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Two Isolates of Cucumber Mosaic Cucumovirus Expressing Mild Mosaic Symptoms on Tobacco Plants

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Two cucumber mosaic virus (CMV) isolates that induce mild mosaic symptoms in tobacco (*Nicotiana tabacum* L.) were characterized by symptom observation, serological tests and the nucleotide sequences of viral RNA. CMV–m2 but not CMV–m1 elicited necrotic etching symptom in the inoculated leaves of tobacco. CMV–m1 and CMV–m2 exhibited different serological properties. Comparison of the nucleotide sequences of RNA3s showed that CMV–m2 belongs to subgroup II of CMV. CMV–m1 was suggested to belong to subgroup I with serological test and symptomatology. The two isolates that induce very mild mosaic symptoms in tobacco but belong to different subgroups of CMV would greatly facilitate the study on the molecular basis of cross–protection of plant viruses.

INTRODUCTION

Cucumber mosaic virus (CMV) is the type member of *Cucumovirus* in the family of *Bromoviridae* which has commonly a functionally divided genome of single–stranded, plus sense RNA molecules, namely RNA1, RNA2 and RNA3, in decreasing order of molecular weight. CMV RNAs 1 and 2 were experimentally demonstrated to be involved in viral RNA replication (Nitta *et al.*, 1988) and expression of both the 3a protein (MP) and the coat protein (CP) encoded by RNA3 was shown to be indispensable for virus transport (Suzuki *et al.*, 1991). CMV is distributed worldwide and exists as a variety of strains or isolates that differ in host range and pathogenicity (reviewed in Palukaitis *et al.*, 1992). CMV is divided into subgroups I and II based on the serological properties and differences in nucleotide sequence.

Many efforts have been made to prevent the enormous damages on agricultural crop production caused by CMV. There are a number of studies that describe the establishment of CMV resistance in transgenic plants which express the gene of CMV coat protein, non–structural protein or satellite RNA (Fitchen and Beachy, 1993). The principle of these strategies is based on "cross–protection" of plant viruses. We are now investigating the molecular basis of the phenomenon using various CMV isolates. In the course of our study, it became necessary to characterize CMV isolates that express attenuated symptoms on tobacco plants.

Several strains (isolates) of CMV have been isolated from tobacco plants (*Nicotiana tabacum* L.) in Japan (Hidaka and Tomaru, 1960; Tomaru and Hidaka, 1960a, b; Tomaru and Udagawa, 1967; 1970). Among the isolates, a mild strain (CMV–C) (Tomaru and Hidaka, 1960a) and a yellow mild mottle strain (CMV–YM) (Tomaru and Udagawa, 1970)

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were reported to induce mild mosaic symptoms in tobacco. Since an isolate of CMV in a sample batch which had originally been isolated and stored as CMV-C by Tomaru and Hidaka (1960a) was revealed to belong to subgroup II in this study, we designated the isolate as CMV-m2. CMV-YM has already been repoted to contain a satellite RNA that was named Y-satRNA and was capable of inducing brilliant yellow mosaic on tobacco (Takanami, 1981). A satellite-free isolate of CMV-YM that had been prepared as described previously (Takanami, 1981) has been shown to induce very mild green mosaic on tobacco (unpublished). Present study also revealed that the satellite-free isolate derived from CMV-YM belongs to subgroup I, and was named CMV-m1. This paper describes the characteristics of the both CMV isolates expressing the attenuated symptoms in tobacco.

MATERIALS AND METHODS

CMV isolates

CMV-m1 and CMV-m2 were as described above. CMV-Y (Tomaru and Hidaka, 1960) and CMV-O (Hidaka and Tomaru, 1960) were used for comparison. Both of the isolates were shown to belong to subgroup I based on the sequencing results (Nitta *et al.*, 1988; Hayakawa *et al.*, 1989). CMV-GT isolated from a tomato plant in Gunma Prefecture belongs to subgroup II (Dr. Hanada, personal communication).

Virus propagation and purification

CMV isolates were propagated in tobacco (*N. tabacum* cv. Xanthi-nc) and purified as described previously (Takanami, 1981).

Serological test

Immunodiffusion tests of CMV were carried out essentially according to Ohki and Inouye (1987) using purified virions of the CMV isolates and antisera against CMV-Y and CMV-P (subgroup II) raised in rabbits. The antisera of CMV-P was provided by Dr. Hanada.

cDNA cloning and sequencing of the cDNA

cDNA cloning and sequencing of CMV-m2 RNA3 was performed according to the methods adopted for RNA3s of CMV-KM and CMV-D8 (Takeshita and Takanami, 1997).

