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Spatial analysis for exclusive interactions between subgroups I and II of *Cucumber mosaic virus* in cowpea

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Abstract

The dynamics of virus interference in *Cucumber mosaic virus* (CMV) infection in cowpea were investigated by tissue-blotting and in situ hybridization. Using co-inoculation assays, we discovered that spatial competition between CMV-LE (subgroup I) and CMV-m2 (subgroup II) occurred in the inoculated leaves. Interestingly, competitive interactions between the two viruses also could be observed in the non-inoculated upper leaf tissues of the plants. Furthermore, the pattern of exclusive distribution was observed between challenge and protecting viruses in the serially inoculated leaves. Taken together, it is suggested that the dynamics of competitive interactions between the two subgroups could be characterized by exclusive infection and multiplication of the individual viruses in cowpea plants.

Keywords: *Cucumber mosaic virus*; Exclusion; Cross-protection; Subgroup; Cowpea

Introduction

Cross-protection was first described by McKinney (1929) and the phenomenon has been characterized generally by competitive interactions between two related plant viruses in a host plant. Cross-protection is an effective strategy to protect plants from virus diseases (Fulton, 1986). The unifying feature of the cross-protection phenomenon is the prevention from further infection by a closely related virus. In an early study by Hull and Plaskitt (1970), it was found that the interval between inoculation of the protecting and the challenge strains affected the extent of cross-protection between the two strains of *Alfalfa mosaic virus*. For decades, it has been speculated that the molecular mechanisms of cross-protection were based on depletion of host-derived precursors indispensable for formation of virus structure, inhibition of uncoating and/or genome amplification of the challenge virus, and induction of extreme host resistance by which multiplication of the challenge virus is suppressed (Hull, 2002).

In the 1980s, it was discovered that plants transformed by nucleotide sequences coding for virus genes showed resistance to the parental viruses (Goldbach et al., 2003). During the last decade, various models for pathogen-derived resistance induced by the transgenic virus RNA or protein have been proposed (Baulcombe, 1996; Goldbach et al., 2003; Lomonossoff, 1995; Palukaitis and Zaitlin, 1997; Sanford and Johnston, 1985). It was suggested that the phenomenon used the same mechanism as cross-protection. In the last few years, it has been recognized generally that posttranscriptional gene silencing (PTGS) plays a key role in viral RNA-mediated resistance in plants (Cogoni and Macino, 2000; Baulcombe, 2002; Goldbach et al., 2003; Ratcliff et al., 1999; Waterhouse et al., 2001).

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Cucumber mosaic virus (CMV) has a tripartite genome of single-stranded RNAs of plus sense that are packaged in small spherical virions (Palukaitis and García-Arenal, 2003; Palukaitis et al., 1992). The two subgroups I and II generally show about 75% sequence similarity to each other, whereas strains in the same subgroup show 90–99% sequence identity. Cross-protection among CMV strains was reported first by Tomaru et al. (1967). Dodds (1982) showed that the level or absence of interference in the inoculated leaves did not affect systemic cross-protection, and that co-inoculation resulted in local and systemic mixed infections and reduction in the synthesis of both strains. Dodds et al. (1985) reported that breakdown of cross-protection occurred only in the leaves directly inoculated with the challenge strain RNA, by detection of the dsRNAs for the two strains of CMV. However, our understanding of cross-protection has been limited by technical barriers due to the similarities between the two subgroups such as extensive sequence similarity of the genomic RNAs, overlapped host range, indistinguishable symptomatic phenotype, and close serological relationships. To overcome such barriers, we have developed subgroup-specific oligonucleotide cDNA probes (Takanami et al., 1999; Takeshita et al., in press). In this study on the competition between CMV-LE (Tomaru and Udagawa, 1967; LE, subgroup I) and CMV-m2 (Takanami et al., 1998; Tomaru and Hidaka, 1960; m2, subgroup II) in cowpea plants, we have employed new tools for hybridization analyses to better understanding the nature of the interference between the two subgroups of CMV.

