The Coat Protein Gene is Essential for the Systemic Infection of Cucumber Mosaic Virus in *Cucumis figareii* at a High Temperature*

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Abstract

Systemic infection of cucumber mosaic virus (CMV) in *Cucumis figareii* at high temperature was investigated using reassortants and chimeric RNAs. Three CMV strains of pepo-, SO-, MY17- and Y-CMV were used: pepo-, SO- and MY17-CMV systemically infected *C. figareii* at 36°C, whereas Y-CMV did not as previously described. Inoculation with *in vitro* transcripts from biologically active cDNA clones of Y-CMV and pepo-CMV RNAs 1, 2 and 3 indicated that only the inocula containing pepo-CMV RNA 3 induced systemic infection at 36°C, suggesting that the resistance against systemic infection by Y-CMV and its breakage by pepo-CMV were mapped to CMV RNA 3. Inoculation with transcripts from the cDNA clones of Y-CMV RNAs 1, 2 and RNA 3 of SO-CMV or MY17-CMV provoked systemic infection at high temperature, indicating that the systemic infection by SO-CMV or MY17-CMV was also mapped to RNA 3. Analysis with chimeras constructed between cDNA clones of Y-CMV and pepo-CMV RNA 3 showed that the resistance and resistance breakage were mapped to the coat protein gene on CMV RNA 3. Comparison of the nucleotide sequence of pepo-, SO- and MY17-CMV RNA 3 with that of Y-CMV RNA 3 revealed that the coat protein of Y-CMV differs from those of pepo-, SO- and MY17-CMV in four amino acids at positions 17 (Leu to Pro), 25 (Ser to Pro), 28 (Ser to Ala) and 129 (Ser to Pro). These results suggested that all or some of the four amino acids play an important role in determining long-distance movement of CMV in *C. figareii* at a high temperature.

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Key words: cucumber mosaic virus, *Cucumis figareii*, virus resistance, long-distance movement, high temperature, coat protein gene.

INTRODUCTION

Many reports have indicated that the inability of a virus to infect a particular plant host results from lack of cell-to-cell or long-distance movement of the virus. Such movement of plant viruses is frequently mediated by viral gene products^{4,9,26}.

In the case of cucumber mosaic virus (CMV), all five gene products were reported to be involved in virus movement. The five CMV genes were expressed with distinct functions from three genomic and two sub-genomic RNAs. RNAs 1 and 2 encode the 1a and the 2a proteins, respectively, involved in replication of the viral genome^{11,17}, which also plays some role in virus movement^{8,12}. The product of an additional gene on RNA 2, known as the 2b gene, also influences virus movement in a host-specific manner⁶. The two gene products encoded by dicistronic RNA 3 are required for

infection in plants. Genomic RNA 3 encodes a non-structural protein of the 3a movement protein (MP) along with the coat protein (CP) that is translated from subgenomic mRNA of RNA 4. The 3a MP is required for cell-to-cell movement^{3,5,15} and possibly for long-distance movement^{1,15}. The CP is also needed for efficient cell-to-cell movement in all hosts tested^{2,3,29} and is a determinant for restriction of systemic spread of the virus^{22,31}.

Cucumis figareii, a wild African relative of *Cucumis melo* (melon), is highly resistant to several viruses, including CMV. A comparative analysis of replication and movement of CMV in *C. figareii* and *C. melo* indicated that the resistance of *C. figareii* to CMV might primarily be caused by the inhibition of virus movement via both cell-to-cell and long-distance²³. Furthermore, we demonstrated that the systemic resistance in *C. figareii* to Y- and two other CMV strains was sustained at a high temperature but the resistance to pepo-, SO- and MY17-

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CMV was breakable at that temperature²⁵.

The objective of this work is to clarify the mechanism for the resistance and long-distance movement of CMV in *C. figareii* plants at a high temperature. In this paper we report that the CP gene on CMV RNA 3 is responsible for the systemic infection and the resistance breakage in *C. figareii* at a high temperature (36°C).

