

キュウリモザイクウイルスY系統(CMV-Y)RNA2の全塩基配列

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Complete Nucleotide Sequence of RNA2 of Cucumber Mosaic Virus Y Strain

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Abstract

cDNA clones covering full-length of cucumber mosaic virus Y strain (CMV-Y) RNA2 were synthesized and the complete nucleotide sequence of the RNA was determined. Specific restriction sites, *Pst*I and *Not*I, were added to the 5' and 3' end of cDNAs, respectively. It consisted of 3,051 nucleotides and contained a long single open reading frame of 2,571 nucleotides. GDD consensus sequence, which is thought to be a "polymerase site", and a common motif surrounding it was found in the amino acid sequence deduced from the RNA. There was almost complete sequence homology between CMV-Y and Fny-CMV RNA2s (98.4% sequence homology at the nucleotide level and 99.5% at the amino acid level). By contrast, lower degree of sequence homology was observed between CMV-Y and CMV-Q RNA2s (71.4% sequence homology at the nucleotide level and 74.4% at the amino acid level). These results suggest that CMV-Y belongs to Subgroup I.

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Key words: cucumber mosaic virus, RNA2, cDNA cloning, nucleotide sequence.

INTRODUCTION

Cucumber mosaic virus (CMV) is a positive-sense RNA plant virus with a tripartite genome consisting of single-stranded RNAs, designated RNA1, 2 and 3 in decreasing order of molecular weight (Mr)^{10,16}; subgenomic RNA4 encoding the coat protein is generated from RNA3 during replication^{8,21}. We have already reported the complete nucleotide sequence of RNA3 of Japanese CMV-Y strain¹⁴, which contains two open reading frames (ORFs) for 3A protein and coat protein. It was demonstrated that inoculation with a mixture of RNA1 and RNA2 was sufficient to induce viral RNA replicase activity and that full-length positive-sense RNA1 and RNA2 were actually produced in tobacco mesophyll protoplasts¹⁵. These observations suggest that the virus replicase would be encoded by either RNA1 or RNA2 or possibly by both RNAs. The first nucleotide sequence of RNA2 of CMV was published on CMV-Q¹⁸, followed by the sequence of Fny-CMV RNA2¹⁹. After nucleotide sequence analysis, significant sequence homology was found among the RNA2s of CMV, alfalfa mosaic virus³ and brome mosaic virus², suggesting that RNA2 encodes the viral replicase. However, to clarify whether putative protein from RNA2 contains replicase activity, it is important that the replicase should be purely isolated and subsequently characterized. Toward this purpose, we first synthesized cDNA clones covering full-length CMV-Y RNA2 and report here the nucleotide sequence of the RNA and compare it with the other known RNA2s of CMV.

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MATERIALS AND METHODS

RNA purification. CMV-Y²³ was propagated in tobacco plants (*Nicotiana tabacum* L. cv. Xanthi nc) and viral RNA was extracted as described²³. CMV RNAs were electrophoresed in a 1.4% agarose gel, and RNA2 was recovered by electroelution from the gel.

cDNA cloning. The cDNAs were synthesized according to Gubler and Hoffman⁷ using a primer complementary to the 3'-terminal 17 nucleotides of CMV-Y RNA3¹⁴, since the extreme 3'-terminal sequences of RNAs 1, 2, 3 and 4 from each three bromoviruses and also from CMV-Q were shown to have extensive intraviral homology in addition to interviral homology¹. The synthesized cDNAs were ligated in *Eco*RI site of λ ZAP (Stratagene) and packaged using Gigapack (Stratagene) *in vitro*. cDNAs inserted into the recombinant phages were sequenced and cDNA clones containing sequences having homology with that of CMV-Q RNA2 were selected. Two specific primers (positions 2219–2235 and 937–953) containing unique restriction sites and complementary to the 5' terminus of the obtained cDNAs were synthesized and used for extension of cDNAs toward 5' end of the RNA2 (Fig. 1). Specific primers used in this study were synthesized by a DNA synthesizer (ABI) and purified by HPLC.

Sequencing. The cDNAs inserted in pBluescript (Stratagene) were deleted by a combination of Exonuclease III and Mung bean nuclease using a deletion kit (Takara Shuzo), which was based on the methods of Henikoff⁸) and Yanisch-Perron²⁴), in order to obtain deletion mutants with various lengths suitable for sequencing. The nucleotide sequence of the RNA was determined on both strands of the cDNAs by dideoxy chain termination methods^{12,20}. The 5' terminal nucleotide sequence of the RNA was determined by direct RNA sequencing¹¹.

