One Amino Acid Change in Cucumber Mosaic Virus RNA Polymerase Determines Virulent/Avirulent Phenotypes on Cowpea

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Accepted for publication 5 August 1999.

ABSTRACT

Karasawa, A., Okada, I., Akashi, K., Chida, Y., Hase, S., Nakazawa-Nasu, Y., Ito, A., and Ehara, Y. 1999. One amino acid change in cucumber mosaic virus RNA polymerase determines virulent/avirulent phenotypes on cowpea *Phytopathology* 89:1186-1192.

The elicitation of the hypersensitive response (HR) is known to depend on the interaction between a resistance gene of a host plant and a corresponding avirulence gene of a pathogen. The cv. Kurodane-Sanjaku of cowpea (*Vigna unguiculata*) has the *Cry* locus that confers resistance against cucumber mosaic virus strain Y (CMV-Y). The resistance is overcome by infection with a legume strain of CMV (CMV-L). RNA 2, which codes for the 2a protein, a subunit of the viral replicase components, has been known to control virulent/avirulent phenotypes. We generated chimeric constructs of full-length cDNA clones of RNA 2 of both strains and

inoculated infectious transcripts to delimit the domain controlling symptoms. A 243-base pair fragment containing a coding region for the GDD RNA-dependent RNA polymerase core sequence was shown to be responsible for the phenotypic differences. From sequence alignment analysis, two amino acids (Phe631 and Ala641) of the HR-type 2a protein encoded in this fragment were specifically exchanged to Tyr and Ser, respectively, in the 2a proteins of resistance-breaking strains. Point mutations introduced into RNA 2 backgrounds of both strains that were designed to change the amino acid at position 631 resulted in a change of symptoms, indicating that a single nucleotide change determines the reactions elicited by both strains. Analysis for one additional mutant RNA 2 showed that symptom determination may be correlated with the nature of the lateral chain of amino acid 631.

The hypersensitive response (HR) is one of the most extensively studied resistance reactions, in which cells invaded by a pathogen develop a diverse array of defense mechanisms including cell wall solidification, accumulation of antifungal compounds, and the activation of a cellular suicide mechanism to delimit pathogens within initial infection sites (8,17). The gene-for-gene hypothesis (7) predicts the existence of a plant-borne resistance (*R*) gene and a corresponding pathogen-borne avirulence (*avr*) gene. An elicitor molecule, which is thought to be a translation product of an *avr* gene or to be a by-product of a reaction catalyzed by an *avr* gene product, is thought to be recognized as a ligand by a receptor encoded by an *R* gene (receptor-ligand model) (1,23). This interaction leads to the activation of a signal transduction cascade necessary for the HR and a series of resistance responses. Many *avr* genes have been identified in, and isolated from, viral, bacterial, and fungal pathogens and, recently, several *R* genes were cloned from several plant species (9). *R* genes have common structural elements such as leucine-rich repeats and, in some cases, nucleotide binding sites, whereas *avr* gene products are diverse, without any apparent common structural element.

Cucumber mosaic virus (CMV) is the type member of the genus *Cucumovirus* of the family *Bromoviridae*. The virus has an icosahedral particle of a diameter of 28 nm and has a segmented genome of three single-stranded RNAs (21; Plant Viruses Online: Descrip-

tions and Lists from the VIDE Database is available on-line from the Australian National University, Canberra). RNA 1 is the largest segment of about 3,350 nucleotides (nt) and codes for the 1a protein. RNA 2 consists of about 3,050 nt and encodes the 2a protein. The 1a and 2a proteins are necessary for replication of CMV RNA (11,20). RNA 3 consists of about 2,200 nt and has two cistrons. The 3a protein, encoded by an open reading frame (ORF) located within the 5' half of RNA 3, is a movement protein involved in cell-to-cell movement of the virus (2,4,24). The second ORF in RNA 3, which codes for the viral coat protein (CP), is located within the 3' half of the RNA molecule and is expressed via the subgenomic RNA 4. Recently, a fifth ORF encoded by CMV RNA, the 2b protein, was shown to be expressed in infected plants and to affect both long-distance movement of the virus and symptomatology (5). Many of the CMV strains described thus far differ in their biological properties. Based on antigenic properties and sequence similarities, they have been divided into two subgroups, I and II (21).

