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Uchiba, Takayuki
Laboratory of Plant Pathology, Division of Plant Pathology and Pestieide science, Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironment Sciences, Kyushu University

Shigemune, Naofumi
Laboratory of Plant Pathology, Division of Plant Pathology and Pestieide science, Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironment Sciences, Kyushu University

Yoshitsugu, Mami
Program of Agronomy, Course of Agricultural Production, Engineering and Economics, School of Agriculture, Kyushu University

Takeshita, Minoru
Laboratory of Plant Pathology, Division of Plant Pathology and Pesticide Science, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University

他

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Subgroup–Specific Detection of *Cucumber Mosaic Virus* RNA in Tobacco Leaves by Tissue–Printing

Takayuki Uchiba*, Naofumi Shigemune*, Mami Yoshitsugu, Minoru Takeshita and Yoichi Takanami**

Laboratory of Plant Pathology, Division of Plant Pathology and Pesticide Science, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University, Fukuoka 812–8581

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Experimental conditions for tissue–printing of *Cucumber mosaic virus* (CMV) RNA were determined to analyze kinetics of viral multiplication in tobacco leaves. Tissue–printing technique usually needs a step of peeling epidermis of the leaves. However, the step is an obstacle to obtain reliable results. In this study, treatments with enzyme and freeze–thaw were used as alternatives of the peeling step. The results obtained here suggest that enzyme treatment is an appropriate substitution for the peeling of epidermis and that the optimum incubation time for enzyme reaction is for 4–6 hours. We found no cross–reactions between viral RNAs of subgroup I and II of CMV and no nonspecific reactions against healthy control in tissue–prints of tobacco leaves.

INTRODUCTION

Cucumber mosaic virus (CMV) is one of the most important plant virus in temperate zone. CMV has a tripartite RNA genome and divided into subgroup I and subgroup II based on serological property and differences in nucleotide sequences (Peden and Symons, 1973; Palukaitis *et al.*, 1992).

We are now investigating the molecular basis of phenomena which would occur in plants dually or serially infected with two closely related CMV strains. For the purpose of subgroup–specific detection of CMV RNA, we previously reported several detection methods using digoxigenin (DIG)–labeled probes (Uchiba *et al.*, 1999; Takeshita *et al.*, 1999; Takanami *et al.*, 1999). To know distribution of virus in leaf tissues, various techniques for tissue–printing have been used. The techniques usually need a step of peeling epidermis of the leaves. In case of tobacco leaves, however, the step is an obstacle to obtain reliable results, because complete peeling of epidermis all over a leaf is rather difficult. In this study, we found that treatments with enzymes and freeze–thaw could be used as alternatives of the peeling step. Experimental conditions for the tissue–printing technique using the treatments and subgroup–specific detection of CMV RNA in tobacco leaves are described.

* Laboratory of Plant Pathology, Division of Plant Pathology and Pesticide science, Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 812–8581

** Program of Agronomy, Course of Agricultural Production, Engineering and Economics, School of Agriculture, Kyushu University

MATERIALS AND METHODS

Viruses and Plants

CMV-Y (Tomaru and Hidaka, 1960) belonging to subgroup I and CMV-m2 (Takanami *et al.*, 1998) belonging to subgroup II were used in this study. *Nicotiana tabacum* L. cv. Xanthi-nc were grown in an air-conditioned greenhouse at 22-28 °C. The plants were mechanically inoculated with viruses suspended in 10 mM sodium phosphate buffer, pH 7.0, at 50 μg/ml. The inoculated leaves and uppermost leaves were sampled 4, 7 and 30 days post inoculation.

DIG-labeled probes

DIG-labeled oligo-deoxyribonucleotide probes Y-2 and m2-2 were complementary to the 3' non-translated regions of CMV-Y (RNA3 : 2131-2175 nt) and CMV-m2 (RNA3 : 2113-2157 nt), respectively. Details of these probes were described before (Takanami *et al.*, 1999).