RESULTS

Symptoms of tobacco plants inoculated with CMV-m1 and CMV-m2

Young tobacco plants (cv. Xanthi-nc) were mechanically inoculated with crude saps of tobacco plants that had been infected with CMV-m1 or CMV-m2, and the symptoms appeared on the plants were observed. CMV-m2 induced necrotic etching symptom but CMV-m1 produced no apparent symptoms on the inoculated leaves of tobacco (Fig. 1). It is well known that the necrotic etching symptom in the inoculated leaves of tobacco is characteristic for subgroup II CMV (Zhang *et al.*, 1994). Both isolates induced very mild green mosaic symptoms which were apparently indistinguishable from those in healthy

control tobacco especially in the latter stages of infection (Fig. 1).

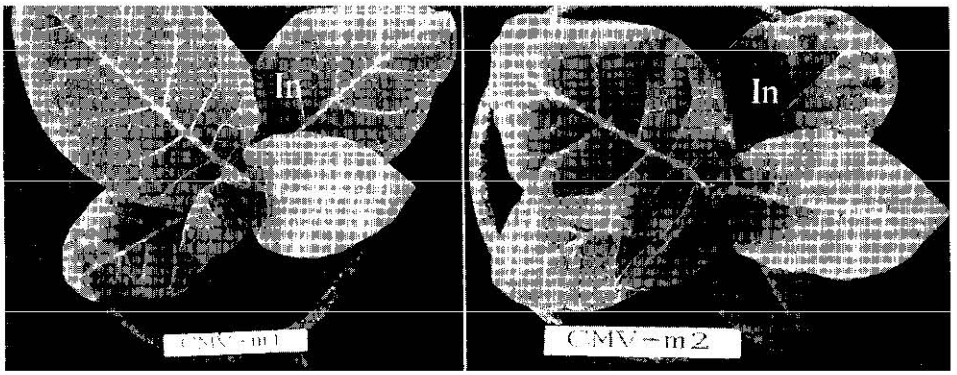

Fig. 1. Symptoms of tobacco (*N. tabacum* cv. Xanthi-nc) inoculated with CMV-m1 (left) and CMV-m2 (right) 14 days after inoculation. (In) indicates the inoculated leaves.

Double-immunodiffusion tests

As shown in Fig. 2A, when the central well was filled with the antiserum against CMV-Y and the outer wells were with the purified CMV virions as antigens, the precipitation lines of CMV-m1 and CMV-Y completely fused each other and those of CMV-m2 and CMV-GT also fused, although spur-precipitin lines were formed between CMV-Y and CMV-m2, CMV-m1 and CMV-m2, and CMV-m1 and CMV-GT. The completely fused precipitation lines were formed between CMV-m2 and CMV-GT and the spur-precipitation lines were between CMV-O and CMV-GT, CMV-O and CMV-m2, CMV-Y and CMV-m2, and CMV-Y and CMV-GT, irrespective as to whether the antiserum

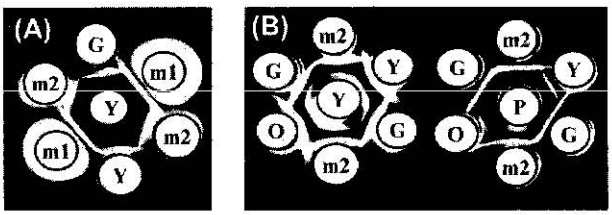

Fig. 2. Double immunodiffusion tests of CMV isolates. (A); the central well was filled with CMV-Y antiserum diluted 6 times with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl (PBS). The peripheral wells contained 1 mg/ml of purified CMV -m1 (m1), -m2 (m2), -Y (Y) and -GT (G). (B); the central wells in the left and right plates were filled with CMV-Y and CMV-P antisera, respectively, which were diluted 6 times with PBS. The peripheral wells contained 1 mg/ml of purified CMV -m2 (m2), -Y (Y), -GT (G) and -O (O). Photographs were taken 24 h after incubation at 28°C.

against CMV-Y or CMV-P was placed in the central well (Fig. 2A). These results clearly demonstrated that CMV-m1 and CMV-m2 belong to subgroup I and subgroup II, respectively, on the basis of serological property.