Many CMV strains can infect cowpea (*Vigna unguiculata* (L.) Walp.) irrespective of their subgroup or strain designation, but the infection may be restricted in and around primary infection sites because of the HR. Confinement of the virus is exhibited as the formation of necrotic local lesions by 24 h after inoculation. Marchoux et al. (18), Hanada and Tochihiro (10), and Edwards et al. (6) showed that RNA 2 was a determinant for symptoms in cowpea by using a pseudorecombinant technique between the HR-inducing and HR-breaking strains. Kim and Palukaitis (15) showed that two amino acids in the 2a protein were responsible for the HR elicitation. Both Phe631 and Ala641 were required for the complete HR elicitation. Ala641 alone could induce the HR, but viral confinement in lesions was not complete and systemic necrosis developed on inoculated plants.

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Publication no. P-1999-1001-01R

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We have also been analyzing interactions between CMV and cowpea. Recently, we found that the *Cry* locus, inherited as a single dominant trait, occurs in a resistant cowpea cultivar as an *R* gene (19). The resistance is overcome by infection with a resistance-breaking strain such as the legume strain of CMV (CMV-L). In our previous report, we used infectious transcripts derived from full-length cDNAs of genomic RNAs of the yellow strain of CMV (CMV-Y, HR-inducing strain) and CMV-L to demonstrate that RNA 2 determined phenotypic differences on cowpea (12). Both CMV-Y and CMV-L belong to subgroup I and highly resemble each other at the nucleotide and amino acid sequence levels. Here, we determined the nucleotide sequence of CMV RNA 2 responsible for the virulent/avirulent phenotypes. Our results both confirmed and differed from those of Kim and Palukaitis (15). Symptom induction in cowpea by CMV infection was further analyzed using several RNA 2 mutants expressing different amino acids at position 631. We suggest the possibility that the nature of the amino acid's side chain at position 631 of the 2a protein correlates with symptom expression.

MATERIALS AND METHODS

Plants and viruses. Cowpea cvs. Kurodane-Sanjaku (genotype: *Cry/Cry*, a cultivar responding with the HR) and PI 189375 (genotype: *cry/cry*, a cultivar without the HR) were used throughout this study. Each cultivar was grown and assayed for CMV infection as described previously (19). CMV-Y, CMV-L, and CMV mutants were maintained in tobacco plants (*Nicotiana tabacum* L. cv. Xanthi nc) and purified as described (19).

cDNA clones, infectious transcripts, and inoculation. Protocols for general recombinant DNA techniques were according to

Sambrook et al. (22) unless mentioned here. A full-length cDNA clone of CMV-Y genomic RNA 1 (pCY1-T7) was a gift from M. Suzuki (Life Science Institute, Japan Tobacco, Inc.; current address: Graduate School of Agricultural and Life Science, The University of Tokyo). The CMV-Y RNAs 2 and 3 clones were described in a previous study (pCY2T7 and pCY3T7, respectively) (12). CMV-L full-length cDNAs (pCL1T7, pCL2T7, and pCL3T7) were obtained as described previously (12). The RNA 2 of both strains show a high degree of sequence identity (over 96%). By using common restriction sites, we constructed several chimeric DNAs between pCY2T7 and pCL2T7 (Fig. 1). cDNA fragments separated by agarose gel electrophoresis were recovered using a Prep-A-Gene DNA Purification Kit (Bio-Rad Laboratories, Hercules, CA). Transcription and inoculation with infectious RNAs were as described elsewhere (12). Transcripts are described as strain name followed by RNA segment name (e.g., Y1 to represent RNA 1 of CMV-Y). All chimeric transcripts were propagated with Y1Y3 transcripts (except as noted) in tobacco plants and virions were prepared.

Site-directed mutagenesis. To introduce point mutations into the Y2 or L2 background, polymerase chain reaction (PCR)-based mutagenesis was carried out using an LA-PCR in vitro Mutagenesis Kit (Takara Shuzo Co., Ohtsu, Japan) and the following mutagenesis primers: L2Y631F-S641A (5'-TTTTCAACATGGAAGCTAAGGTGATGGAACCTGCAGTACC-3') for pCL2FA, L2Y631F (5'-CACGACTCTTTCAACATGG-3') for pCL2F, L2S641A (5'-ACCTGCAGTACCATATTTGC-3') for pCL2A, Y2F631Y-A641S (5'-CTCTTTACAAACATGGAAGCCAAGGTGATGGAACCTTCAGTA-3') for pCY2YS, Y2F631Y (5'-CACAACCTCTTTACAACATGG-3') for pCY2Y, Y2A641S (5'-GTGATGGAACCTTCAGTACC-3') for pCY2S, and Y2W(UGG) (5'-CACAACCTCTTTGGAACATGG-3')