MATERIALS AND METHODS

Plants, CMV strains, CMV inoculation and detection of CMV in tissues *C. figareii* plants were grown in a growth chamber at 25°C with a 12-h light/12-h dark cycle. Four CMV strains in subgroup I, Y-CMV, pepo-CMV^{18,24}, SO-CMV^{24,25} and MY17-CMV^{24,25} were used. Three expanded true leaves of *C. figareii* were inoculated 30 days after sowing with purified CMV at a concentration of 2 mg/ml in 100 mM phosphate buffer at pH 7.0. The plants were then grown in growth chambers at the determined temperatures. CMV in tissues was detected by tissue printing and northern blotting as previously described^{23,25}. In both cases, the ³²P-labeled RNA probe complementary to the 3' 200 nucleotides of pepo-CMV RNA 3 was used²⁵.

cDNA clones for Y-, pepo-, SO- and MY17-CMV RNAs Full-length cDNA clones of Y-CMV RNAs 1 (Y1: pCY1-T7), 2 (Y2: pCY2-T7) and 3 (Y3: pCY3-T7)²⁹ were obtained from C. Masuta, Hokkaido University.

To construct the cDNA clone of pepo-CMV RNA 1, two overlapping fragments were synthesized. The 3' proximal 1541-3361 fragment was amplified by 5'-CCGGATCCTGGTCTCCTTTTGGAGGCC-3' and 5'-CGCCTAATACGACTCACTATAGTTTTATTTACAAGAGCG-3'. This fragment was then digested with *Eco*RI and *Bam*HI, and ligated into the same restriction sites of pCRII (Invitrogen), creating pCP13IB. The 5' proximal 1-1620 fragment amplified by 5'-CCGGATCCTAATACGACTCACTATAGTTTTATTTACAAAGAGCG-3' and 5'-GCGGATCCCGGATGATATCACGTCCC-3' was digested with *Bam*HI and ligated into the *Bam*HI site of pCRII, creating pCP15BV. pCP1TP1 (P1) containing full-length cDNA of pepo-CMV RNA 1 was obtained by ligating an *Eco*RI fragment of pCP15BV into the same restriction site of pCP13IB.

Full-length cDNA clone of pepo-CMV RNA 2 (P2: pCP2TP1) was prepared by the method described elsewhere²⁴. cDNA clones for pepo-CMV RNA 3 (P3: pCP3TP2), SO-CMV RNA 3 (S3: pCS3TP1) and MY17-CMV RNA 3 (M3: pCM3TP3) were also used²⁴.

Construction of reassortants between pepo- and Y-CMV; Y-CMV and MY17- or SO-CMV pCP1TP1 (P1), pCP2TP1 (P2) and pCP3TP2 (P3) were linearized with *Bam*HI, whereas pCY1-T7 (Y1), pCY2-T7 (Y2) and pCY3-T7 (Y3) were linearized with *Not*I. The RNA transcripts from the full-length cDNA clones of pepo- and Y-CMV RNAs 1-3 were combined to form the parental RNA. The RNA 3s transcribed from *Eco*RI-linearized pCS3TP1 (S3) or pCM3TP3 (M3) were

combined with RNA transcripts from pCY1-T7 (Y1) and pCY2-T7 (Y2) to form the parental RNA. Individual RNAs of CMV strains Y- (Y), pepo- (P), SO- (S) and MY17- (M) are designated as 1, 2, and 3; e.g., Y1Y2P3 contains RNA 1 plus 2 of Y-CMV and RNA 3 of pepo-CMV. These reassortants were then propagated in tobacco plants (*Nicotiana tabacum* cv. Xanthi nc), because *C. figareii* can be infected only with CMV at a high concentration. The reassortants purified from systemically infected leaves were used for inoculating *C. figareii*.

