; Mo, mottle or mosaic; (Mo), weak mottle or mosaic; N, necrosis; and NS, necrotic spots.

TABLE 2. The serological variability of clover yellow vein virus (CIYVV) and bean yellow mosaic virus (BYMV) isolates in Japan as determined by double-antibody sandwich-enzyme-linked immunosorbent assay (ELISA) and triple-antibody sandwich-ELISA

Isolates	Monoclonal antibody to								
	Polyclonal antiserum to			CIYVV-90-1				CIYVV-92-3	
	CIYVV-90-1	CIYVV-92-3	BYMV-90-2	1C8	1A2 ^a	3G9	5F2	5D6	3H8 ^b
CIYVV- 90-1	+++ ^c	++	++	+++	+++	+++	++	+	-
MB3	+++	++	++	+++	+++	+++	++	+	-
No30	+++	++	++	+++	+++	+++	++	+	-
NFU	+++	++	++	+++	+++	+++	++	-	-
N	+++	++	++	+++	+++	+++	++	+	-
NC	+++	++	++	+++	+++	+++	++	-	-
I89-1	+++	++	++	+++	+++	+++	++	-	-
92-3	+	+++	-	-	+++	-	+++	+	+++
94-6	++	+++	-	-	+++	-	++	+	+++
P180	+	+++	-	-	+++	-	++	+	+++
BYMV- 90-2	+	+	+++	-	-	++	++	-	-

^a Results obtained with an additional monoclonal antibody, 2H8, were identical.

^b Results obtained with two additional monoclonal antibodies, 2D6 and 6F8, were identical.

^c Serological reactivity, measured as the absorbance at 405 nm (A) after 1 h of substrate incubation: -, A < 0.05; +, 0.05 < A < 0.5; ++, 0.5 < A < 1.5; and +++, A > 1.5.

RESULTS

Biological variability. While significant biological variabilities were observed among CIYVV isolates from Japan, the 10 CIYVV isolates studied here were separated into two subsets (subset 1: 90-1, MB3, No30, NFU, N, NC, and I89-1; and subset 2: 92-3, 94-6, and P180) by the reactions of *C. amaranticolor*, *C. quinoa*,

N. benthamiana, *N. clevelandii*, *V. faba*, and *T. repens* (Table 1). CIYVV isolates in subset 1 induced systemic symptoms on *C. amaranticolor*, *C. quinoa*, and *N. clevelandii*. Those in subset 2 incited only local infection in these plants. The isolates in subset 1 induced systemic mosaic and necrotic symptoms on *N. benthamiana*, while those in subset 2 produced systemic chlorotic spots on this plant. On *V. faba*, the isolates in subset 1 induced systemic