In a recent study, Dietrich and Maiss (2003) analyzed the distribution of viruses carrying monopartite genome RNA at the cellular level in *Nicotiana benthamiana* plants. Spatial separation between the viruses was observed in the plants by co-inoculation of either the same viruses differently labeled or different potyviruses. In the mixed inoculation assays with LE and a reassortant LLm consisting of RNA1 and RNA2 from LE, and RNA3 from m2, we have observed that LE RNA3 and RNA4 accumulated predominantly in cowpea (cv. Kurodane-sanjyaku) (Takeshita et al., in press). In this work, we determined the type of spatial interactions between LE and m2 in another cultivar of cowpea (cv. PI 189375), which they both can infect systemically. To the best our knowledge, this is the first report that provides the molecular biological evidence for spatial competition between subgroups I and II of CMV in host plants. Finally, the biological significance of the interfering events between the viruses is discussed.

Results

Separate distributions of LE and m2 in the inoculated leaves

To compare the cell-to-cell movement of LE with that of m2, simultaneously or serially inoculated cowpea leaves (PI 189375) were collected and then were subjected to tissue-blot analysis using the subgroup-specific probes (Fig. 1). From the blotting patterns in Figs. 1A and H, LE and m2 appeared to spread randomly in the co-inoculated leaves. The data in Fig. 1O, which are overlays of Fig. 1A over Fig. 1H, implied random effects in the initial distribution of both of the strains in the infection foci. The ability of the second virus to infect leaves inoculated previously with protecting virus also was investigated (Figs. 1B vs. I, and 1C vs. J). Leaves inoculated with LE (or m2) and challenged with mock inoculation showed efficient spread of the virus throughout the leaves (Figs. 1D and L), whereas those inoculated with LE (or m2) and challenged with m2 (or LE) showed less efficient cell-to-cell movement of the protecting virus (Figs. 1B and J). The localization and rate of spread of the challenge virus LE (or m2), in the leaves inoculated previously with the protecting virus m2 (or LE) (Figs. 1C and I), were restricted compared with those of the challenge virus LE (or m2) following mock inoculation (Figs. 1F and N). Furthermore, the data in Figs. 1P and Q, which are overlays of Fig. 1I over Fig. 1B, and Fig. 1C over Fig. 1J, respectively, showed that LE (or m2) in the serially inoculated leaves prevented m2 (or LE) from co-infecting almost all the infected areas. The results also indicated that not only was efficient spread of the challenge virus limited by the preexisting protecting virus, but also that further spread of the protecting virus was limited by infection of the challenge virus.

Random effects in the initial distribution in the co-inoculated leaves, and exclusive distribution in the serially inoculated leaves were observed similarly between RNA3 and RNA4 from LE and those from m2 in another cowpea cultivar (cv. Kurodane-sanjyaku) inoculated with LE and the reassortant LLm (data not shown).

In situ spatial exclusion between LE and m2 in cowpea

To analyze the in situ spatial correlation between accumulation of LE RNA and m2 RNA in more detail, thin sections of the infected tissues were prepared and were subjected to in situ hybridization (Figs. 2 and 3). Forty-two sets of serial sections from the inoculated leaves and 40 sets of those from the non-inoculated upper leaves were analyzed. As seen in Figs. 2A and B, the serial sections from the co-inoculated leaves showed that accumulation of LE RNA was found throughout almost all the areas, whereas that of m2 RNA was highly localized. In specific area (a) in Figs. 2A and B, LE and m2 RNAs apparently coexisted, although subsequent cell-to-cell spread of m2 RNA was suppressed. Furthermore, spatial exclusion between LE and m2 occurred in another area (b) in Figs. 2A and B. In the area (b), m2 (or LE) could not spread in cells in which LE (or m2) had accumulated to a high level. Interestingly, both viruses reached a vein system in area (b) from opposite sides. The other sections from the co-inoculated leaf, however, exhibited clear exclusion between LE and m2 RNAs (Figs. 2C and D) that was different from what was seen in Figs. 2A