Tissue-printing

Fully expanded tobacco leaves were removed 4, 7 and 30 days post inoculation and lower sides of the leaves were abraded with celite using a brush. After being rinsed in distilled water, the leaves were incubated in W5 solution containing 0.7% cellulase and 0.25% macerozyme (Szilassy *et al.*, 1999) for appropriate time at 30 °C or frozen at -70 °C. Then the leaves were placed onto nylon membrane (Hybond-N^+, Amersham) with the abraded side downwards. The partially degraded or frozen leaves were tissue-printed to the membrane using vacuum gel dryer (Rapidry-mini, ATTO). The dryer was operated at ambient temperature for 30 min. The membrane was subsequently rinsed with 2×SSC to remove the leaf debris, dried, and baked at 80 °C. The viral RNA was detected with DIG-labeled probes and a chemiluminescence reagent, CSPD (Roche diagnostics) as described before (Takanami *et al.*, 1999).

RESULTS AND DISCUSSION

To know the optimum time for the enzyme treatment, incubation was performed for various incubation hours. As shown in Fig. 1, incubation for 4-6 hours gave the highest and stable signals of hybridization. In contrast, when incubation time was shorter than the time, hybridization signals was very weak or sometimes undetectable. On the other hand, prolonged incubation time was unsuitable, because it led to degradation of whole leaf tissue, and made the next blotting-step difficult or almost impossible.

The availability of freeze-thaw treatment was also evaluated as an alternative for the enzyme treatment. However, the results showed that the freeze-thaw method was insufficient compared to the enzyme treatment (Fig. 2).

Another point in this experiment is in the step of tissue-printing to nylon membrane. Usually this step has been performed by hammering or by roller pressing. In this study, we used a vacuum gel dryer to obtain the tissue-prints on nylon membrane. This idea gave a stable and high sensitivity because of its equal high pressure to a layer of leaf tissue and membrane and efficient transportation of leaf sap to membrane.

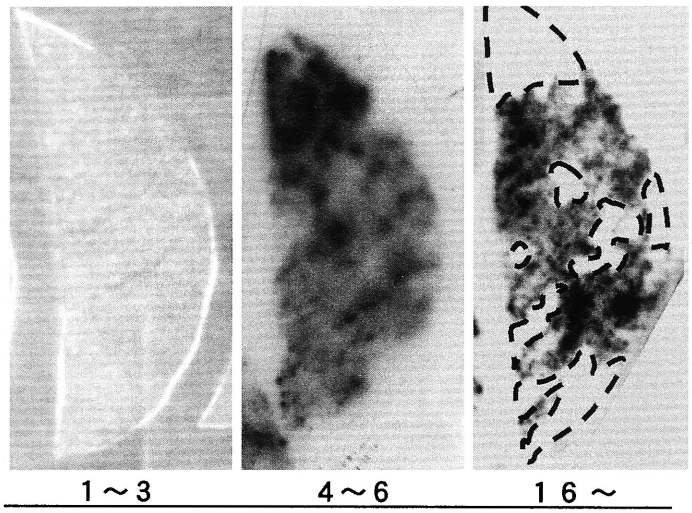

Fig. 1. Effect of incubation time for enzyme treatment on detection of CMV RNA in tobacco leaves. Tobacco plants were inoculated with CMV–Y, and the uppermost leaves were sampled about 30 days post inoculation. After incubation with enzyme at 30 °C, the blotted membranes were subjected to hybridization. Black dotted lines indicate portions of degraded tissue because of prolonged incubation time.

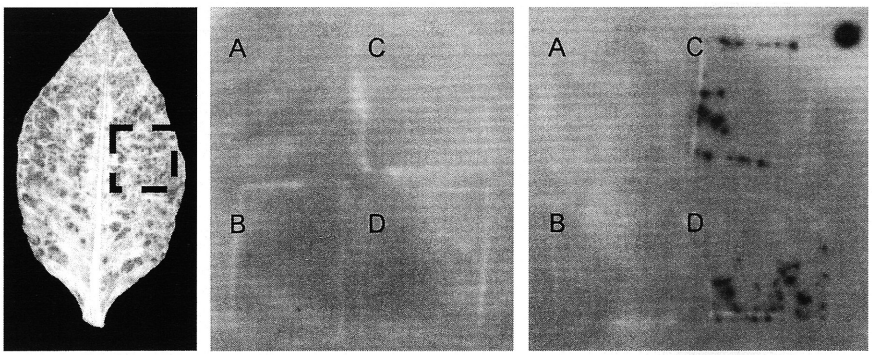

Fig. 2. Efficiency of freeze–thaw treatment for detection of CMV RNA in tobacco leaves. Tobacco plants were inoculated with CMV–Y, and the uppermost leaves were sampled about 30 days post inoculation. After freeze–thaw treatment, the blotted membranes were subjected to hybridization. Black dotted line indicates the portions of the infected leaves which was subjected to tissue–printing.
A; no treatment, B; celite treatment, C; freeze–thaw treatment, D; celite and freeze–thaw treatment. A blot on upper–right of the right panel is positive control.