Nucleotide sequence of CMV-m2 RNA3

The complete nucleotide sequence of CMV-m2 RNA3 was submitted to the DDBJ/GenBank/EMBL DNA data base as the accession number of AB006813. The sequences of the 5' noncoding regions in RNA3s of CMV-m2, CMV-Y (Nitta *et al.*, 1988) and CMV-Q (Davis and Symons, 1988) were aligned in Fig. 3. There found long insertions and deletions in the 5' regions among the isolates and the structural feature of

```
                        10         20         30         40         50
CMV-Y RNA3       1 GUAAUCUAAC CAC------- ---------- ---------- ------CUGU
CMV-m2 RNA3      1 GUAAUCUUAC CACUUUCUUU UUCACGUCGC GUCGCGUCAG UCCACGCUGU
CMV-Q RNA3       1 GUAAUCUUAC CACUUUCUUU --CACGUCGU GUCGCGUCAG UCCACGCUGU

                        60         70         80         90        100
CMV-Y RNA3      51 GUGUGUGUGU GUGUGUAUCG AGUCGUGUUG UCCGCACAUU -------UGA
CMV-m2 RNA3     51 GUGUGUGUGU GUGU------ ---------- ---------- UAGUUAGUGU
CMV-Q RNA3      51 GUGUGUGUGU GUGU------ ---------- ---------- UAGUUAGUGU

                       110        120        130        140        150
CMV-Y RNA3     101 GUCGUGUUGU CCGCACAUAU AUAUUUAUUU CGUUGUACAG UGUGUUAGAU
CMV-m2 RNA3    101 GUCGUGUU-- ---------- ---------- ---------- -----UAGAU
CMV-Q RNA3     101 GUCGUGUU-- ---------- ---------- ---------- -----UAGAU

                       160
CMV-Y RNA3     151 UUCCCGAGGC -AUG
CMV-m2 RNA3    151 UACG-GAGGU UAUG
CMV-Q RNA3     151 UACG-AAGGU UAUG
```

Fig. 3. Alignment of the 5' noncoding regions in RNA3s of CMV-Y, CMV-m2 and CMV-Q. The sequence data of CMV-Y and CMV-Q were refereed to Nitta *et al.* (1988) and Davis and Symons (1988), respectively. Dots (-) represent deletions and nucleotides common to the three isolates are shown in bold letters. Underlines show the start codons of the open reading frame for 3a protein.

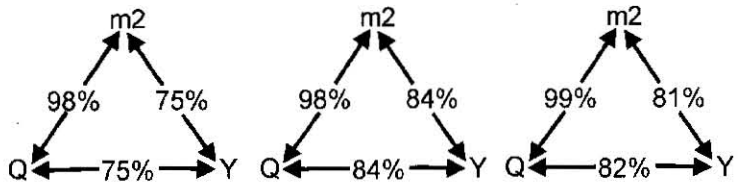

Fig. 4. Similarity in entire nucleotide sequences of RNA3s and amino acid sequences of the 3a and coat proteins of three CMV isolates. The sequence data of CMV-Y and CMV-Q were refereed to Nitta *et al.* (1988) and Davis and Symons (1988), respectively.
m2 : CMV-m2, Y : CMV-Y, Q : CMV-Q.

the region of CMV–m2 was almost the same as that of CMV–Q belonging to subgroup II. In addition to the serological property of the isolate, the alignment suggested that CMV–m2 is a member of subgroup II of CMV. Comparison of the entire nucleotide sequences of RNA3s and amino acid sequences of the 3a and coat proteins of the isolates further confirmed the result (Fig. 4).

DISCUSSION

CMV–m2 was found in the samples that had been long time stored as a mild strain, CMV–C, under lyophilized conditions (Tomaru and Hidaka, 1960). Our present study clearly demonstrated that CMV–m2 belongs to subgroup II of CMV. A serological study on the CMV isolates (Kiriyama, 1972), however, showed that CMV–C used in his study formed precipitation lines completely fused with those of CMV–Y and CMV–O, suggesting that the virus would be of subgroup I. On the other hand, a study on interference among CMV strains (Tomaru *et al.*, 1967) reported that only CMV–C showed incomplete cross–protection against other CMV strains, suggesting that CMV–C used in this study would have properties somewhat different from those of the other isolates. Furthermore, the original report concerning CMV–C (Tomaru and Hidaka, 1960) recorded the induction of the necrotic etching symptoms (originally described as necrotic lesions with irregular shape) in the inoculated leaves of several tobacco varieties by CMV–C. The samples stored as CMV–C might contain CMV belonging to both subgroup I and II, which commonly induced mild mosaic symptoms in tobacco.

CMV–m1 produced very mild systemic symptoms in tobacco indistinguishable from those of CMV–m2 although the present study suggested that it belongs to subgroup I from the serological test and symptoms in the inoculated leaves of tobacco. Nucleotide sequencing of genome RNAs of CMV–m1 has not yet finished but CMV–m1 RNAs was probed to hybridize specifically with synthetic DNA probe designed for detection of subgroup I CMV RNAs (will be described elsewhere).

The two CMV isolates that induce very mild mosaic symptoms in tobacco but belong to different subgroups would greatly facilitate the study on the molecular basis of cross–protection of plant viruses.

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