CIYVV-	90-1	SDKEKLNAGE	QQKSKDKESR	QRDQEGENSN	RQIIPDRDIN	AGTTGTFSVP	KLKKISGKLS	LPTIRGKGLL
	MB3			S.....				..K.K.....
	No30	V..						..K.K.....
	NFU	...D.....		S.....				..K.K.....
	N	S.....	P.					..K.K.....
	NC	D.	...L.....					..K.K.....
	I89-1							..K.K.....
	B		...F...P.					..K.K.....
	92-3	·G·Q·	...P·DP·	·E·S·A·	K·		T·	..K·KE·
	94-6	·G·Q·	...P·DP·	·E·S·A·	K·	·S·	R·	..K·KE·
	P180	·G·KQ·	...P·DP·	·E·S·A·	K·		T·	..K·KE·
	NZ	·G·Q·	...PR·DPK	·E·P·A·	K·		R·	..K·K·
BYMV-	90-2	·Q·	KK·D·DEN	PSKSDGQ·S	·V·V·	T·V·	R·A·N	I·K·G·IV·
			^					
			RV					
CIYVV-	90-1	NLDHLLVYVP	NQDDISNNIA	TQEQLAWHE	GVKNAYEVDD	QQMEIICNGL	MVWCIENGTS	GDLQGEWTMM
	MB3							
	No30				E.			
	NFU							
	N	·G·			N.			..K.K.....
	NC							
	I89-1			..K.				
	B				...S.			
	92-3	L.			S.			
	94-6	L.			S·S.			
	P180	I·	L.		S.	I.		
	NZ	L.						
BYMV-	90-2	D·N.	P·	·A·F·YN	·Q·E.	P·G·L.		
								140
CIYVV-	90-1	DGEKQVTFPL	KPILDFAKPT	LRQIMAHFSQ	AAESYIEFRN	STERYMPRYG	LQRNLADYGL	ARYAFDFYRL
	MB3						...T.	
	No30				K.		...T.	
	NFU						...T.	
	N	·D·					...T.	
	NC						...T.	
	I89-1	·N.					...T.	
	B						...PT.	
	92-3	·D·	·V.			A.	...T.	
	94-6	·D·	·V.			G.	...T.	
	P180	·D·	·V.	F.	D.	A.	...T.	G.
	NZ	·D·	·V.		C.		...T.	
BYMV-	90-2	·E·Y.	·N.	·F·S.	·V·A·K.	A.	...T.	
								210
CIYVV-	90-1	TSKTPARARE	AHMQMKAAT	RGKSNHMEGL	DGNVGTDEEN	TERHTANDVN	RNMHHIAGAR	F*
	MB3							..
	No30							..
	NFU							..
	N							..
	NC						K.	
	I89-1							..
	B							..
	92-3	T.						..
	94-6	T.		I.				..
	P180	T.						..
	NZ	T.						..
BYMV-	90-2	V.	V.	RL.		G.	·D·TML·V.	I.
								271

Fig. 1. Alignment of deduced coat protein amino acid sequences of clover yellow vein virus (CIYVV) and bean yellow mosaic virus (BYMV) isolates from Japan, Australia, and New Zealand. The sequences of CIYVV-No30 (35), NFU (28), NC (28), B (34), and NZ (7) have been published. Amino acids of each isolate that differ from those of CIYVV-90-1 are indicated.

mosaic and necrosis and a final lethal infection, while those in subset 2 induced systemic mosaic and necrotic spots on this plant. The isolates in subset 2 did not infect *T. repens*, which is an indicator plant for CIYVV and BYMV. Typically, CIYVV, but not BYMV, is capable of systemically infecting *T. repens* (2,4,5,19).

Serological variability. The CIYVV and BYMV isolates in Japan were characterized serologically by DAS-ELISA using polyclonal antisera to CIYVV-90-1, CIYVV-92-3, and BYMV-90-2 (Table 2). All CIYVV and BYMV isolates reacted with the antisera to CIYVV-90-1 and CIYVV-92-3, but the isolates in subset 2 did not react with the antiserum to BYMV-90-2. Although all CIYVV and BYMV isolates reacted with the antisera to CIYVV-90-1 and CIYVV-92-3, the reactivities among CIYVV and BYMV isolates were not identical. The isolates in subset 1 reacted more strongly with the antiserum to CIYVV-90-1 than did the isolates in subset 2 and BYMV-90-2. In contrast, the isolates in subset 2 reacted more strongly with the antiserum to CIYVV-92-3 than did the isolates in subset 1, and BYMV-90-2 reacted weakly with the antiserum to CIYVV-92-3.

To obtain more information about the serological differences between the isolates in subsets 1 and 2, TAS-ELISA was performed using five MAb to CIYVV-90-1 and four MAb to CIYVV-92-3 (Table 2). TAS-ELISA using MAb revealed differential reactivities among CIYVV and BYMV isolates, and none of these MAb reacted with several other potyviruses such as pea seedborne mosaic virus, watermelon mosaic virus 2, lettuce mosaic virus, papaya ringspot virus W-type, and potato virus Y (data not shown). One MAb (MAb-1C8) reacted only with the isolates in subset 1. In contrast, three MAb (MAb-3H8, MAb-2D6, and MAb-6F8) reacted only with the isolates in subset 2. Two MAb (MA-1A2 and MAb-2H8) reacted with the isolates in both subsets, but did not react with BYMV-90-2. Although MAb-5D6 reacted with some CIYVV isolates in subset 1 and all CIYVV isolates in subset 2, their absorbance values were very low (Table 2). MAb-3G9 reacted with the isolates in subset 1 and BYMV-90-2, and MAb-5F2 reacted with all CIYVV and BYMV isolates.

