A New Technique to Select Mild Strains of *Cucumber mosaic virus*

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ABSTRACT

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An efficient technique to select a good attenuated virus to control *Cucumber mosaic virus* (CMV) disease was developed. Preliminary screenings were conducted to assess the virulence of virus recovered from dark-green islands and yellow tissues of mosaic leaves of *Nicotiana rustica* after co-inoculation with an attenuated mutant P2bR46C of CMV and its original severe isolate Pepo. All single-lesion isolates (SLIs) obtained from dark-green islands had the attenuated P2bR46C phenotype, but the SLIs from yellow tissue had either the virulent Pepo or the P2bR46C phenotype. When Pepo-infected *N. rustica* and tomato plants were grown at 15 or 36°C for 30 days, 17 of 288 SLIs obtained from the treated leaves elicited mosaic and dark-green spots without malformation. Dark-green tissue from each plant infected with 1 of these 17 SLIs then was used to inoculate one plant of *N. rustica*. All 17 plants had either very mild mosaic or no visible symptoms. One of these potential mild strains, 36R37, had an amino acid substitution on the 2b gene encoding the 2b protein. Isolate 36R37 also was highly cross-protective, and its symptom attenuation was stable for three serial host passages. After cold or heat treatment, the dark-green tissue proved to be a good source for isolating mild strains of the virus.

Additional keywords: cross-protection, temperature treatment

Pre-infection with one virus can prevent symptom expression on plants after subsequent infection with closely related strains of the same virus. Known as cross-protection (22), this ability has been harnessed to control virus diseases in several crops. However, the selection and production of mild strains for this control strategy has been dependent upon various empirical methods (15). Thus, this time-consuming process for obtaining stable mild strains has been a major factor prohibiting our progress in using mild strains to control viral diseases. Development of an effective selection method should further our progress in finding mild strain candidates.

Cucumber mosaic virus (CMV) is the type member of the *Cucumovirus* group, which contains a tripartite single-stranded and plus-sense RNA genome and infects

more than 1,000 plant species (21). CMV encodes at least five proteins. The 2b protein encoded on the RNA 4A, subgenomic RNA from the RNA 2 (3), is a determinant of virulence and a suppressor of RNA silencing (2,4,8,27).

CMV causes severe diseases and economic losses in cereal, fruit, vegetable, and ornamental plants worldwide (10,21). Therefore, it is important to obtain useful mild strains of CMV for cross-protecting these crops. In this study, we describe an efficient method to select mild strains of CMV.

MATERIALS AND METHODS

Viruses. Two isolates of CMV, an attenuated point mutant P2bR46C (T. Kobori, Y. Kosaka, and T. Natsuaki, unpublished data) and its original severe isolate Pepo (23), were used for tests to select mild strains. These isolates were propagated in *Nicotiana rustica*, and infected leaves were stored at -80°C before being used as standard inoculum. The virus also was purified from the infected leaves for use as inoculum. The inoculum was extracted or diluted in 0.1 M phosphate buffer (PB; pH 7.0).

Temperature treatment and single-lesion isolations. *N. rustica* and tomato (*Lycopersicon esculentum* cv. Momotaro-T93) were inoculated with the Pepo isolate at the third- to fifth-leaf stage and at the cotyledon stage, respectively. Immediately,

the plants were grown in a growth chamber at 15, 24, or 36°C for 30 days. All the uninoculated upper leaves were harvested, and these leaf extracts in 0.1 M PB were used to mechanically inoculate *Chenopodium quinoa* leaves. Single local lesions (SLLs) that developed on *C. quinoa* at 2 or 3 days after inoculation were ground individually in 0.1 M PB and transferred to *N. rustica* seedlings at the third- or fourth-leaf stage. The plants then were grown in a temperature-controlled (25°C) greenhouse.

Cross-protection assay. Cotyledons of 10-day-old tomato seedlings were mechanically inoculated with mild isolates 36R37 and SRO (28). Control seedlings were inoculated with 0.1 M PB only. At 15 days post inoculation (dpi), the third true leaves were mechanically inoculated with the challenge isolate Pepo at a concentration of purified virus of 25 µg/ml. Symptom development was observed for 25 days after the challenge inoculation. The plants were scored for disease severity according to the following scale: 0 = no visible symptoms; 1 = mosaic or mild filiform leaf; 2 = mosaic and mild filiform leaf; and 3 = mosaic, mild filiform leaf, and stunting. Disease severity or percentage of diseased plants was statistically analyzed with the Kruskal-Wallis analysis of variance test or the Fisher's exact test or χ^2 test of independence at $P = 0.01$ (Bonferroni adjusted probabilities).