Fig. 1. Construction of chimeric cDNAs for RNA 2 and inoculation on cowpea cultivars. Infectious transcripts used for inoculation are listed in the inocula column. Portions derived from cucumber mosaic virus strain Y (CMV-Y) RNA 2 are shown by white bars and those from legume strain (CMV-L) RNA 2 by black bars in the RNA 2 constructions column. Borders in which RNA 2 are combined are indicated by restriction sites in each construction pair. Positions of the two amino acids showing a specific difference in the 2a proteins are indicated by solid black lines. All inoculations resulted in systemic symptoms on cv. PI 189375 (genotype: *cry/cry*) (data not shown). Symptoms on cv. Kurodane-Sanjaku (*Cry/Cry*) are indicated as those on inoculated leaves/those on noninoculated trifoliates. NLL = necrotic local lesions, CS = chlorotic spots, NS = necrotic spots, SM(N) = systemic mosaic with necrosis, and - = no visible symptoms.

for pCY2W(UGG). Mutant transcripts were propagated with L1L3 or Y1Y3 transcripts, and virions were recovered. All mutant clones were sequenced both at the cDNA and virion RNA levels using a DyeTerminator Cycle Sequencing FS Kit (Perkin-Elmer Applied Biosystems, Foster City, CA).

Analysis for HR-specific cowpea gene expression. As a molecular marker for elicitation of the HR, we analyzed CpPR3 mRNA gene expression. CP8 is a cDNA clone for CpPR3 (DDBJ/GenBank/EMBL accession no. AB027154) (25) that is a pathogenesis-related (PR) protein whose accumulation begins 8 to 9 h postinoculation and is abundant in 'Kurodane-Sanjaku' cowpea. Accumulation of CpPR3 mRNA in CMV-inoculated cowpea primary leaves was investigated by reverse-transcriptase (RT)-PCR, in which 2 µg of total RNA prepared by the hot-phenol method was used as a template as described previously (12).

Western blot analysis for CP accumulation in cowpea plants. Accumulations of the CP in inoculated leaves 5 days post-inoculation (dpi) and in noninoculated first trifoliate at 14 dpi were assayed by Western analysis of 5 µg of crude leaf protein soluble in 10 mM Tris-HCl, pH 7.5. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16), blotted onto Hybond-ECL membrane (Amersham International plc., Buckinghamshire, United Kingdom), and probed with anti-CMV-Y CP monoclonal antibody (MAb) 4 (S. Hase, A. Karasawa, and Y. Ehara, *unpublished data*), which was shown to react to CMV-Y and CMV-L with similar intensity under our standard assay conditions. CMV CP was specifically detected

by the ECL Detection System (Amersham International plc.) according to the manufacturer. After exposure to X-ray films, signals were compared with one for 10 ng of virion as a standard and estimated to be low (signals weaker than the standard) or high (signals stronger than the standard).

RESULTS

The sequence between the *HindIII*-*EcoRI* sites and induction of symptoms. CMV-Y and CMV-L are both subgroup I strains, which implies that both strains share a high degree of homology to each other. In fact, sequence analyses of both CMVs show homology values of more than 94% at the nucleotide level (12), irrespective of extreme differences in pathogenicity on cowpea (Fig. 2A and E). Our target molecules, RNA 2 and the encoded 2a proteins, share 96.5 and 98.1% identities, respectively, and there are many common recognition sites for restriction endonucleases in their cDNAs (data not shown). To identify the symptom-determining domain on RNA 2 sequence, we constructed several chimeric plasmids between pCY2T7 and pCL2T7 (12). Resultant hybrids were assayed with pCY1-T7 and pCY3T7 for the ability of their respective transcripts to induce symptoms on cowpea plants. All transcripts were inoculated onto tobacco plants as a propagation host, and purified virions (10 µg/ml) were used for assay for symptoms and accumulation of the CP on cowpea. Hereafter, nucleotide positions for restriction endonucleases indicate the 5' termini of the recognition sites. RNA segments of CMV are abbreviated as L2, Y2, and so on.