Synthesis of 3' and 5' terminal cDNAs. To construct a full-length cDNA clone of CMV-Y RNA2 in future and use in various experiments, cDNAs complementary to the terminal regions of the RNA were synthesized as follows to contain specific restriction sites at the both ends. First strand cDNA synthesis was primed with a synthetic oligonucleotide which was complementary to the 3' region of the RNA and was designed to create a *Not*I site (Fig. 1). After synthesis of first strand cDNA containing 5' region of RNA2 sequence, template

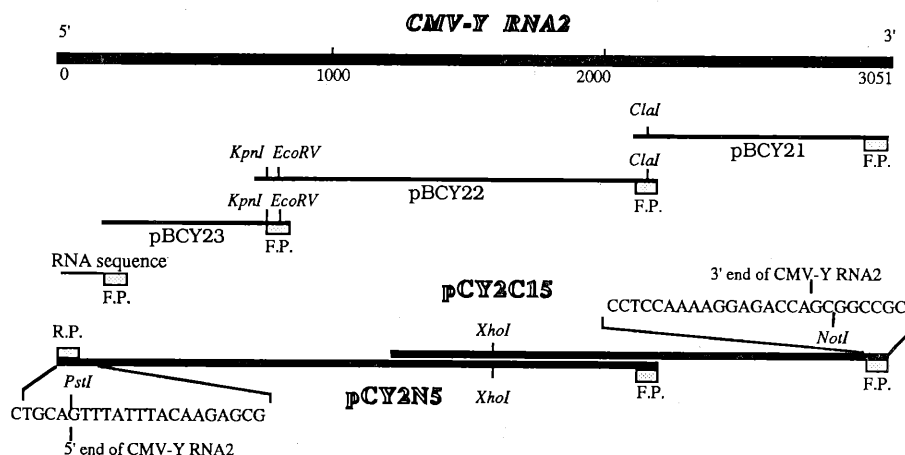


Fig. 1. Schematic representation of the cloning and sequencing strategies for CMV-Y RNA2. CMV-Y RNA2, the first set of overlapping cDNA clones used for sequencing and the second set of overlapping cDNA clones covering full-length RNA2 are indicated by broad, thin and middle lines, respectively. Specific primers used for the synthesis of first strand cDNA (F.P.) and second strand cDNA (R.P.) are represented by dotted area. The nucleotide sequences of 5' and 3' terminal primers are shown.

m7GpppGUUUUUUACAAGAGCGUACCGGUUACAACCCUCCUCCUCCUAAAACUCCUAGACUUGAAUUCUUCUUCUAGUUCUUUUU

88 AUG GCU UUC CCC GCC CCC GCA UUC UCA CUA GCC AAU CUU UUG AAC GGC AGU UAC GGU GUC GAC ACU CCC GAG GAU GUG GAA CGU UUG CGA
Met Ala Phe Pro Ala Pro Ala Phe Ser Leu Ala Asn Leu Leu Asn Gly Ser Tyr Gly Val Asp Thr Pro Glu Asp Val Glu Arg Leu Arg

178 UCU GAG CAA CGC GAA GAG GCU GCU GCG GCU UGU CGU AAU UAC AGC CCC CUA CCC GCU GUG GAU GUC AGC GAG AGU GUC ACA GAG GAC CGC
Ser Glu Gln Arg Glu Glu Ala Ala Cys Arg Asn Tyr Arg Pro Leu Pro Ala Val Asp Val Ser Glu Ser Val Thr Glu Asp Ala

268 CAU UCC CUC CGA ACU CCU GAC GGA GCU CCC GCU GAA GCG GUG UCU GAU GAG UUU GUA ACU UAU GGU GCU GAA GAU UAC CUU GAA AAA UCU
His Ser Leu Arg Thr Pro Asp Gly Ala Pro Ala Glu Ala Val Ser Asp Glu Phe Val Thr Tyr Gly Ala Glu Asp Tyr Leu Glu Lys Ser

358 GAU GAU GAG CUC CUU GUC GCU UUU GAG ACG AUG GUC AAA CCC AUG CGU AUC GGA CAA CUA UGG UGC CCU GCG UUU AAU AAA UGU UCU UUU
Asp Asp Glu Leu Leu Val Ala Phe Glu The Met Val Lys Pro Met Arg Ile Gly Gln Leu Trp Cys Pro Ala Phe Asn Lys Cys Ser Phe