Construction of RNA 3 chimeras between Y- and pepo-CMV

RNA 3 chimeras between Y- and pepo-CMV were constructed as follows. The cDNA clone of pepo-CMV RNA 3 (P3: pCP3TP2) was cut with *Xba*I and *Hind*III, blunt-ended with T4 DNA polymerase and then self-ligated, creating P33 (pCP3TP3). The first pair of reciprocal chimeras was constructed using the common *Sal*I site, located 1297 nucleotides from the 5' end of Y-CMV RNA 3 and 1294 nucleotides from the 5' end of pepo-CMV RNA 3, and the *Sac*I cloning site in Y3 or P33. These chimeras were constructed by exchanging *Sal*I/*Sac*I fragments, creating YP (pCY3P3Sal) and PY (pCP3Y3Sal). The third chimera was constructed using *Sal*I and the *Nru*I site which was located at nucleotide 1377 in Y-CMV RNA 3 and nucleotide 1374 in pepo-CMV

Fig. 1. Systemic infectivity of reconstructed cucumber mosaic virus (CMV) on *Cucumis figareii* at 36°C. CMV contained RNAs 1 and 2 from Y-CMV (Y1 and Y2) and RNA 3 derived from Y-CMV (Y3), pepo-CMV (P3) or chimeras formed between cDNA clones of Y-CMV RNA 3 (open rectangles) and pepo-CMV RNA 3 (filled rectangle), using the restriction sites *Sal*I, *Nru*I or *Xho*I. The infectivity of virus containing the wild-type or chimeric RNA 3 is indicated by "+" or "-" with the number of systemically infected plants over the number of inoculated plants. Systemic infectivity was assayed by symptoms and verified by tissue printing and northern blotting analysis.

RNA 3, and by exchanging *SalI/NruI* fragment of Y3 with that of P3, creating YPY-SN (pCY3PcpSN). The fourth chimera was constructed using *NruI* and the *XhoI* site which was located at nucleotide 1839 in Y-CMV RNA 3 and nucleotide 1836 in pepo-CMV RNA 3, and by exchanging *NruI/XhoI* fragment of Y3 with that of P3, creating YPY-NX (pCY3PcpNX). The fifth chimera was constructed by exchanging *XhoI/SacI* fragment of Y3 with that of P3, creating YP3t (pCY3P3Xho) (Fig. 1).

Y1, Y2 and PY were linearized with *NotI*, Y3, YP, YPY-SN, YPY-NX, YP3t with *EcoRI* and P3 with *BamHI*. The RNA transcripts from Y1, Y2 and full-length cDNA clones of each RNA 3 were combined to form pseudorecombinants which were then propagated in tobacco plants as described previously. The purified reassortants were used as the inocula. The preserved nucleotide sequence for each RNA construct was further confirmed for RNA 3 recovered from the purified reassortants.

Comparative studies on the nucleotide sequence among CMV isolates were assisted by computer software program GENETYX (Software Development).

RESULTS AND DISCUSSION

Mapping resistance breakage to CMV RNA 3

Whereas Y-, pepo-, SO- and MY17-CMV locally infect *C. figareii* at 24°C, only pepo-, SO- and MY17-CMV systemically infect the plant at 36°C²⁵. To map the resistance against systemic infection by Y-CMV and its breakage by some CMV strains in *C. figareii* at 36°C, systemic infectivity of the reassortants was tested.

Table 1. Development of systemic symptoms in *C. figareii* infected with 10 CMV reassortants at different temperatures

CMV reassortants ^{a)}	Incubation temperature (°C)	
	24	36
Y1Y2Y3	0/15 ^{b)}	0/15
Y1P2Y3	0/15	0/15
Y1Y2P3	0/15	15/15
Y1P2P3	0/15	9/15
P1Y2Y3	0/15	0/15
P1P2Y3	0/15	0/15
P1Y2P3	0/15	8/15
P1P2P3	0/15	15/15
Y1Y2S3	0/22	16/22
Y1Y2M3	0/18	11/20

a) Individual RNAs of CMV strains Y- (Y), pepo- (P), SO- (S) and MY17- (M) are designated as 1, 2, and 3; e.g., Y1Y2P3 contains RNA 1 plus 2 of Y-CMV and RNA 3 of pepo-CMV.

b) Number of plants systemically infected/number of plants tested at 14 days after inoculation. Data reflect infectivity ascertained by symptoms and verified by tissue printing and northern blotting analysis of noninoculated upper leaves.