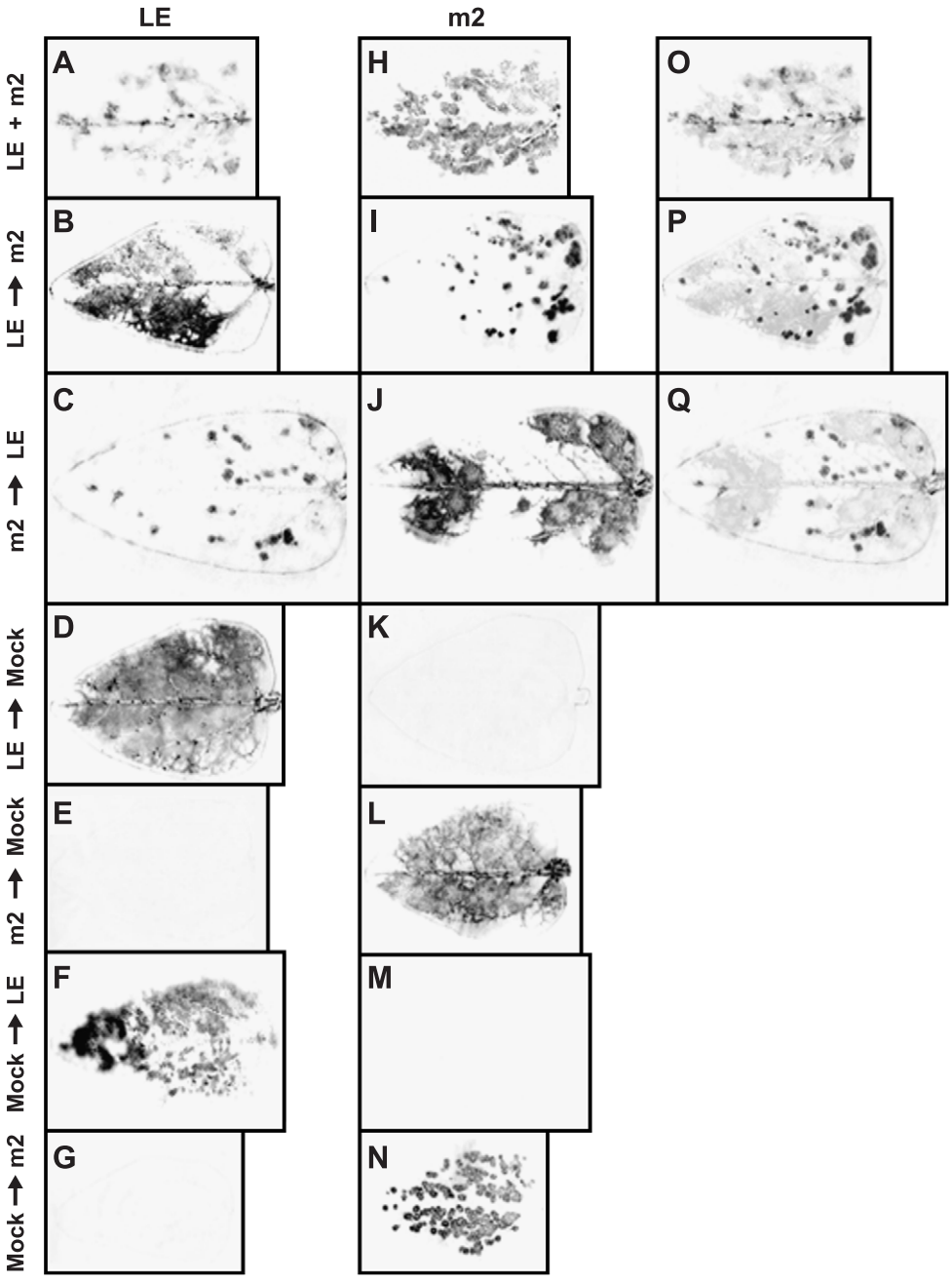

Fig. 1. Tissue-blot hybridization analysis of the primary leaves of cowpea (cv. PI 189375) inoculated with the CMV isolates. Primary leaves were co-inoculated with LE plus m2 (A and H) or were inoculated serially with m2 after LE (B and I), LE after m2 (C and J), mock after LE (D and K), mock after m2 (E and L), LE after mock (F and M), or m2 after mock (G and N). The inoculated leaves were detached at 8 days post-inoculation (dpi) (4 dpi of challenge inoculation). The same tissue-blots of the leaf inoculated are shown in A and H, B and I, C and J, D and K, E and L, F and M, and G and N. Overlays of A over H, I over B, and C over J are shown as O, P, and Q. Virus distribution was detected by the subgroup II-specific probe (H to N). Then, the same membranes were stripped and were probed for the subgroup I-specific probe (A to G). CMV RNAs in the blots were detected as described in the text.

and B. In the serial sections, distribution of LE and m2 RNAs were divided into two areas, implying that further spread of both viruses was restricted at the border cells. Virus distribution in single infections was detected in almost all the areas in the inoculated leaves (Figs. 2E and F).