Viral cDNA synthesis. Simple direct tube-reverse transcription (SDT-RT) was used for cDNA synthesis from infected leaves (24). Ground leaf sap with 10 mM phosphate-buffered saline (PBS) was added to a microcentrifuge tube. After removing the sap, the tube was washed twice with PBS containing 0.5% Tween 20. RNase-free water added to the tube was used as the template for the RT reaction. First-strand cDNA was synthesized using primer CMV3'NSR (5'-CCCCCG-CATGCGGCCGCTGGTCTCCTTTTAGA-GG-3'), complementary to the 3' terminal common sequence of CMV RNAs, and the First-Strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ) at 37°C for 60 min.

RT-polymerase chain reaction-based single-strand conformation polymorphism analysis. RT-polymerase chain reaction-based single-strand conformation polymorphism (RT-PCR-SSCP) analysis

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(20) was used to detect any nucleotide mutations on the *2b* gene of 17 attenuated candidates obtained after temperature treatments. The *2b* gene was amplified from viral cDNA by PCR using primers SSCP-2bF (5'-CCAATAAACAGCGAA-AGAAATATG-3') and SSCP-2bR (5'-CGGAAGGGGAGGTTTCA-3') that were designed from Pepo RNA 2 (accession no. AB124835). The PCR products (2 µl) mixed in 18 µl of loading buffer (10 ml of formamide, 0.4 ml of 0.5 M EDTA [pH 8.0], 0.01% [wt/vol] xylene cyanol, 0.01% [wt/vol] bromophenol blue) were treated for 5 min at 85°C and then were transferred immediately to ice. The samples were separated by electrophoresis for 60 min at 200 V on 6% Tris-borate-EDTA (TBE)-polyacrylamide gel at 10°C. The band pattern on the gel was visualized by silver staining (Silver Stain KANTO III; KANTO KAGAKU, Toyko, Japan). The PCR product having nucleotide mutations indicates a different band pattern from that of Pepo.

RT-PCR restriction fragment length polymorphism analysis. RT-PCR-based restriction fragment length polymorphism (RT-PCR-RFLP) analysis was used to identify Pepo and P2bR46C (18). A PCR of 30 cycles was performed using KOD Dash (TOYBO, Osaka, Japan) with primers R2-F (5'-ACTCCAACCTGGCTCG-TATGG-3') and CMV3'NSR and the following parameters: denaturation at 95°C for 30 s, annealing at 58°C for 1 min, and

extension at 68°C for 1 min. The PCR products were treated using restriction enzyme *Hha*I and then electrophoresed with 3% TBE-agarose gel; two fragments of about 500 and 100 bp were produced for P2bR46C, but none for Pepo.

Determination of nucleotide sequence on *2b* gene. The *2b* gene was amplified by PCR using primers SSCP-2bF and SSCP-2bR as described. The PCR product then was separated using 3% TBE agarose gel. The product at the expected size was sliced from the gel and eluted by GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences). The sequence reaction was performed using CEQ DTCS Quick Start kit (Beckman Coulter, Fullerton, CA), and the resultant samples were analyzed with a CEQ-8000 (Beckman Coulter). Nucleotide sequence analysis was carried out by GENETIX-MAC (Software Development, Tokyo, Japan).

RESULTS

Selection of mild isolates from dark-green islands. We first inoculated *N. rustica* plants with a mixed inoculum of an attenuated point mutant P2bR46C and its original severe Pepo isolate. Dark-green islands (DGIs) and yellow tissues on mosaic leaves were carefully detached with razor blades at 13 dpi, and leaf extracts from each region then were used to inoculate *C. quinoa*. Extracts from DGI tissue produced far fewer SLLs than did yellow-tissue extracts (*data not shown*). When *N.*

rustica then was inoculated with 1 of 20 SLLs, all SLLs originating from the DGIs developed a phenotype identical to that of P2bR46C at 14 dpi (Table 1). In addition, these phenotypes corresponded with results from the concomitant RT-PCR-RFLP analysis. On the other hand, all SLLs obtained from DGIs on leaves infected with Pepo alone caused severe mosaic and malformations (Table 1). These results indicated that attenuated variants within diseased plants could be efficiently isolated from DGIs on the mosaic leaves, but the virus concentration was low.

Selection of mild variants from dark-green tissue after cold or heat treatment. We obtained 17 out of the total of 288 SLLs by treatments at 15 and 36°C. These 17 SLLs caused milder symptoms, such as mosaic and dark-green spots without malformation (Table 2; Fig. 1), than did Pepo. On the other hand, all SLLs from a control treatment at 24°C expressed severe mosaic and symptoms similar to Pepo. More mild isolates tended to be produced by the cold treatment (7.4%) than by the heat treatment (3.0%).