All *C. figareii* plants inoculated with reassortants between Y- and pepo-CMV and incubated at 24°C or 36°C showed bright yellow chlorotic spots on the inoculated leaves. None of the plants inoculated with reassortants and incubated at 24°C developed systemic symptoms on the upper leaves. Furthermore, none of the plants inoculated with Y1Y2Y3, Y1P2Y3, P1Y2Y3 and P1P2Y3 and incubated at 36°C developed systemic symptoms on the upper leaves. On the other hand, all or most of the plants inoculated with Y1Y2P3, Y1P2P3, P1Y2P3 and P1P2P3 incubated at 36°C showed yellow chlorotic spots on the upper leaves (Table 1). The systemic infectivity of the reassortants containing P3 was verified by detecting viral RNAs with tissue printing and northern blotting from the noninoculated upper leaves (data not shown). These results indicated that the viral factor involved in the resistance against systemic infection by Y-CMV and its breakage by pepo-CMV at 36°C was located in CMV RNA 3.

C. figareii inoculated with reassortants containing RNA 3 from the SO- and MY17-CMV (Y1Y2S3 and Y1Y2M3) and incubated at 36°C also showed prominent chlorotic spots on the upper leaves (Table 1). These results further suggested that the resistance breakage of *C. figareii* at 36°C to some CMV strains was determined by CMV RNA 3.

There are some reports that temperature effects on CMV infection were variable and dependent on host plant and virus strains. On the cultivar-specific temperature sensitivity, Roossink (1991)²¹ examined the temperature effect on CMV infection in muskmelon (*Cucumis melo* cv. Iroquis), using a fast strain of Fny and slow strain of Sny. The pseudorecombination between the two strains showed that the temperature sensitivity was mapped to Sny-CMV RNA 1. There are several works reporting that one or more gene products are needed for the long-distance movement of CMV in specific cucurbit species^{3,8,14,15,21,27}. Nevertheless, our results indicated that CMV RNA 3, encoding the 3a protein and the CP, is solely essential for the systemic infection of CMV in *C. figareii* at 36°C.

Localization of resistance breakage within the 3' half of the pepo-CMV RNA 3

To delimit which region within CMV RNA 3 determines the systemic infection, five chimeric recombinants were constructed between the cDNA clones of Y- and pepo-CMV RNA 3. Infectivity of the viruses with the chimeric recombinants was shown in Fig. 1. The purified virus containing the 3' region of pepo-CMV RNA 3 (YP) induced systemic symptoms, whereas the reciprocal chimera (PY) did not. Furthermore, none of the plants inoculated with the reassortants containing RNA 3 transcripts from YPY-SN, YPY-NX and YP3t developed systemic symptoms at 36°C. Systemic infection of *C. figareii* plants inoculated with reassortants containing P3 and YP were further analyzed by tissue printing (Fig. 2). These results suggested that two or more regions of the *SalI/NruI*, the *NruI/XhoI* and the region from the *XhoI*

Fig. 2. Analysis by tissue printing of CMV RNAs in leaves of *C. figareii* inoculated with the reconstructed CMVs and incubated at 36°C. The inocula contained RNAs 1 and 2 from Y-CMV (Y1 and Y2) and RNA 3 derived from Y-CMV (Y3), pepo-CMV (P3) or chimeras formed between cDNA clones of Y-CMV RNA 3 and pepo-CMV RNA 3 as indicated in Fig. 1. Sample leaves from individual plants for each reconstructed CMV were analyzed at 7 days after inoculation by the ^{32}P -labeled RNA probe complementary to the 3' 200 nucleotides of pepo-CMV RNA 3. I, inoculated leaves; 1-6, non-inoculated upper leaves in descending order from top; mock, leaves treated with buffer.

site to the 3' end were necessary for the resistance breakage.

Localization of specific CP sequences involved in resistance breakage

We determined the nucleotide sequence of RNA 3 of pepo-, SO- and MY17-CMV and compared them to that of Y-CMV RNA 3²⁴⁾. There are some differences in the nucleotide sequence among pepo-, SO- and MY17-CMV RNA 3, though all three strains can systemically infect *C. figareii* at 36°C. Among the predicted 279 amino acids of Y-, pepo-, SO- and MY17-CMV 3a protein, only one amino acid at position 238 was different: Val for pepo-CMV, and Ala for Y-, SO-, MY17-CMV.