Most interestingly, alignment of the sections prepared from the non-inoculated upper leaves also exhibited a pattern of exclusive infection between LE and m2 (Fig. 3). Figs. 3A and B showed that LE spread into the right area of the leaf tissue from the right half of a class I vein system, whereas m2 spread in the opposite area from the left half of the same vein system. In Figs. 3C and D, the distribution of LE was clearly separated by cells infected with m2, which had spread from a class III vein system. On the other hand, the sections from the leaves singly inoculated with LE or m2 showed that both viruses accumulated throughout almost all

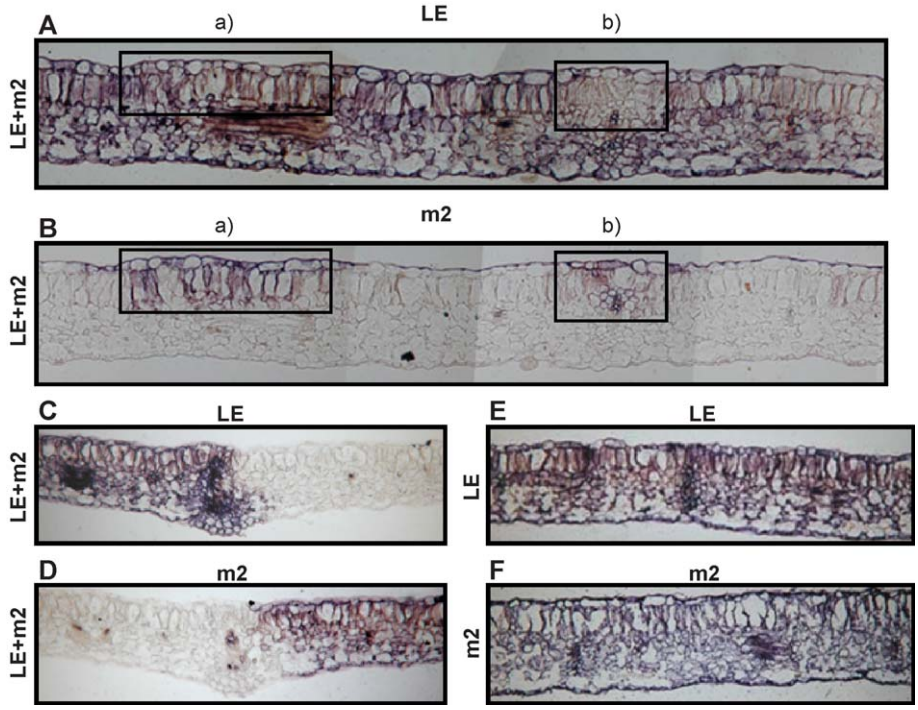

Fig. 2. Spatial analysis of CMV RNA in the leaves of cowpea (cv. PI 189375) co-inoculated with LE plus m2. Serial sections prepared from the leaf tissues co-inoculated with LE plus m2 (A and B, and C and D), and the other sections from those inoculated with LE (E) or m2 (F) were subjected to in situ hybridization. The sections were analyzed by the subgroup I-specific oligonucleotide cDNA probe (A, C, and E), and the subgroup II-specific oligonucleotide cDNA probe (B, D, and F), respectively. A and B show that LE and m2 mixedly infected in specific area (a), and that spatial exclusion between LE and m2 occurred in another area (b). The inoculated leaves were detached at 6 dpi. The purple and/or blue precipitate indicates positive reaction for virus RNA. CMV RNAs in the sections were detected as described in the text.