Nucleotide sequence of the 3'-terminal region of CIYVV-90-1, CIYVV-92-3, and BYMV-90-2. The complete sequences of clones CY9013 (CIYVV-90-1), CY9233 (CIYVV-92-3), and BY9021 (BYMV-90-2) were determined (data not shown). These sequences included part of the nuclear inclusion b gene, the complete CP gene, and the entire 3'-NCR. The 3'-NCR of both CIYVV-90-1 and CIYVV-92-3 were 175 nucleotides long, while that of BYMV-90-2 was 172 nucleotides. The 3'-NCR of CIYVV-92-3 had 94 and 73% nucleotide identity with the 3'-NCR of CIYVV-90-1 and BYMV-90-2, respectively.

Comparison of the CP gene of CIYVV and BYMV isolates. Synthetic primers 5' CY-I and 3' CY-I were used to amplify the complete CP genes of five CIYVV isolates (MB3, N, I89-1, 94-6,

and P180), and up to three clones of each isolate were sequenced (data not shown). The nucleotide sequences and the deduced amino acid sequences of these five CP genes were compared with the published data of the same regions of three other Japanese isolates, No30, NC, and NFU (28,35), the Australian isolate CIYVV-B (34), and the New Zealand isolate CIYVV-NZ (7) (Fig. 1 and Table 3). The CP of 12 CIYVV isolates were identical in size (271 amino acids), whereas the CP of BYMV-90-2 was 273 amino acids, two amino acid residues longer. The CP of the isolates in subset 1, including CIYVV-B, showed 93 to 98% nucleotide identity and 97 to 99.6% amino acid identity, whereas the CP of the isolates in subset 2, including CIYVV-NZ, showed 92 to 98% nucleotide identity and 95 to 98% amino acid identity. In contrast, the CP among the isolates in different subsets showed 82 to 84% nucleotide identity and 90 to 94% amino acid identity, and most of the nucleotide differences between subset 1 and subset 2 occurred in wobble bases (data not shown). However, the CP of BYMV-90-2 showed 71 to 73% nucleotide identity and 73 to 77% amino acid identity when compared with 12 CIYVV isolates.

When the amino acid sequences of the CP among CIYVV isolates including CIYVV-B and CIYVV-NZ were compared, most of the differences were located in the N-terminal 31 amino acids, which is well-known to be the most variable region of the potyvirus CP (31,32) (Fig. 1 and Table 4). Among the isolates in the same subset, the N-terminal region of the CP appeared highly conserved (the amino acid identity was 87 to 100%), but, among the isolates in different subsets, the N-terminal region of the CP appeared diversified (the amino acid identity was 61 to 74%). In contrast with the divergence of the N-terminal region, the C-terminal regions of these 12 CIYVV isolates were highly conserved.

DISCUSSION

Taxonomy of the bean yellow mosaic subgroup of the genus *Potyvirus* has been confusing, because the viruses in this subgroup have many properties in common including host reactions and serological reactions (3,4,21). For some time, CIYVV was considered a strain or a serotype of BYMV, because of an overlapping host range and strong serological interrelationships between CIYVV and BYMV (3,4,20,21). In contrast, some CIYVV isolates have different biological and serological properties. Inouye (20) pointed out that two isolates of BYMV-N (later identified as CIYVV) have differences in pathogenicity. In our preliminary experiments, polyclonal antiserum to CIYVV-90-1 did not react with one of Inouye's BYMV-N isolates (P180) in sodium dodecyl sulfate agar double-diffusion tests (27).