Several findings demonstrate that changes in the sequence of the 3a protein can affect the ability to regulate the long-distance movement of CMV strains. The restricted movement in cucurbit hosts has been mapped to two sequence changes in the codons of amino acids 51 and 240 of the Sny-CMV 3a gene¹⁴⁾. Sequences between amino acids 209 and 236 contain a domain critical for function for the 3a protein, and amino acids 237-279 are not required for the movement-associated functions of the 3a protein¹⁵⁾. We found one amino acid substitution among the four CMV strains at position 238 of the 3a protein in the region not required for movement.

Figure 3 shows an alignment of the predicted amino

Amino Acid Positions										
	SalI		NruI				XhoI			
	<div>Coat Protein Gene</div>									
	8	17	25	28	47	65		129		205
Y-CMV	S	L	S	S	A	K		S		E
Pepo-CMV	G	P	P	A	S	K		P		E
SO-CMV	S	P	P	A	S	K		P		E
MY17-CMV	S	P	P	A	A	R		P		I

Fig. 3. Difference in amino acid sequences in coat protein gene for Y-, pepo-, SO- and MY17-CMV. Encoded amino acids which differ from Y-CMV are indicated with the relative positions. The three restriction sites are also indicated as Fig. 1.

acid sequence of Y-, pepo-, SO- and MY17-CMV CP. Among 218 amino acids of the CP, changes were found at eight positions. Four amino acids at positions 17, 25, 28 and 129 were different between Y-CMV and pepo-, SO-, MY17-CMV (Fig. 3, shown in bold). These results suggested that the changes in the CP gene of Y-CMV RNA 3 at four positions 17 (Leu to Pro), 25 (Ser to Pro), 28 (Ser to Ala) and 129 (Ser to Pro) were possibly required for long-distance movement of CMV in *C. figareii* at a high temperature. We cannot deny involvement of the 3' end sequence as yet. Whether all four amino acids are responsible for the movement remains unclear. We have so far known that CMV containing the Y-CMV RNA 3 derivatives with only one amino acid substitution (17 Pro, 25 Pro, 28 Ala and 129 Pro) could not systemically infect at 36°C (unpublished data).

Wikoff *et al.* (1997)²³⁾ suggested that the N-terminal 22 amino acids of CMV CP featuring a high density of Arg residues, known as the internal R-domain, may interact with the viral RNA in either assembling or stabilizing CMV particles. Kaplan *et al.* (1998)¹⁶⁾ also demonstrated that the deletion of some of the basic amino acids within the amino-terminal 26 amino acids of the CMV CP (amino acids 15-40) had an effect on virion stability as well as RNA-binding stability by the CP. N-terminal amino acids of *Brome mosaic virus*²⁰⁾ and *Alfalfa mosaic virus*³²⁾, which are classified in *Bromoviridae* along with CMV, are required for regulating virus-host interaction. Therefore, the first two amino acids (17 Pro and 25 Pro) or three amino acids (17 Pro, 25 Pro or 28 Ala) in the CP are possibly important for the virus movement.

Another amino acid substitution was Ser (Y-CMV) to Pro (pepo-, SO- and MY17-CMV) at amino acid 129 (Fig. 3). The amino acid at position 129 is reported to influence symptom expression^{28,30)}, aphid transmissibility¹⁹⁾, or a break in host resistance: changes in the CP gene of M-CMV RNA 3 at both positions 129

(Leu to Pro) and 162 (Thr to Ala) were required to overcome resistance against M-CMV in maize²². Thus, the amino acid difference at this region in the CP may have some effect on restricting movement of Y-CMV at a high temperature in *C. figareii*.