Fig. 2. Symptoms induced on primary leaves of resistant cv. Kurodane-Sanjaku infected with parental cucumber mosaic virus (CMV) strains or CMV mutants. Each mutant contained legume strain (CMV-L) RNAs 1 and 3 and mutated RNA 2 and was used as purified virions at 10 µg/ml. Photograph were taken at 5 days after inoculation. **A**, CMV-L; **B**, L2FA mutant; **C**, L2F mutant; **D**, L2A mutant; **E**, CMV-Y; **F**, Y2YS mutant; **G**, Y2Y mutant; and **H**, Y2S mutant. Necrotic local lesions were induced in **B**, **C**, **E**, and **H**, whereas chlorotic spots were in **A**, **D**, **F**, and **G**.

KpnI recombinant LY2K, which consists of L2 from its 5' terminus to the *KpnI* recognition site (nucleotide position 837 of Y2) ligated to Y2 from the *KpnI* recognition site to the 3' terminus, induced the HR on the resistant cv. Kurodane-Sanjaku (Fig. 1). However, YL2K, the reverse construct of LY2K, induced systemic symptoms on 'Kurodane-Sanjaku'. Next, we used an *XbaI* recognition site to combine two DNAs at approximately the center of the 2a proteins. LY2XBBH, which has the 5' terminus to the *XbaI* recognition site (nucleotide position 1,210) of L2 and *XbaI* to the 3' terminus of Y2, induced the HR on the resistant cowpea, indicating that the 3' terminal 1,800 nt were responsible for inducing the HR. When the *HindIII* recognition site (nucleotide position 1,897) was used for recombination, the resultant YL2HD, which contains a Y2-derived sequence less than 40% of the full length, could induce the HR. However, inoculation with LY2ERI tran-

scripts resulted in systemic infection of 'Kurodane-Sanjaku' plants, showing that a region from the *EcoRI* site (nucleotide position 2,134 of Y2) to 3' terminus was not sufficient for induction of the HR. All of these results suggested that the sequences between the *HindIII* and *EcoRI* recognition sites determined the infection phenotype.

To confirm the responsibility of the sequence between the *HindIII*-*EcoRI* sites for symptom changes, the fragments were exchanged reciprocally. LY2HE is a recombinant having the *HindIII*-*EcoRI* fragment of Y2 in the L2 background and was inoculated with L1 and L3 to the *Cry/Cry* cultivar. This recombinant induced development of necrotic local lesions on the inoculated primary leaves, whereas no symptoms were observed in the noninoculated upper leaves (Fig. 1). In contrast, YL2HE, which has the corresponding region of L2 in the Y2 background, systemically infected the resistant cowpea when inoculated with Y1 and Y3 (Fig. 1). It was evident that the 243 base region of Y2 was necessary and sufficient for induction of the HR on the *Cry/Cry* cowpea.

Sequence analysis of *HindIII*-*EcoRI* fragments of several CMV strains. The *HindIII*-*EcoRI* fragment of CMV-Y RNA 2 cDNA is 243 base pairs (bp) long and codes for a portion of the 2a protein. This region of the 2a protein includes the conserved RNA-dependent RNA polymerase core sequence (GDD sequence). When compared with CMV-L RNA 2 cDNA, there are substitutions of four nucleotides, three of which result in changes at the amino acid level (Fig. 1). A Gly at position 625 of the CMV-Y 2a protein is encoded by the codon GGU. The first G residue of this codon is different from that in the Y2 sequence originally reported by Kataoka et al. (13), as well as from the L2 sequence. In 2a protein sequences, a Ser625 is conserved in all strains or isolates except for the CMV-Y 2a protein (Fig. 3), suggesting that Ser/Gly625 is not likely to be the determinant for symptoms on cowpea. On the contrary, amino acid 631 is a Phe in the CMV-Y 2a protein, but it is a Tyr in the CMV-L 2a protein. An alanine of the CMV-Y 2a protein at position 641 is substituted with Ser in the CMV-L 2a protein. Interestingly, these amino acid substitutions were conserved in all of the 2a proteins of CMV correlating with the symp-

Fig. 3. Alignment analysis for the 2a proteins of several cucumber mosaic virus (CMV) strains comparing amino acids between positions 622 and 644. Strain names are indicated at the left side. L, MB-8, and B are CMVs that infect cowpea systemically, whereas Y, O, Fny, and Q are those infecting with a hypersensitive response. Shaded areas are amino acids that are the same as those of the legume strain (CMV-L). Amino acid substitution at position 625 is specific for CMV strain Y (CMV-Y), but differences at positions 631 and 641 are conserved among CMV strains corresponding with symptoms on cowpea. Accession numbers of nucleotide sequences are L, D16406; MB-8, D86613; B, U59740; Y, this article; O, D10209; Fny, D00355; and Q, X00985.