When the leaf extract from each dark-green spot produced by these mild SLLs was used to inoculate *N. rustica*, the inoculated plants either did not develop any visible symptoms or the symptoms were a very mild mosaic (Fig. 2). To determine the stability of such symptom attenuation, 2 of 17 mild isolate candidates, 15T3 and 36R37, selected by screening from cold- and heat-treated plants, respectively, underwent three serial passages through *N. rustica* and single-lesion isolations using *C. quinoa*. Both isolates were stable and maintained their mild symptom phenotype during the host passages. In addition, all plants inoculated with each of 15 SLLs from 15T3 or 36R37 induced mild symptoms similar to the intact isolate. These results suggest that mild variants are effective

Table 1. Symptom expression on leaves of *Nicotiana rustica* after inoculation with single-lesion isolates (SLLs) from dark-green islands or yellow tissues from mosaic symptoms on *N. rustica* after co-infection with severe and mild isolates

Inoculum	Region ^x	No. tested ^y	No. of SLLs ^z	No. of SLLs with phenotype of isolate ^w	
				P2bR46C	Pepo
Pepo + P2bR46C	Y	20	20	6 (6)	14 (0)
	DGI	20	7	7 (7)	0 (NT)
Pepo	Y	20	20	0 (NT)	20 (NT)
	DGI	50	50	0 (NT)	50 (NT)

^w Number of SLLs with P2bR46C pattern in reverse-transcription polymerase chain reaction restriction fragment length polymorphism (RT-PCR-RFLP). Symptoms were observed nearly every day for 3 weeks. Viral RNA extracts from third and fourth upper leaves were used for RT-PCR-RFLP; NT = not tested.

^x Region on mosaic leaves; Y = yellow tissue and DGI = dark-green-island tissue.

^y Number of single lesions tested; single lesion was used to inoculate *Nicotiana rustica*.

^z Viral infection was checked by enzyme-linked immunosorbent assay.

Table 2. Selection efficiency of mild variants of *Cucumber mosaic virus* from the plants of *Nicotiana rustica* infected with a virulent Pepo isolate after cold or heat treatment

Temperature (°C) ^y	Plant	No. of SLLs tested ^z	Symptoms on <i>N. rustica</i> ^x	
			SM/Mal	MM/GS
36	Tomato	52	50	2
	<i>N. rustica</i>	48	47	1
15	Tomato	88	81	7
	<i>N. rustica</i>	100	93	7
24	<i>N. rustica</i>	30	30	0

^x Symptoms were observed nearly every day for 3 weeks. SM = severe mosaic, Mal = leaf malformation, MM = mild mosaic, GS = dark-green spot.

^y Plants were held at each temperature for 30 days after inoculation with the Pepo isolate.

^z SLLs = single-lesion isolations.

Fig. 1. Typical dark-green spots on second upper leaf of *Nicotiana rustica* 15 days after inoculation with a single local lesion isolate obtained after heat treatment.

tively obtained by dark-green tissue isolation after cold or heat treatment.

The 2b gene sequence and cross-protective ability of a promising mild variant. The 2b gene encoding the 2b protein of CMV has numerous functions associated with the virulence (2,4,8). In RT-PCR-SSCP analysis of the 2b gene of 17 mild candidates obtained in our selection trials, only isolate 36R37 yielded a band pattern different from that of Pepo (Fig. 3A). This result indicated that 36R37 had a nucleotide mutation or mutations on the 2b gene. We then determined the nucleotide sequence of 2b gene of this mild variant 36R37. Although the 2b gene nucleotide lengths of 36R37 and Pepo were the same (333 nucleotides), 36R37 had a nucleotide mutation at position 122 on the 2b open reading frame, which involved an amino acid substitution at position 41 (Fig. 3B; Pro to Leu) in a putative phosphorylation domain (16).

We also examined the cross-protective ability of 36R37 in tomato. An attenuated SRO isolate with a useful protective ability (28) was used as a positive control. At 25 days after the challenge inoculation, none of the isolates caused any visible symp-

toms on tomato plants, and isolate 36R37 had a high degree of cross-protection that was similar to that of SRO (Table 3).

DISCUSSION

In this study, we isolated mild variants from dark-green leaf tissue after cold or heat treatments. Other researchers also have obtained mild isolates for practical use after both cold and heat treatments, which can induce mutations on viral genomic RNAs (7,11–13,17,19). In our experiments, mild isolates appeared to be more efficiently selected by cold treatment than by heat treatment (Table 2). Szitty et al. (25) have reported that low temperature inhibits RNA silencing-mediated resistance against virus. Moreover, several groups have reported that the loss of silencing suppression is associated with viral symptom attenuation (5,14). For mild variants in diseased plants that were maintained at low temperature, low temperature may provide better conditions for survival than for those at high temperature.

