Protection against cucumber mosaic virus (CMV) strains O and Y and chrysanthemum mild mottle virus in transgenic tobacco plants expressing CMV-O coat protein

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Since the first report that transgenic plants expressing the tobacco mosaic virus (TMV) coat protein (CP) showed a delay in disease development caused by TMV infection (Powell Abel et al., 1986), this strategy has been widely adopted for protecting plants against other viruses (Beachy, 1990). These studies have shown that CP plays a significant role in protection against the virus, and also that the degree of protection depends on the similarity between the amino acid sequence of CP of the challenging virus and that expressed in the transgenic plants. The CP-mediated protection was more effective against closely related strains than less related strains (Loesch-Fries et al., 1987; Van Dun & Bol, 1988).

However, Stark & Beachy (1989) showed that the CP gene of soybean mosaic virus (SBMV) expressed in transgenic tobacco confers high protection against infection by two other potyviruses, tobacco etch virus (TEV) and potato virus Y (PVY). TEV shares 61% and PVY shares 58% identity in their CP amino acid sequences with that of SBMV. Ling et al. (1991) also showed a broad spectrum of protection to potyviruses using transgenic tobacco expressing the CP of papaya ringspot virus. Furthermore, Nejidat & Beachy (1990) showed CP-mediated protection against tobamoviruses with a low degree of similarity in their CP amino acid sequences. They suggested that structural similarities, as determined by conserved primary amino acid sequences, are important in conferring protection against infection. Recently Quemada et al. (1991) and Namba et al. (1991) have reported that transgenic tobacco plants expressing the CP of cucumber mosaic virus (CMV) showed a significant level of resistance to infection by various CMV strains. However, it is not known whether this protection would extend to another member of the cucumovirus group.

We have made transgenic tobacco plants expressing the CP gene of CMV strain O (CMV-O). We conducted a series of virus resistance assays using two strains of CMV, strain O and strain Y (CMV-Y), and chrysanthemum mild mottle virus (CMMV). CMV-O and CMV-Y belong to subgroup I and show a high degree of identity in their nucleotide sequences (Hayakawa et al., 1989a). CMV-Y, however, shows more severe disease symptoms than CMV-O. CMV is a member of the cucumovirus group but has no serological relationship to CMV (Tochihara, 1970; Hanada & Tochihara, 1980).

Cloning and sequencing of the cDNA of CMV-O RNA 3 have been reported elsewhere (Hayakawa et al., 1989b). A binary vector, pLAN500, was made from pGA482 (An, 1986) by replacing the ampicillin resistance gene with that for spectinomycin resistance (Fling et al., 1985). A fragment containing the CP coding region, the cauliflower mosaic virus 35S promoter (kindly provided by V. Walbot, Stanford University, Stanford, Ca., U.S.A.) and the nopaline synthase polyadenylation signal from pBI221 (Jefferson, 1987) was inserted into pLAN500, resulting in pLAN501. The plasmid was transferred to Agrobacterium tumefaciens EHA101 (Hood et al., 1986) by a direct transformation method (Hofgen & Willmitzer, 1988).

Plant transformation was performed using leaf discs from Nicotiana tabacum cv. Petite Havana SR1 (SR1)
After clarification, extracted proteins were separated on mercaptoethanol and 0.1 M-PMSF and boiled for 5 min. Buffer (50mM-Tris HCl pH7.5, 2% SDS, 2mM-2-
were transferred to hormone-free MS medium containing leaf tissue according to Richards (1987) and Chomczynski & Sacchi (1987), respectively. Southern and Northern blot analyses were performed by methods
for an (0.5 mg/ml), kanamycin sulphate (0.3 mg/ml) and system (2 to 3 weeks), plantlets were transferred to soil in a growth chamber set for 10 h at 21 °C in the dark and 14 h at 26 °C with light. The leaf discs were incubated with EHA101 containing pLAN501 in MS medium (Murashige & Skoog, 1962) for 48 h at 25 °C. The leaf discs were transferred to LS medium (Linsmaier & Skoog, 1965) containing benzylaminopurine (0.2 μg/ml), clorforan (0.5 mg/ml), kanamycin sulphate (0.3 mg/ml) and 0.1% agarose. Kanamycin-resistant regenerated shoots were transferred to hormone-free MS medium containing 50 μg/ml of kanamycin. After developing a root system (2 to 3 weeks), plantlets were transferred to soil in a growth chamber set for 10 h at 21 °C in the dark and 14 h at 26 °C with light.