			Symptoms on Kurodane-Sanjaku (Inoculated/Non-inoculated)
CMV-Y RNA 2	1950- GGUGACCCGGUAAAUUCACAAUCUUUUCAACAUUGGAAGCCAAGGUGAUGGAACCGCAGUACCAUUAU	-2020	
CMV-Y 2a protein	621- G D P S K F T T L F N M E A K V M E P A V P Y	-645	NLL/-
Y2YS transcript	-----G-----A-----AU-----		CS/M
Y2Y transcript	-----G-----A-----UG-----		CS/M
Y2S transcript	-----G-----U-----AU-----		NLL/-
CMV-L RNA 2	1948- -----A-----A-----AU-----	-2018	
CMV-L 2a protein	621- - - - G - - - - Y - - - - S - - - -	-645	CS/M
L2FA transcript	-----A-----U-----UG-----		NLL/-
L2F transcript	-----A-----U-----AU-----		NLL/-
L2A transcript	-----A-----A-----UG-----		CS/M

Fig. 4. Nucleotide and amino acid sequences of mutated cucumber mosaic virus (CMV) RNA 2 and symptoms on cv. Kurodane-Sanjaku cowpea. Sequences of strain Y (CMV-Y) RNA 2 and the 2a protein are shown on the top two lines, and those of the legume strain (CMV-L) or each mutant are indicated below. Sequences that are the same as CMV-Y are shown by -; only those different from CMV-Y are given. Symptoms induced on cv. Kurodane-Sanjaku cowpeas inoculated with CMV-L RNAs 1 and 3 together are shown as symptoms on inoculated leaves in Figure 2. Symptoms on noninoculated upper leaves are indicated as follows: NLL = necrotic local lesion, CS = chlorotic spot, - = no symptoms, and M = mosaic.

tom induction pattern; an F631 and an A641 were common in the 2a proteins of CMV strains that infected cowpea with the HR, whereas a Y631 and a S641 were conserved in the 2a proteins of CMV-L, MB-8 (12), and strain B (15) (Fig. 3). These results suggest the responsibility of such amino acid substitutions for the phenotype of CMV on cowpea plants.

Analysis of site-directed mutants. To localize the sequence necessary for phenotype determination, we introduced point mutations into RNA 2 cDNAs of both strains by a PCR-based site-directed mutagenesis protocol. The resultant mutagenized clones were sequenced and confirmed to have the native sequences in the nonmutagenized regions preserved. All mutant transcripts were recovered as virions and sequenced at virion RNA level.

Transcripts from pCL2FA, which contained a Phe at amino acid position 631 and an Ala at position 641 in the L2 background (Fig. 4), were propagated with L1 and L3 in tobacco and inoculated onto the primary leaves of cowpea as purified virions (10 µg/ml). At 1 dpi, we observed necrotic local lesions on inoculated leaves of the *Cry/Cry* cowpea. The lesions enlarged slightly over the experimental period (Fig. 2B), as seen on CMV-Y-infected cowpeas. No symptoms appeared on the noninoculated upper leaves. Necrotic local lesion symptoms on inoculated leaves were indistinguishable from those induced by CMV-Y (Fig. 2E). To identify which mutations introduced into L2FA were responsible for the

TABLE 1. Summary of analyses for coat protein (CP) accumulation in cowpea infected with cucumber mosaic virus (CMV) RNA 2 mutants

CMV strains or RNA 2 mutants ^a	CP ^b			
	Inoculated leaves		Noninoculated leaves	
	Detection ^c	Amount ^d	Detection	Amount
CMV-Y	8/8	+	0/8	–
CMV-L	8/8	++	8/8	++
L2FA	2/8	+	0/8	–
L2F	6/8	+	0/8	–
L2A	8/8	++	6/8	++
Y2YS	8/8	++	8/8	++
Y2Y	8/8	++	8/8	++
Y2S	5/8	+	0/8	–

^a Each RNA 2 mutant was transcribed from mutated cDNAs and used as inoculum along with L1 and L3 transcripts. Progeny virions were used for assay.