448 AUU UCC AGC AUU GCU AUG GCC AGA GCU UUG UUG UUG GCA CCU AGA ACA UCC CAC CGA ACC AUG AAG UUU GAA GAC CUG GUC GCG GCU
Ile Ser Ser Ile Ala Met Ala Arg Ala Leu Leu Leu Ala Pro Arg Thr Ser His Arg Thr Met Lys Cys Phe Glu Asp Leu Val Ala Ala

538 AUU UAC ACU AAA UCU GAU UUC UAU UAC AGU GAA GAG UGU GAA GCC GAC GAC GCU CAG AUA GAU AUC UCG UCU CGC GAU GUA CCC GGU UAU
Ile Tyr Thr Lys Ser Asp Phe Tyr Tyr Ser Glu Glu Cys Glu Ala Asp Asp Ala Gln Ile Asp Ile Ser Ser Arg Asp Val Pro Gly Tyr

628 UCU UUC GAA CCG UUG UCC CGA ACG UCU GGA UUU GAA CCG CCG CCC AUU UGU GAA GCG UGC GAC AUG AUC UAC CAG UGC CCG UGU UUU
Ser Phe Glu Pro Trp Ser Arg Thr Gly Phe Glu Pro Pro Ile Cys Glu Ala Cys Asp Met Ile Met Tyr Gln Cys Ser Phe

718 GAU UUC AAU GCU UUA AAG AAA UCG UGC GCU GAG AGG ACU UUC GCU GAU GAU UAU GUU AUU GAA GGU UUA GAU GGU GUU GAU AAU GCG
Asp Phe Asn Ala Asp Val Lys Lys Ser Cys Ala Glu Arg Thr Phe Ala Asp Asp Tyr Val Ile Glu Gly Leu Asp Gly Val Asp Asn Ala

808 ACU CUG UUG UCG AAU UUA GGU CCA UUU UUG GUA CCU GUG AAA UGU CAA UAU GAA AAA UGU CCA ACA CCA ACC AUG GCG AUU CCU CCG GAU
Thr Leu Leu Ser Asn Leu Gly Pro Phe Leu Val Pro Val Lys Cys Gln Tyr Glu Lys Cys Pro Thr Pro Thr Ile Ala Ile Pro Pro Asp

898 UUA AAU CGU GCU ACU GAU CGU GUU GAU AUU AAU UUA GUU CAA UCC AUU UGU GAC UGC ACU CUG CCC ACU CAU AGU AAU UAC GAC GAC UCU
Leu Asn Arg Ala Thr Asp Arg Val Asp Ile Asn Leu Val Gln Ser Ile Cys Asp Ser Thr Leu Pro Thr His Ser Asn Tyr Asp Asp Ser

988 UUU CAU CAA GUG UUC GUC GAA AGU GCA GAC UAC UCU AUA GAU CUG GAU CAU GUU AGA CUU CGA CAG UCU GAU CUU AUC GCA AAA AUU CCA
Phe His Gln Val Phe Val Glu Ser Ala Asp Asp Tyr Ser Ile Asp Leu Asp His Val Arg Glu Ser Arg Val Leu Ile Ala Lys Ile Pro

1078 GAU UCA GCG CAU AUG AUA CCG GUU CUG AAG GCG AGC GGU CAC AAG AGA GUA GGU ACA CCG AAG GAG GUC CUU ACA GCA AUU AUC GAA AAA
Asp Ser Gly His Met Ile Pro Val Leu Asn Thr Gly Ser Gly His Lys Arg Val Gly Thr Thr Lys Glu Val Leu Thr Ala Ile Lys Lys

1168 CGU AAU GCU GAC GUU CCA GAG CUA GGU GAU UCC GUU AAU CUG UCU AGA UUG AGU AAA GCU GUG GCU GAG AGA UUC UUU AUU UCA UAC AUC
Arg Asn Ala Asp Val Pro Glu Glu Ser Arg Leu Ser Lys Ala Val Ala Glu Phe Ser Val Ser Asp Phe Val Ser Asp Phe Tyr Ile

1258 AAU GGU AAC UCU CUA GCA UCC AGU AAC UUU GUC AAU GUC GUU AGU AAU UUC CAC GAU UAC AUG GAA AAG UGG AAG UCC ACA GGU CUU UCU
Asn Gly Asn Ser Leu Ala Ser Ser Asn Phe Val Asn Val Val Ser Asn Phe His Asp Tyr Met Glu Lys Trp Lys Ser Ser Gly Leu Ser