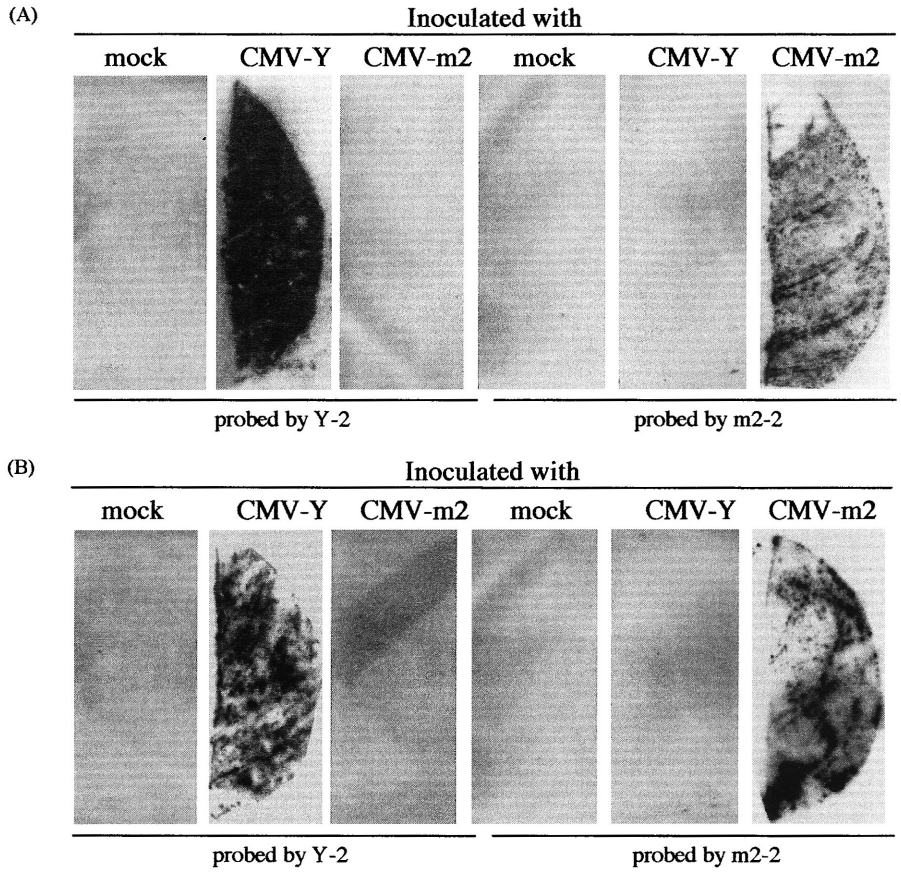

Fig. 3. Subgroup–specific detection of CMV RNA in tobacco leaves. The plants were inoculated with CMV–Y, CMV–m2 or mock–inoculated. The inoculated leaves were sampled 4 days post inoculation and the uppermost leaves were sampled 7 days post inoculation. Halves of the leaves were subjected to tissue–printing. (A), uppermost leaves, (B), inoculated leaves

To verify availability of the technique described above, inoculated leaves and uppermost leaves were sampled form infected or mock–inoculated tobacco plants. The leaves were subjected to the tissue–printing and CMV RNAs in the prints were probed with probe Y–2 and probe m2–2. As shown in Fig. 3, each probe was specifically hybridized with its corresponding strain of CMV. Multiplication and spread of CMV–Y were superior to those of CMV–m2 in uppermost leaves sampled 7 days post inoculation. This result indicated a possibility that there were some differences between CMV subgroup I and II in efficiency of replication, cell–to–cell movement, or long distance movement.

In this study, we report an availability of simple tissue–printing method for detection of plant virus RNA. This method would also facilitate analysis of expression pattern or

localization of a particular plant mRNA.

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