Several studies have been reported in which the DGIs on leaves of diseased plants, protected from infection by closely

related viruses, contained little or no virus (1,6). However, no evidence of the presence of mild strains in DGIs has been reported (9).

In our screening test of the mild mutant P2bR46C from DGIs on mosaic leaves that had been co-infected with the original severe isolate Pepo, only P2bR46C was isolated from the DGIs. On the other hand, mild variants were not isolated from DGIs on leaves with severe mosaic of plants inoculated with Pepo alone, but mild variants were isolated from these tissues after cold or heat treatment. These results suggest that using inoculum containing mild variants such as the cold- or heat-treated sample is necessary to select mild strains from DGIs.

The 36R37 isolate had an amino acid substitution in the 2b protein. A mutation that reduced RNA-silencing suppression also attenuated symptoms, as described earlier (5,14). In addition, the 2b-defective CMV caused mild symptoms (4). Therefore, the amino acid substitution on 36R37 2b protein may reduce the suppression of silencing, resulting in symptom attenuation. However, other mild isolates obtained in this study did not have the mutation on the 2b gene, suggesting that a viral factor or factors other than the 2b protein also is associated with symptom attenuation. In our study, two mechanisms are possible: symptom attenuation may be due to a mechanism other than RNA silencing, and a viral factor other than the 2b protein also may suppress RNA silencing because the 2b-defective CMV variant has suppressed amplicon-mediated RNA silencing (26).

This is the first report of the isolation of a mild strain from dark-green islands. Although we used CMV as a model in this study, most plant viruses cause mosaic symptoms on more than one host. Therefore, dark-green tissue isolation after mutagenesis is a technique that may be

Fig. 2. Symptoms on upper leaves on *Nicotiana rustica* 15 days after inoculation with various isolates of *Cucurbit mosaic virus* (CMV). Pepo = Pepo isolate, 15T3 = mild strain isolated after cold treatment, and 36R37 = mild strain isolated after heat treatment. Mild isolates were isolated from dark-green spots. Bars = 2 cm.

Fig. 3. Nucleotide sequence analysis of the 2b gene of 36R37 isolate. **A**, Reverse-transcription polymerase chain reaction-based single-strand conformation polymorphism (RT-PCR-SSCP) analysis of the 2b gene of mild strain candidates. The 2b gene of each isolate was amplified by RT-PCR. The PCR products were separated by electrophoresis (200 V for about 60 min) on 6% Tris-borate-EDTA-polyacrylamide gel at 10°C. The band pattern was visualized by silver staining. 36R37 = Mild strain isolated after heat treatment, 15T3 = mild strain isolated after cold treatment, 36R38 = virulent strain obtained after heat treatment, and Pepo = Pepo isolate. **B**, Partial nucleotide sequence (from nucleotide position 106 to 138) of the 2b open reading frame on 36R37 (DDBJ/EMBL/GenBank accession no. AB182559) and Pepo (accession no. AB124835), and the translational amino acid residues (amino acid position from 35 to 46) is shown. Asterisk (*) indicates matching nucleotide position. Bold lettering indicates a different amino acid residue. Residues in italics indicate a putative phosphorylation domain (16).

Table 3. Cross-protection ability of mild *Cucumber mosaic virus* (CMV) 36R37 isolate against Pepo isolate of CMV in tomato

Pre-inoculated isolate ^x	No. of plants tested	Diseased plants (%) ^y	Disease severity ^z
36R37	41	0.0 a	0 a
SRO	44	4.5 a	0.1 a
Noninoculated	30	76.7 b	1.8 b

^x Purified virus was used as inoculum at a concentration of 50 µg/ml. The plants infected with 36R37 or SRO expressed no visible symptoms during testing.

^y Symptoms were observed 25 days after the challenge inoculation with Pepo at a concentration of 25 µg/ml. The different letter indicates a significant difference, based on the Fisher's exact test or χ^2 test of independence at $P = 0.01$ (Bonferroni adjusted probabilities).

^z Plants were scored for disease severity on a scale of 0 to 3, where 0 = no visible symptoms; 1 = mosaic or mild filiform leaf; 2 = mosaic and mild filiform leaf; and 3 = mosaic, mild filiform leaf, and stunting. Means within the column followed by different letters were significantly different at $P = 0.01$ in Kruskal-Wallis analysis of variance test.

applicable for several plant viruses. This alternative technique has promise in furthering our progress in controlling viral diseases through cross protection with attenuated viruses.

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