^b CP in inoculated cowpea was detected by ECL Detection System (Amersham International) and CMV-Y- and CMV-L-specific monoclonal antibody (MAb) 4 as described in text. This table represents two to three replicates.

^c Eight independent cowpea plants were inoculated in each inoculation. Number of plants accumulating CP/number of plants analyzed.

^d Amount of CP: – = not detected, + = low level, and ++ = high level, based on comparison with 10 ng of purified virions co-electrophoresed as a standard.

Fig. 5. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for CpPR3 mRNA expression in cowpea leaves inoculated with cucumber mosaic virus (CMV). Strain Y (CMV-Y; lane 1), a legume strain (CMV-L; lane 5) and CMV RNA 2 mutants were used to inoculate primary leaves of resistant cv. Kurodane-Sanjaku, and total RNAs were extracted from each plant at 24 h after inoculation. Total RNA from each inoculation (2 µg) was used for RT-PCR analysis and portions of the reaction were electrophoresed on 1% agarose gel in Tris-borate-EDTA buffer (22), followed by staining with ethidium bromide. CMV mutants: lane 2, Y2YS; lane 3, Y2Y; lane 4, Y2S; lane 6, L2FA; lane 7, L2F; and lane 8, L2A. PCR products of expected size are shown by arrowhead on the left side. Lane M, molecular weight marker (100-base pair ladder; New England Biolabs, Inc., Beverly, MA).

HR elicitation, each mutation was introduced separately (Fig. 4). L2F transcripts expressed a Phe at position 631 of the CMV-L 2a protein and induced the HR on the inoculated leaves (Fig. 2C), whereas L2A transcripts, which had an Ala at position 641 of the CMV-L 2a protein, failed to induce formation of necrotic local lesions. Instead, it caused systemic symptoms on the resistant cowpea similar to those induced by CMV-L (Fig. 2D). High levels of CP were detected by Western blot analysis in both inoculated and noninoculated leaves of the L2A-inoculated plants as well as in the CMV-L-inoculated cowpea (Table 1). Trace amounts of CP were found in the leaves inoculated with virions containing either L2FA or L2F RNAs, but not in the noninoculated leaves (Table 1). These results indicate that the mutation at nucleotide position 1,977 in L2 directing the expression of Phe at amino acid 631 was responsible for inducing the HR.

Subsequently, we developed a second set of site-directed mutants; these mutants had nucleotide substitutions in the Y2 background. Y2YS is a double-point mutant directed to have amino acids identical to the CMV-L 2a protein at amino acid positions 631 and 641 (Fig. 4). When this mutant transcript was used as inoculum with L1 and L3, we observed chlorotic spots on the inoculated cowpea leaves (Fig. 2F). Later, mosaic symptoms developed on noninoculated upper leaves (data not shown). Thus, symptoms observed on the L1Y2YSL3-inoculated cowpeas were identical to those induced by the CMV-L infection (Fig. 2A).

To evaluate the ability of each nucleotide substitution to induce systemic symptoms, we constructed the pCY2Y and pCY2S point mutants. The clone pCY2Y had a nucleotide substitution (T to A) at position 1,979, which resulted in the amino acid substitution of a Phe to a Tyr at position 631 (Fig. 4). Another mutant clone, pCY2S, was a derivative of pCY2T7 that had an AT dinucleotide at nucleotide position 2,007 to 2,008 instead of a TG. This mutant was predicted to express the 2a protein with a Ser at amino acid position 641. When Y2Y transcripts with L1 and L3 were used to inoculate cowpea primary leaves, systemic mosaic symptoms were observed (Fig. 2G) similar to those caused by Y2YS infection, whereas the symptoms induced by L1Y2SL3 infection were the same as those induced by CMV-Y infection (Fig. 2H). CMV CP accumulated to high levels both in inoculated and noninoculated leaves in plants in which systemic infection (Y2YS and Y2Y) as well as CMV-L infection was observed, whereas, in leaves showing the HR, trace amounts of the CP were detected, similar to that observed for CMV-Y infection (Y2S) (Table 1).