In the current study, CIYVV isolates in Japan, collected from different hosts and locations, were largely classified into two subsets by host reactions and serological properties. Typical CIYVV

TABLE 3. Percent identities among coat protein nucleotide and amino acid sequences of clover yellow vein virus (CIYVV) and bean yellow mosaic virus (BYMV) isolates^a

Isolates	CIYVV-												BYMV-
	90-1	MB3	No30	NFU	N	NC	I89-1	B	92-3	94-6	P180	NZ	90-2
CIYVV- 90-1	...	97	96	96	96	96	96	93	82	82	83	83	72
MB3	99	...	98	98	96	97	96	94	84	84	84	83	72
No30	98	99	...	97	96	97	95	94	83	83	83	83	72
NFU	98	99.6	98	...	95	98	97	95	84	84	83	83	72
N	97	98	97	97	...	96	97	94	84	84	84	83	72
NC	98	99	98	98	97	...	96	94	84	83	83	82	73
I89-1	98	99	98	99	98	98	...	94	84	83	84	83	72
B	97	98	97	98	97	98	98	...	83	83	83	82	72
92-3	93	94	93	94	93	93	93	93	...	98	98	94	72
94-6	92	93	92	93	92	92	92	93	98	...	96	94	72
P180	90	92	90	92	91	91	91	91	98	96	...	92	71
NZ	93	94	93	93	93	93	93	93	97	97	95	...	72
BYMV- 90-2	77	77	77	77	76	77	77	77	76	75	73	76	...

^a Identities are presented for a coat protein amino acid sequence (below the diagonal) and for a coat protein nucleotide sequence (above the diagonal).

infects *C. quinoa*, *N. clevelandii*, and *T. repens* systemically, and these plants were used for the indicator host plants of CIYVV (2,4,5,17,19). CIYVV isolates in subset 1 systemically infected these plants, but those in subset 2 incited local infection or no infection on these plants. The host reactions incited by the isolates in subset 2 were closer to those of the BYMV isolate than to those of the isolates in subset 1. In contrast with the similarity of the host reaction between the isolates in subset 2 and the BYMV isolate, those in subset 2 were serologically closer to the isolates in subset 1 than to BYMV. The polyclonal antiserum to BYMV-90-2 reacted moderately with the isolates in subset 1 and did not react with those in subset 2.

When the nucleotide and amino acid sequences of the CP gene were compared, CIYVV isolates in Japan also could be divided into two subsets, and the molecular variations of the CP gene were well correlated with biological and serological variabilities of CIYVV. CP sequence comparisons are widely accepted as the molecular basis of distinction between different *Potyvirus* species, and it is well established that CP amino acid sequence identities range from 38 to 70% among different *Potyvirus* species, and 90 to 99% among strains of the same *Potyvirus* species (11,31–33). The amino acid identities between the CP of viruses in subsets 1 and 2 are above the threshold of 90% identity, indicating that the viruses in subset 2 are isolates of CIYVV and not a different *Potyvirus* species. As mentioned above, the identities of CP amino acid sequences among different *Potyvirus* species so far reported are less than 70%. That between CIYVV and BYMV, however, was 73 to 77%. Therefore, differences between these two viruses are smaller than those between other *Potyvirus* species.

We propose that the two subsets of Japanese CIYVV isolates described here actually comprise two strains, according to Barnett's definition (1). The existence of different strains of CIYVV has been reported not only in Japan, but also in other countries. Barnett et al. (3) reported that CIYVV isolates from Australia were closely related to each other and distantly related to the Canadian isolate (CIYVV-Pratt) on the basis of molecular hybridization using random cDNA probes to the whole CIYVV genome and ELISA using polyclonal antibodies to BYMV and CIYVV. High-performance liquid chromatography peptide profiles have also been used to show that the CP sequences of CIYVV isolates from the United States and Australia were very similar to each other and more distant to the Canadian isolate (23). Host range and nucleotide sequence comparisons of the isolates of strain 1 and the Australian isolate CIYVV-B (34) indicated that CIYVV-B may be considered a member of strain 1, although its serological properties have not been compared with those of the Japanese CIYVV isolates. Although the nucleotide sequence identity between the New Zealand isolate CIYVV-NZ (7) and the isolates of strain 2 indicated that CIYVV-NZ should be considered as strain 2, host range of CIYVV-NZ differed from that of the isolates of strain 2. In fact, the CIYVV-NZ isolate possesses

a host range and serological properties similar to those of CIYVV-Pratt (10). Thus, the tentative classification of the Japanese CIYVV isolates reported in this paper cannot be applied directly to the New Zealand and Canadian isolates. More detailed experiments are needed to clarify the relationships among CIYVV isolates of strain 2, the New Zealand isolate, and the Canadian isolate.