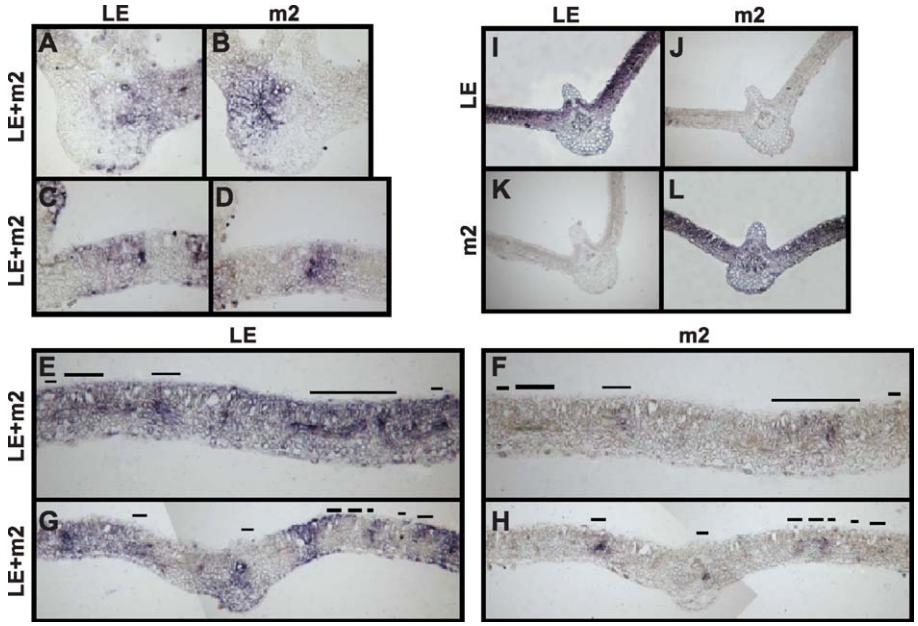

Fig. 3. Spatial analysis of virus RNA in the non-inoculated upper leaves of cowpea (cv. PI 189375) co-inoculated with LE plus m2. Independent serial sections prepared from the tissues co-inoculated with LE plus m2 (A and B, C and D, E and F, and G and H), and the other sections from the tissues inoculated LE (I and J) or m2 (K and L) were subjected to in situ hybridization. The sections were analyzed by the subgroup I-specific oligonucleotide cDNA probe (A, C, E, G, I and K), and the subgroup II-specific oligonucleotide cDNA probe (B, D, F, H, J and L). The non-inoculated upper leaves were detached at 9 dpi. The purple and/or blue precipitate indicates positive reaction for virus RNA. Bars in E, F, G, and H indicate areas in which accumulation of m2 RNA was detected. CMV RNAs in the sections were detected as described in the text.

the areas (Figs. 3I and L). Furthermore, Figs. 3E, F, G, and H indicated that in mixed infections LE spread predominantly in the non-inoculated upper leaves, and that m2 localized in the vein systems could not spread efficiently into the areas where LE had accumulated. LE, however, did not show a pattern of uniform distribution throughout the non-inoculated upper leaf tissues, indicating that LE could not invade via the vein systems to the areas in which m2 previously had reached and accumulated.

Discussion

Spatial analyses on the interactions between subgroups I and II of CMV resulted in the discovery of exclusive distribution of the viruses in both the inoculated and the non-inoculated upper leaves of cowpea plants (Figs. 1, 2 and 3). Spatial structuring of viral populations in the infected host plants has been reported by other groups using different experimental systems and approaches. Hall et al. (2001a) documented that cross-protection, vector transmission bottlenecks, and subdivided virus populations within a plant contribute genetic isolation of individual viral lineages of *Wheat streak mosaic virus* (WSMV) strains. Hall et al. (2001b) also suggested that the spatial subdivision of WSMV populations will enhance the probability of fixation of a mutation. Furthermore, severe population bottlenecks also have been shown in tobacco leaves infected systemically with Tobacco mosaic virus by a quantitative estimate (Sacristán et al., 2003). Our data strongly suggested that establishment of reassortant viruses between the subgroups is not favored due to the predominant infection and the spatial separation of the parental viruses in the non-inoculated upper leaf tissues. Interestingly, LE and m2 spread exclusively in the non-inoculated upper leaves from the same vein systems (Fig. 3), implying that each virus independently invaded different vascular cells in the same vein systems. It is unlikely that the network of vein systems provided an opportunity for genetic exchange either by reassortment of genomic segments or by recombination between the viruses.