Genomic DNA and RNA were isolated from young leaf tissue according to Richards (1987) and Chomczynski & Sacchi (1987), respectively. Southern and Northern blot analyses were performed by methods supplied by Amersham. A fragment of the cDNA of the CP coding region was labelled with [32P]dCTP (111 TBq/mmol) by using a multiprime labelling kit (Amersham), and was used as a probe for hybridization. For Western blot analysis, leaf tissue was ground in Tris buffer (50 mM-Tris-HCl pH 7.5, 2% SDS, 2 mM-2-mercaptoethanol and 0.1 mM-PMSF) and boiled for 5 min. After clarification, extracted proteins were separated on 10% SDS-polyacrylamide gels and transferred onto a nylon membrane (Immobilon, Millipore). Non-specific binding to the membrane was blocked by 3% skim milk in TBS buffer (50 mM-Tris-HCl pH 7.5, 150 mM-NaCl), and the membrane was treated with rabbit antibody to CMV CP. The bound protein was detected using alkaline phosphate-linked goat anti-rabbit antibody (Perbal, 1988). Protein was quantified as described by Bradford (1976).

We obtained 23 Northern blot-positive transformants out of 38 Southern blot-positive ones. Two lines of transformants, 4-4 and 2-29, were used to evaluate the protection against virus infection. The CP expression levels of the progeny of 4-4 and 2-29 were calculated to be 0.04% and 0.01% of the total soluble protein, respectively (Fig. 1). For each virus protection assay, five to 17 progeny (6 to 7 weeks old) of 4-4 and 2-29 were inoculated on Carborundum-dusted leaves with various concentrations of purified viruses.

When CMV-Y was inoculated at a concentration of 1 μg/ml, disease symptoms developed in systemic leaves of non-transformed plants in a few days, whereas the progeny of 4-4 and 2-29 showed a significant level of resistance at least 12 days after inoculation (Fig. 2). Similar results were obtained when 5 μg/ml of CMV-O was inoculated (data not shown). However, when increased concentrations of inoculum (10 and 50 μg/ml) were used for the protection assay, a different level of resistance was observed, i.e. stronger against CMV-O than against CMV-Y (Fig. 3). Similar observations have been seen in the case of transgenic tobacco expressing the CP of CMV-C (Quemada et al., 1991).

If the CP-mediated protection depends on physical blockage of the site where uncoating of CP takes place (Register & Beachy, 1988), the secondary structure of the protein should be important for the protection. A recent report identifying a specific region in the CP of alfalfa mosaic virus that is critical for virus protection suggests that the N-terminal region is important for CP-mediated protection (Tumer et al., 1991). CMV-O and CMV-Y (Hayakawa et al., 1989a; Nitta et al., 1988) have seven amino acid differences of the 218 amino acids of CP. The substitution of amino acid residues could lead to a conformational change of CP and affect virus susceptibility. The substitution of Pro(18) (CMV-O) to Leu(18) (CMV-Y) causes a conformational change from a turn structure to β-sheet as assessed by the method of Garnier et al. (1978).

We also tested protection against another member of the cucumovirus group, CMMV, which was obtained from the gene bank of the National Institute of Agrobiological Resources. The amino acid sequence of CMMV has not been determined and the degree of similarity between CMMV and CMV is not yet known.
Fig. 3. Symptom development in transgenic plants after inoculation with CMV-O or CMV-Y. Progeny of 4-4 (Δ) and 2-29 (☐) and non-transformed seedlings of SR1 (○) were visually scored after inoculation with 10 μg/ml (a) and 50 μg/ml (b) of CMV-Y, or 10 μg/ml (c) and 50 μg/ml (d) of CMV-O. Numbers in parentheses are the sample size.

Judging from the double diffusion serological test, the similarity should be quite low (Tochihara, 1970; Hanada & Tochihara, 1980). However, when 10 μg/ml and 50 μg/ml of CMMV were inoculated, the transformants expressing CP of CMV showed a significant level of protection against CMMV (Fig. 4). Comparison of the developing pattern of disease symptoms caused by CMMV with those of CMVs suggests that the infectivity of CMMV is lower than that of CMV (Fig. 3 and 4). As the number of plants in the virus protection assay was quite small, further experiments are needed to assess fully the CP-mediated protection by CMV CP against the serologically unrelated CMMV. Furthermore, the mechanism of protection needs to be investigated particularly to ascertain whether it is a simple tolerance or a true resistance to virus. The primary sequence conserved in CMMV and CMV, which does not correspond to an antigen recognition site, also remains to be determined.

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References


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