1348 UAU GAU GAU CUU CCG GAU CUU CAU GCU GAG AAU UUG CAG UUU UAU GAC CAC AUG AUA AAA UCC GAU GUG AAA CCU GUG GUG AGC GAC ACA
Tyr Asp Asp Leu Asp Asp Leu His Ala Glu Asn Leu Gln Phe Tyr Asp His Met Ile Lys Ser Asp Val Lys Pro Val Val Ser Asp Asp Thr

1438 CUC AAU AUC GAC AGA CCG GUU CCA GCU ACU AUA ACG UAU CAU AAG AGU AUA ACC UCC CAG UUC CCA CCG UUA UCA CCG ACA CCG CUA UUC
Leu Asn Ile Asp Arg Pro Val Pro Ala Thr Ile Thr Tyr His Lys Lys Ser Ile Thr Ser Gln Phe Ser Pro Leu Phe Thr Ala Leu Phe

1528 GAG CGC UUC CAG AGA UGC CUU CGA GAA CGC AUU AUU CUU CCU GUU GGU AAG AUU UCA UCC CUU GAG AUG GCA GGA UUU GAU GUC AAA AAC
Gln Phe Glu Lys Leu Leu Phe Ser Gly Arg Glu Ile Ile Leu Pro Val Gly Lys Ile Ser Ser Leu Glu Met Ala Glu Phe Asp Val Lys Asn

1618 AAA CAC UGC CUC GAG AUU GAU UUG UCU AAG UUU GAU AAG UCU CAA GGU GAA UUU CAC UUG CUA AUC CAG GAA CAC AUU UUG AAU GGU CUA
Lys His Cys Leu Glu Ile Asp Leu Ser Lys Phe Asp Lys Ser Gln Gly Glu Phe His Leu Leu Ile Gln Glu His Ile Leu Asn Gly Leu

1708 GGU AUC AUC GCU CCG AUA AUC UGG UGG UGU GAU UUC CUA UUC UCU UAC AUC AGA GAC CGU GCU GGU GUU GGU AUC CUC AUU
Gly Cys Pro Ala Pro Ile Thr Lys Trp Trp Cys Asp Phe His Arg Phe Ser Tyr Ile Arg Arg Arg Arg Ala Gly Val Gly Met Pro Ile

1798 AGU UUC CAG AGA CGA ACU GGU GAU GCA UUC ACU UAU UUU GGC AAU ACC AUU GUC ACC AUG GCU GAG UUU GGC UGU UAU GAC ACC GAC
Ser Phe Gln Arg Arg Thr Gly Asp Ala Phe Thr Tyr Phe Gly Asn Thr +++ +++ +++ +++ +++ +++ +++ +++ +++ +++ +++ +++

1888 CAA UUC GAA AAG CUU UUA UUC UCA GGC GAU GAU UCU CUA GGA UUU UCA CUG CUU CCC CCU GUU GGU GAC CCG AGU AAA UUC ACA ACU CUU
Gln Phe Glu Lys Leu Leu Phe Ser Gly Asp Ser Ser Leu Leu Pro Pro Val Gly Asp Pro Ser Lys Phe Thr Thr Leu
+++ +++ +++ +++ Polymerase site +++ +++ +++ +++ +++ +++ +++ +++ +++ +++ +++ +++

1978 UUC AAC AUG GAA GCC AAG GUG AUG GAA CCU GCA GUA CCA UAU AUU UGU UCG AAG UUC UUA CUC UCU GAC GAG UUC GGU AAC ACA UUU UCC
Phe Asn Met Glu Ala Lys Val Met Glu Pro Ala Val Pro Tyr Ile Cys Ser Lys Phe Leu Leu Ser Asp Glu Phe Gly Asn Thr Phe Ser

2068 UGU CCA GAU CUA UUG CCG GAG GUU CCA CCG UUA GGA ACA AAG AAU AUU CCC UAU UCU GAC AAU GAU GAA UUC UUG UUU GCU CAC UUC AUG
Val Pro Asp Pro Leu Arg Glu Val Gln Arg Leu Gly Thr Lys Lys Ile Pro Tyr Ser Asp Asn Asp Glu Phe Leu Phe Ala His Phe Met