As another indicator for the HR, we investigated HR-specific gene expression. CP8 is a cDNA clone of the cowpea PR protein CpPR3. CpPR3 mRNA expression was detected in a HR-specific

Fig. 6. Symptoms induced by infection with cucumber mosaic virus RNA 2 mutant Y2W after inoculation with Y1 and Y3. **A**, Systemic necrosis observed on resistant cv. Kurodane-Sanjaku; **B**, symptoms on systemic cv. PI 189375.

manner in cowpea (12) using specific primers based on the CP8 sequence. In cowpea primary leaves inoculated with CMV isolates having point mutations in RNA 2, expression of CpPR3 mRNA was also correlated with the appearance of local necrotic lesions (Fig. 5). Faint DNA bands of about 600 bp were visible in several lanes, but these were confirmed to be amplified from genomic DNA contamination in total RNA samples (data not shown). CpPR3-specific PCR products 514 bp in size were detected in L2FA-, L2F-, and Y2S-inoculated cowpeas, as well as in those inoculated with Y2, whereas there were no specific PCR products in lanes in which systemic infections were analyzed.

Taking all results together, we concluded that the infection phenotype of CMV on the cowpea cv. Kurodane-Sanjaku was determined by one nucleotide change resulting in an amino acid replacement at position 631 of the 2a protein.

Correlation of induction of systemic symptoms with the nature of the lateral chain of the amino acid at position 631 of the 2a protein. The amino acid Tyr or Phe that occurs at position 631 of CMV 2a proteins both have side chains with hydrophobic and aromatic moieties. In fact, these two amino acids differ only in the presence of a hydroxyl group attached to the phenol ring of the Tyr lateral chain. Thus, Tyr is classified as a hydrophobic, aromatic, and polar amino acid, whereas Phe is a hydrophobic and aromatic amino acid. Trp is also classified as being a hydrophobic, aromatic, and polar amino acid. We constructed a mutant Y2W(UGG), which expressed a W631 specified by a UGG codon, to investigate the effect on symptom induction in cowpeas. When cowpea cvs. Kurodane-Sanjaku and PI 189375 were inoculated with Y1Y2W(UGG)Y3, cv. Kurodane-Sanjaku developed necrotic symptoms (Fig. 6). These symptoms were indistinguishable with those induced by Y1L2Y3 recombinants (12), indicating that induction of systemic mosaic symptoms on cowpea inoculated with CMV may be correlated with the nature of the lateral chains of amino acid 631 of the 2a protein.

CMV-Y RNA 3 is responsible for the induction of systemic necrosis on cv. PI 189375. 'PI 189375' developed systemic necrosis when infected with CMV-Y (19) (Table 2), although systemic mosaic without necrosis was observed on 'PI 189375' inoculated with CMV-L (Table 2). To identify the viral determinant or determinants responsible for the elicitation of systemic necrosis, we inoculated plants with pseudorecombinants between CMV-Y and CMV-L (Table 2). Systemic necrosis was observed on plants inoculated with inocula containing Y3, but not Y2. On 'PI 189375' cowpeas infected with CMVs containing Y2, necrotic areas were occasionally observed, but such symptoms were very weak, limited within inoculated leaves, and appeared 2 to 3 days later (Table 2) (data not shown). Thus, necrosis observed on 'PI 189375' was different from the unlimited HR against an avirulent RNA 2 as suggested by Kim and Palukaitis (15).

DISCUSSION

CMV RNA 2 has long been postulated to be a determinant for symptoms on cowpea plants since the pseudorecombinant experiments carried out by Marchoux et al. (18), Hanada and Tochiwara

(10), and Edwards et al. (6). Indeed, using genetically pure inocula, we also found that RNA 2 determines symptoms (12). In addition, alignment analysis of the 2a proteins of several CMV strains sequenced thus far, including CMV-L, MB-8, and CMV-Y, revealed differences of three amino acids that correlated with symptoms. The 2a proteins of resistance-breaking CMV strains have a Tyr512, Tyr631, and Ser641, whereas those of the HR-inducing CMV strains have a His, Phe, and Ala, respectively (12). Kim and Palukaitis (15) extended knowledge of the molecular details of the HR inducibility with their molecular analysis of RNA 2 of several CMV strains. They concluded that both amino acids F631 and A641 of the 2a protein were necessary to form authentic necrotic local lesions on cowpea. A641 was the sole *avr* factor to elicit the HR, but viral confinement in lesions was incomplete in the Y631-A641 combination, with the result being a systemic necrosis on infected plants.