Roberts et al. (1997) and Santa Cruz et al. (1998) have shown that virus downloading along the major veins in sink leaves occurred at discrete points along the veins, from which discrete infection foci arise. They found that symplastic unloading of virus in developing sink leaves of *N. benthamiana* occurred predominantly from the class III vein network. Roberts et al. (1997) also determined that virus movement into the minor veins (classes IV and V) occurred by cell-to-cell transport through the mesophyll in the plants. In the non-inoculated upper leaves of the plants co-inoculated with LE and m2, we demonstrated that distribution of LE (or m2) was separated clearly by cells infected with m2 (or LE) (Fig. 3). It was interesting that m2 could not spread efficiently from almost all the class III vein systems in the non-inoculated upper leaf tissues where LE had accumulated (Fig. 3). The restriction of m2 egress out of the class III vein systems indicated a significant feature of the spatial exclusion between LE and m2 in the non-inoculated upper leaves. Dietrich and Maiss (2003) reported that populations of either the same viruses differently labeled or different potyviruses were replicating predominantly in discrete areas and that co-existence was restricted to only a few cells at the border of these clusters. Our results from subgroups I and II of CMV, therefore, support the conclusion of Dietrich and Maiss (2003) that spatial competition is a phenomenon widely observed between closely related plant viruses.

The rate of virus spread appeared to affect the dynamics of the spatial exclusion between the two subgroups of CMV in cowpea plants because m2 could not spread efficiently in the non-inoculated upper leaves of the plants co-inoculated with LE and m2 (Fig. 3). Faster movement of LE in both the inoculated and the non-inoculated upper leaf tissues in mixedly infected plants seemed to result in the confinement of m2 egress to within several cells from the vein systems. The fate of the two co-inoculated viruses may depend on their genetic differences involved in cell-to-cell movement that would determine the pattern of accumulation between LE and m2 in the plants. Given the superiority of LE in the rate of spread, m2 might be excluded eventually from almost all the non-inoculated upper leaf tissues of a cowpea plant co-inoculated with these two viruses.

In the serially inoculated leaves, the pattern of exclusive localization of the subgroups I and II clearly demonstrated that restriction of the movement of the challenge virus was due to the prior spread of the protecting virus (Fig. 1). Spatial competition limiting co-infection of the subgroups I and II in the same areas might indicate that CMV cannot counter the host defense responses induced by infection by another subgroup, and that the challenge virus does not co-infect the same cells to breakdown cross-protection. We suggest that establishment of initial infection by the challenge virus in areas that are spatially separated from those infected with the protecting virus is indispensable for breakdown in cross-protection. There could be due to any or all of the following: subsequent cell-to-cell movement in mesophyll cells and loading in minor vein systems such as class IV and/or V, unloading from the upper classes of vein systems (e.g., class III) in areas that have not been infected with the protecting virus and subsequent cell-to-cell spread in mesophyll cells, and sequential occurrence of subdivided challenge virus populations based on the same steps.

Valkonen et al. (2002) speculated that the differences in cross-protection abilities between *Potato virus A* (PVA) isolates and mutants may be associated with virus-induced gene silencing (VIGS) because amino acid mutations in the helper component proteinase, the potyvirus-encoded suppressor gene of PTGS, enhanced the accumulation of PVA and the ability to overcome cross-protection. The experiments of Kalantidis et al. (2002) demonstrated that expression of CMV-derived dsRNA in transgenic tobacco can trigger sequence-specific gene silencing of the virus.

VIGS might be responsible for intracellular defense that acts as a rapid breakdown system of virus RNA in a sequence-specific manner. Such interactions between the virus and host would result in a considerable reduced opportunity for co-infection in the same cell, and may determine the nature of cross-protection between the two subgroups. We have detected short RNA derived from minus-stranded RNA3 of CMV that accumulated in the inoculated leaves of cowpea (Takeshita et al., in press). The two subgroups of CMV generally show about 75% sequence similarity to each other, whereas a region of 40 nt in the 3' non-coding regions of the virus RNA segments shows 100% sequence identity. The highly homologous 3' non-coding regions may be accessible as a target for RNA silencing. The short RNAs might be derived from such a common region of the two subgroups, and target challenge virus RNA for VIGS in the same host cells. Characterization of the short RNAs from cowpea plants infected with subgroups I and/or II of CMV will provide direct evidence that viruses from one taxonomically subgroup can induce a PTGS-like defense response against viruses from another subgroup.