Although it is now well established that strains of the same *Potyvirus* species have above 90% amino acid identity in the CP region, no clear sequence thresholds exist to delineate given isolates as distinct strains of a virus in support of biological or serological evidence. One approach to circumvent this problem was to compare the N-terminal region of the CP (39). Xiao et al. (38) reported that comparison of the N-terminal region of seven sugarcane mosaic virus strains revealed five different sequence patterns, and these could be grouped into three subsets that correlated with the host ranges of the strains. In this study, the amino acid variability of the CIYVV CP was particularly evident in the N-terminal region of the CP. The amino acid identities of the N-terminal region among CIYVV isolates of each strain ranged from 87 to 100%, whereas a range of 61 to 74% identity was found between CIYVV isolates from different strains. Similar divergences have been reported in the N-terminal region between different biological strains of bean common mosaic virus (22), plum pox virus (8,37), and potato virus Y (36). These results strongly suggest that divergence in the N-terminal region may provide a reliable criterion to establish *Potyvirus* strain relationships, and that distinct potyvirus strains may be identified by some degree of the divergence in the N-terminal region.

The existence of different types of CIYVV has sometimes led to difficulty in classifying these isolates with respect to their strain designations, and hampered the development of reliable diagnostic techniques of CIYVV. Our results revealed the serological variabilities of CIYVV isolates in Japan and demonstrated that MABs can distinguish CIYVV from other bean yellow mosaic subgroup viruses (i.e., BYMV), and bean yellow mosaic subgroup viruses (CIYVV and BYMV) from several other potyviruses. MAB-1A2 and MAB-2H8 reacted with all CIYVV isolates tested, but did not react with BYMV and other potyviruses, indicating that there is at least one epitope unique to CIYVV. MABs 1C8, 3H8, 2D6, and 6F8 were both strain-specific and CIYVV-specific. MAB-5F2 defined an epitope common to both the CIYVV and BYMV isolates used in this study. This confirms the close relationship of CIYVV with BYMV (3,4,21), but also showed that there is a serological difference of at least three epitopes in the CP of CIYVV and BYMV isolates.

It is well-known that virus-specific epitopes are generally located in the variable N-terminal region of potyviral CPs (15,29,30). Hewish et al. (15) reported that epitopes for CIYVV-specific MABs were located in the N-terminal region of the CIYVV CP, whereas epitopes for potyvirus cross-reactive MABs were located

TABLE 4. Percent identities among amino acid sequences in the hypervariable region (residues 1 to 31) of the coat protein of clover yellow vein virus (CIYVV) and bean yellow mosaic virus (BYMV) isolates

Isolates	CIYVV-												BYMV-
	90-1	MB3	No30	NFU	N	NC	I89-1	B	92-3	94-6	P180	NZ	90-2
CIYVV- 90-1	...	97	97	94	94	94	100	94	71	71	68	65	45
MB3		...	94	97	90	90	97	90	74	74	71	65	45
No30			...	90	90	90	97	90	68	68	65	61	42
NFU				...	87	87	94	87	71	71	71	61	42
N					...	87	94	94	71	71	68	65	42
NC						...	94	90	68	68	65	61	42
I89-1							...	94	71	71	68	65	45
B								...	74	74	71	74	45
92-3									...	100	97	90	42
94-6										...	97	90	42
P180											...	87	39
NZ												...	39
BYMV- 90-2													...

in the core region of the CP. Although we made no attempt to localize the epitopes recognized by our strain-specific MAbs, the variation in N-terminal sequences of the CPs of our CIYVV isolates is consistent with a possible N-terminal location for the strain-specific epitopes.

In conclusion, CIYVV isolates in Japan could be grouped into two strains after comparison of their biological and serological properties. These results were also confirmed by nucleotide sequence analysis of the CP gene. Amino acid sequence differences among CIYVV isolates of different strains were located mostly at the N-terminal regions of the CP.

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