Possible mechanisms for the break in resistance

CMV requires its CP for both cell-to-cell and long-distance movement. The CP also has host-specific determinants for systemic spread^{13,27,31}. Y-CMV as well as pepo-CMV cannot infect *C. figareii* systemically at 24°C, whereas pepo-CMV infects systemically at 36°C²⁵. In this study, we suggested that the CMV CP gene had an active role in the long-distance movement of CMV infection in *C. figareii*, which occurred only at a high temperature (36°C). This CMV-*C. figareii* relationship is considered as a non-necrotic resistance²³, in which the entrance or exit of viruses through the bundle-sheath/phloem parenchyma barrier is regulated^{7,10}. In a separate study, we demonstrated that pepo-CMV was detectable in the vascular system as well as in the parenchyma cells of inoculated leaves of *C. figareii* incubated at 36°C but not at 24°C and that Y-CMV was detected only in the parenchyma and bundle-sheath cells at both temperatures (unpublished data). Recently, a model was presented that CMV moved into minor sieve elements as a ribonucleoprotein (RNP) complex that contained the viral RNA, the CP and the 3a MP¹. In this model, a host factor in addition to the CP may be needed for forming a stable RNP complex. Both the R-domain^{16,33} and the amino acid at position 129¹⁹ in CP may affect its ability to interact with the host factor. All four changed amino acids in pepo-, SO- and MY17-CP are hydrophobic, whereas the three changed amino acids in Y-CP are not (Fig. 3). This difference probably modifies the stability of the CPs.

Taken together, we speculate that the major mechanisms for the resistance breakage are as follows: (1) The host factor for viral movement is induced in the *C. figareii* plant. (2) The movement of Y- as well as pepo-CMV is inhibited at 24°C, because another host factor that prevented the interaction between the CP and the host factor or the formation of the RNP complex is induced in the plant at 24°C. (3) The second host factor, suppressed by heat shock at 36°C, enables pepo-CP to interact with the host factor or to form the RNP complex. The CP-host factor interaction or RNP-formation is not launched for Y-CMV, on the other hand, because the Y-CP accumulation in the inoculated leaves is notably low (unpublished data). (4) The host factor for the viral movement is induced only at a high temperature, which is effective for pepo-CMV. It does not work with Y-CMV because of the CP shortage. (5) The pepo-CP specifically elicits host responses necessary for viral movement. (6) The pepo-CP and Y-CP are structurally different, because the amino acids in the R-domain and at position 129 are important for conformation. Only pepo-CP is stable enough to be recognized by the host factor at 36°C. The long-distance movement of CMV

may be regulated by some of the mechanisms of (1), (2), (3) and (6). To verify these possibilities, further clarification of these factors in the host, as well as in the virus, is needed.

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和 文 摘 要

斉藤宏昌・藤原正幸・大木 理・尾崎武司：*Cucumis figareii* の高温条件でのキュウリモザイクウイルスによる全身感染には外被たんぱく質遺伝子が必要である

Cucumis figareii の高温条件でのキュウリモザイクウイルス (CMV) による全身感染のウイルス側の因子を調べるために、reassortment 実験とキメラ RNA による実験を行った。高温条件で全身感染する pepo-CMV の RNA 1-3 の感染性 cDNA クローン由来の RNA と、全身感染しない Y-CMV RNA 1-3 の cDNA 由来の RNA とを組み合わせ、接種したところ、36°C 条件では pepo-CMV RNA 3 を含む組み合わせのみで全身感染が認められたことから、全身感染にかかわる因子は CMV RNA 3 にあることがわかった。全身感染する SO-と MY17-CMV の RNA 3 の cDNA クローン由来 RNA と Y-CMV RNA 1-2 の接種でも全身感染したことから、これらの系統についても全身感染にかかわる因子は CMV RNA 3 にあることが示された。また、Y-CMV RNA 3 と pepo-CMV RNA 3 を部分的に組み換えたキメラ RNA による実験で、全身感染にかかわる因子が CP 遺伝子にあることが示された。さらに、pepo-, SO-, MY17-CMV の RNA 3 の塩基配列を Y-CMV RNA 3 と比較した結果、Y-と pepo-, SO-, MY17-の CP 領域には 17 (Leu → Pro), 25 (Ser → Pro), 28 (Ser → Ala), 129 (Ser → Pro) 番目の 4 箇所のアミノ酸相違部位が認められた。これらのアミノ酸が高温条件における CMV の長距離移行に重要と考えられる。