2158 AGC UUU GUU GAU CGA UUG AAG UUU UUG GAC CGA AUG UCU CAG UCG UGU AUC GAU CAA CUU UCG AUU UUC UUC GAA UUG AAA UAC AAG AAG
Ser Ile Phe Phe Glu Leu Lys Tyr Lys Lys Arg Ile Ile Leu Glu Ala Ala Leu Met Leu Gly Ala Phe Lys Lys Tyr Thr Ala Asn Phe Gln

2248 UCU GCG GAA GAG GCU GCU UUA AUG CUA GGC GCC UUU AAG AAG UAU ACC GCU AAU UUC CAG UCC UAC AAA GAA CUC UAU UAU UCA GAU CGU
Ser Gly Glu Glu Ala Ala Leu Met Leu Gly Ala Phe Lys Lys Tyr Thr Ala Asn Phe Gln Ser Tyr Lys Glu Leu Tyr Tyr Ser Asp Arg

2338 CGU CAG UGC GAA UUG AUC AAU UGU UGU ACA GAG UUC AGG GUU GAG CGU GUA AAU UCC AAU AAA CAG CCA AAG AAA UAU GGA AUU
Arg Gln Cys Glu Leu Ile Asn Ser Phe Cys Ser Thr Glu Phe Arg Val Glu Arg Val Asn Ser Asn Lys Gln Arg Lys Lys Tyr Gly Ile

2428 GAA CGU AGG UGC AAU GAC AAA CGU CGA ACU CCA ACU GGC UCG UAU GGU GGA GGC GAA GAA GCA GAG ACG AAG GUC UCA CAA ACA GAA UCG
Glu Arg Arg Cys Asn Asp Lys Arg Arg Thr Pro Thr Gly Ser Tyr Gly Gly Glu Glu Ala Glu Thr Lys Val Ser Gln Thr Glu Ser

2518 ACG GGA ACG AGG UCA CAA AAG UCC CAG CGA GAG AGC GCG UUC AAA UCU CAG ACU GUU CCG CUU CCU ACC GUU CUA UCA AGU AGA UGG UUC
Thr Gly Thr Arg Ser Gln Lys Ser Gln Arg Glu Ser Ala Phe Lys Ser Gln Thr Val Pro Leu Pro Thr Val Leu Ser Ser Arg Trp Phe

2608 GGA ACU GAC AGG GUC AUG CCG CCA UGC GAA CGU GGC GGA GUC ACC CGA GCC UGA GGCCUUCUGUUUAGAGUUAUCGCGGGAAGACCAUGAUUUUAGACGAUA
Gly Thr Asp Arg Val Met Pro Pro Cys Glu Arg Gly Gly Val Thr Arg Ala END

2709 CAGAUUGGUUCCCGGUAACGAUUGGCGGAAGUGCUUUCUGAAUCCUCCUCCUCCGUAUUCUCCUCCGUGUUUUCUGGCGGAGUCAGUUGGCAGUAUUGCUUAAUUCUUCUGA

2828 AGUCACUAAACACUUGUGGUGAAGCGGUUGUCCUACCGAGUUCUACGUGUAGAGAAUUCUACGCCAGCAGACUUAACAGUCUCUAGGCGACCUUUGAAACCAUUCU

2947 CCUAGGUUUCUUCGGAAGGACUUCGUGCGGUGUACUUCAGCAACCGGUGUAGUUCUUCAGGUGUAGGUGUCCGCCACUUCUGGCGGCGUCCAAAGAGACCA

Fig. 2. Nucleotide sequence of CMV-Y RNA2 and amino acid sequence of the protein encoded by the RNA. "Polymerase site" (GDD and TGxxxTxxxNT consensus sequences) are boxed by heavy line. Hydrophobic residues flanking to the "polymerase site" are underlined by (+++). Amino acid replacements found in CMV-Fny RNA2 are indicated by (==).

RNA was digested by RNaseH. Second strand cDNA was synthesized using the Klenow fragment in the presence of a specific primer which was complementary to the 3' region of the first strand and contained additional nucleotides to create a unique restriction site, *Pst*I (Fig. 1).

Computer analysis. The Microgenie sequence analysis program (Beckman) was used in the analysis of the CMV-Y RNA2.