Our results, described in this article, confirmed those of Kim and Palukaitis (15). We also focused on the amino acids at positions 631 and 641 of the 2a protein by analyzing chimeric cDNA constructs and the 2a protein sequences. However, we found a discrepancy among analyses for the 2a proteins. The F631 of the CMV-Y 2a protein was necessary and sufficient for full induction of necrotic local lesions on cowpea of the *Cry/Cry* background. From an inoculation test with our additional point mutant Y2W(UGG), we concluded that the nature of the side chain of amino acid 631 may play an important role for symptom determination. Computer-aided structural analysis (3) of the 2a proteins of CMV-Y and CMV-L showed that the amino acid at position 631 was in a β -sheet that joins a turn and a helix in either case, and Phe-to-Tyr or Tyr-to-Phe exchanges in CMV-Y or CMV-L 2a proteins, respectively, at this position did not result in any structural changes (data not shown). Thus, the polarity of the amino acid at position 631 may affect the structure of the 2a protein. Differences in the nature of side chains could also affect accessibility of host factors to the 2a protein. This idea fits recent findings for the receptor-ligand model (1,14).

Another discrepancy between our results and those of Kim and Palukaitis (15) is in regard to the induction of systemic necrosis in the F/A mutant-inoculated plants. The F/A mutant in their study was designed to have a Y631 instead of an F631 in the Fny-CMV RNA 2 background; this corresponds to our Y2Y mutant. However, we have never seen systemic necrosis in the Y2Y-inoculated cowpea, whereas similar necrotic responses were observed on L1L2Y3-inoculated plants (12). Recently, we mapped the necrosis-inducing activity to the CP gene (A. Karasawa, H. Takanami, Y. Chida, K. Akashi, S. Hase, H. Takahashi, and Y. Ehara, *unpublished data*). Systemic necrosis may be induced by two factors with systemic symptoms determined by L-RNA 2 (Y631 of the 2a protein) and necrosis by Y-RNA 3 (CP gene).

The reason(s) for the inconsistency observed between the work reported by Kim and Palukaitis (15) and our findings as described in this article are unclear. They may reflect the specificity of cultivar-CMV strain interactions. The cowpea cultivar used by Kim and Palukaitis (15) was not reported. We used the cv. Kurodane-Sanjaku cowpea (*Cry/Cry*), which responds hypersensitively to CMV-Y infection. Kim and Palukaitis (15) suggested that *Cry* was a gene involved in viral confinement in lesions, but was not a resistance gene involved in the HR elicitation. Their suggestions were based on the development of systemic necrosis on the susceptible cv. PI 189375 (*cry/cry*) infected with CMV-Y (19). However, as shown in Table 2, systemic necrosis on 'PI 189375' depended on the presence of Y-RNA 3, but not Y-RNA 2. This means that even if the systemic necrosis on CMV-Y-infected 'PI 189375' represents the HR without viral localization in lesions, it responds to the Y-RNA 3 sequence, which is not an *avr* factor in 'Kurodane-Sanjaku' cowpea. Thus, cowpea cultivars with or without the *Cry* locus have factor(s) that cause induction of necrotic responses. Therefore, we propose that the *Cry* locus confers specificity only to the 2a protein.

TABLE 2. Symptoms observed on 'PI 189375' cowpea inoculated with pseudorecombinants

Pseudorecombinants	Symptoms ^a	
	Inoculated leaves	Noninoculated leaves
Y1Y2Y3	NS, CS	N
L1L2L3	CS	M
Y1L2L3	CS	M
L1Y2L3	CS	M
L1L2Y3	NS, CS	N

^a NS = necrotic spots, CS = chlorotic spots, N = necrosis, and M = mosaic.

ACKNOWLEDGMENTS

This work is supported, in part, by the Sasakawa Scientific Research Grant from The Japan Science Society and by Grant-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan. We thank H. Takahashi, Tohoku University, and S. Nakamura, Miyagi Agricultural Research Center, for their help and critical reading of the manuscript; M. Suzuki, Graduate School of Agricultural and Life Science, The University of Tokyo, for his kind gift of CMV-Y RNA 1 transcription vector pCY1-T7; and T. Ishimizu, Division of Protein Chemistry, Institute for Protein Research, Osaka University, and M. Watanabe, Faculty of Agriculture, Iwate University, for their useful suggestions on protein structure. We also thank B. E. Hazen for her efforts to correct our English.

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