We found that expression of a cowpea-encoded RNA-dependent RNA polymerase gene (*VuRdRp1*) mRNA was induced in the plants infected with CMV (Takeshita et al., in press), although the biological activity of *VuRdRp1* remains to be clarified. Further spatial analyses of CMV-inducible host resistant genes would provide a better understanding of competitive interactions between the two subgroups that might be associated with VIGS and/or distinct virus-resistant responses.

Materials and methods

Virus and Host plants

Two CMV isolates, LE and m2, were used. LE which infects leguminous plants systemically was isolated from a tobacco plant showing mosaic (Tomaru et al., 1967). m2 was isolated from a tobacco plant showing mild systemic mosaic originally as a mild strain (CMV-C) by Tomaru and Hidaka (1960) and was renamed CMV-m2 (Takanami et al., 1998).

Cowpea (*Vigna unguiculata* (L.) Walp. cv. PI 189375, Nasu et al., 1996) plants were maintained in an air-conditioned greenhouse at 22–28 °C.

cDNA clones of CMV RNAs and preparation of inocula

The cDNA clones of LE RNAs1, 2, and 3 (Takeshita et al., in press) were used to prepare wild-type LE. The complete nucleotide sequence of RNA3 of LE can be found in the DDBJ data bases (Accession no. AB119091).

Capped transcripts derived from the full-length cDNA clones of CMV RNAs were synthesized in vitro according to Suzuki et al. (1991). The wild-type combination of the in vitro transcripts from the full-length cDNA clones of RNAs1, 2, and 3 of LE was abbreviated to be LE. LE and m2 were propagated in tobacco (*Nicotiana tabacum* L. cv. Xanthi-nc) and purified essentially according to Takanami (1981). Fully expanded primary leaves of the cowpea plants were inoculated with the purified viruses (50 μg/ml). For the co-inoculation assays, equal amounts of inoculum of LE (100 μg/ml) and m2 (100 μg/ml) were mixed to adjust final concentration to 50 μg/ml each. Control plants were mock-inoculated with 10 mM phosphate buffer, pH 7.0. For each inoculum, a set of six plants was used. The data were obtained from the experiments repeated twice.

Leaf-tissue blotting

The accumulation and spread of CMV RNAs were detected with tissue-blot hybridization analyses on nylon membranes (0.45 μm, BIODYNE PLUS, Pall BioSupport, NY). Leaf-tissue blots on membranes were prepared according to Takeshita et al. (2001). Sampled tissues were processed for tissue-blot hybridization. The tissue-blots on the membranes were probed with the DIG-labeled oligo cDNA probes as described by Takanami et al. (1999). The hybridized probes were immunodetected with alkaline phosphatase-conjugated antibody against digoxigenin and visualized with a chemiluminescent substrate, CDP-*Star* (TROPIX) on X-ray films according to Takanami et al. (1999).

Preparation of the probes

Subgroup-specific 5′-DIG labeled oligonucleotide cDNA probes complementary to the sequences near the 3′ ends of the noncoding region of CMV RNA3s and capable of detecting evenly all RNA segments of CMV RNA were essentially as described by Takanami et al. (1999) and Takeshita et al. (in press). The sequences of the 5′-DIG labeled oligonucleotide cDNA probes are as follows: for subgroup I; 5′-GTACCCTRAAACTAGCACGTTGTGCT-AGARGTAGACGGACCGAA-3′, and for subgroup II; 5′-CGCACTCTTGGTAATATCAGTGTATTACCGTGCAC-GAGCTTCTCA-3′. In these sequences, R indicates A or G.

In situ hybridization

The serial paraffin sections of the leaf tissues to detect target RNAs by in situ hybridization were prepared. The subgroup-specific oligonucleotide cDNA probes to detect CMV RNA were used for in situ hybridization at 45 °C and were detected with alkaline phosphatase-conjugated anti-digoxigenin antibody, as described by Havelda and Maule (2000).

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