RESULTS AND DISCUSSION

The cloning and sequencing strategies for CMV-Y RNA2 were shown in Fig. 1. The first set of overlapping cDNA clones, designated pBCY21, pBCY22 and pBCY23, were used for the determination of the nucleotide sequence for the RNA. Last four nucleotides at the 3' terminus, 5'-ACCA_{OH}, were lost in pBCY21, possibly excised by the exonuclease activity of *E. coli* DNA polymerase I used for the synthesis of a second strand cDNA, and pBCY23 was shown to lack about 150 nucleotides at the 5' terminus of the RNA by sequence determination. Therefore, the second set of overlapping clones, designated pCY2N5 and pCY2C15, were synthesized to create a full-length clones of RNA2. These two clones would facilitate any future reengineering of the inserts, such as the transfer of them to transcription vectors, pUT118 or pUT118-GG⁹), for the precise start of *in vitro* transcription after these clones were connected at the position of *Xho*I (Fig. 1).

The complete nucleotide sequence of CMV-Y RNA2 is shown in Fig. 2. It consisted of 3,051 nucleotides long and contained a long ORF of 2,571 nucleotides encoding a 96,720 Mr protein (857 amino acids). It is known that there is a GDD consensus amino acid sequence flanked by hydrophobic residues and preceded by the sequence TGxxxTxxxNT, so called "polymerase site", in deduced protein sequences from both animal and plant viruses¹³). This common sequence are also found in the protein encoded by CMV-Y RNA2 and the conserved amino acids are boxed in Fig. 2. This fact suggests that the protein encoded by CMV-Y RNA2 would be involved in virus RNA replication.

Nucleotide sequence alignment of three CMV RNA2s is shown in Fig. 3. Variations found among these CMV RNA2s were summarized in Table 1 in nucleotides and amino acids. Comparing Y-RNA2 with Fny-RNA2, homologies are calculated to be 98.4% at the nucleotides and 99.5% at the amino acids of the protein. The amino acid replacements are found at only 4 positions, which are also mapped by underlines in Fig. 2, and only one nucleotide deletion is found in the 5' non-coding region of Fny-RNA2. By contrast, many variations are found between Y-RNA2 and Q-RNA2, and the nucleotide sequences and corresponding translation products of the RNAs of the both strains have overall homologies of 71.4% and 74.4%, respec-

Table 1. Differences found in the RNA2s of CMV-Fny and CMV-Q compared to that of CMV-Y

Region	5' non-coding		ORF		3' non-coding		Total	
Length ^{a)}	87		2571		393		3051	
CMV strain	Fny	Q	Fny	Q	Fny	Q	Fny	Q
Length ^{b)}	86	92	2571	2517	393	426	3050	3035
Insertion	0	5	0	4	0	53	0	62
Deletion	1	0	0	58	0	20	1	78
Base Change	2	23	38	669	4	105	44	797
A.A. Rep. ^{c)}	—	—	4	201	—	—	4	201

a) Number of nucleotides in CMV-Y RNA2.

b) Number of nucleotides in RNA2s of CMV-Fny (Fny) and -Q (Q) strains.

c) Amino acid replacement.

tively. The strains of CMV are reported to fall into two Subgroups I and II on the basis of serology⁴⁾ and nucleic acid hybridization^{5,17)}, and CMV-Fny and CMV-Q have been grouped to Subgroups I and II, respectively¹⁹⁾. From the nucleotide sequence homology, CMV-Y is considered to belong to Subgroup I.

We are now constructing a full-length cDNA using the clones of pCY2N5 and pCY2C15, in order to produce biologically-active RNAs *in vitro*. This construct will be useful for understanding the function(s) of CMV RNA2.

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和 文 摘 要

片岡二郎・増田 税・高浪洋一：キュウリモザイクウイルス Y 系統 (CMV-Y) RNA2 の全塩基配列

CMV RNA2 の完全鎖長に相同な連続した cDNA をおもにガブラー & ホフマン法により作出し、その全塩基配列をダイデオキシ法により決定した。5' および 3' 末端の cDNA にはそれぞれ制限酵素 *Pst*I と *Nor*I の認識配列を合成プライマーにより付加した。RNA2 は 3,051 塩基よりなり、その翻訳産物には GDD で示される polymerase site と一致する配列が存在し、その近傍の配列の特徴もよく保存されていた。他の CMV 系統 RNA2 と塩基配列の比較を行ったところ、Y 系統は Fny 系統ときわめて高い相同性 (98.4%) を示したが Q 系統との相同性 (71.4%) はあまり高くなかった。これらの結果から CMV-Y は CMV-Fny が属する Subgroup I に分類されるものと考えられる。