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Characterization of Transgenic Tobacco Plants Expressing the Coat Protein Gene of Cucumber Mosaic Virus from Taiwan

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ABSTRACT

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Cucumber mosaic virus (CMV) causes great losses in several crops in Taiwan, especially in tobacco, tomato and pepper. Lack of resistant source makes the problem difficult to solve. Thus, CP-mediated protection provides an innovative strategy to control CMV in these crops. The objective of this study was directed to construct and evaluate transgenic tobacco plants expressing the CP of a local tomato-infecting CMV-T isolate as the first step for testing the feasibility of the approach. The coat protein (CP) gene of the virus was cloned from the DNA fragment amplified by polymerase chain reaction (PCR) and further constructed in a Ti-binary vector. Twenty eight putative transgenic plant lines of *Nicotiana tabacum* cv. Havana that expressing the CP of CMV-T were produced via *Agrobacterium*-mediated transformation. The integration and expression of the CP gene was confirmed by PCR, Western blotting and indirect ELISA. Three transgenic lines expressed the CP gene at higher levels as reflected in ELISA and Western blotting. Resistance of the transgenic lines against CMV-T infection was evaluated by mechanical inoculation with the virus. Four and five transgenic lines were subjected to challenge inoculations in winter and summer, respectively. In general, the transgenic lines showed a significant delay in symptom development, and the higher expressors showed better resistance than the lower expressors. Some plants of lines 1-10, 1-23, and 1-28, and all plants of line 1-17 did not show any symptoms when tested under low inoculum pressure and low temperature conditions. The production of CP transgenic tobacco resistant to CMV infection provides a model system for control of the diseases caused by CMV in other crops.

Key words: cucumber mosaic virus, transgenic plant, CP-mediated protection.

INTRODUCTION

Cucumber mosaic virus (CMV), the type member of the cucumovirus group, is a small plus-sense RNA virus that is the causal agent for virus diseases affecting many important crop species, including tomato, pepper, legume, and cucurbit (8,30). The virus has a tripartite genome which is comprised of three RNAs that contain genes encoding four proteins (38). The larger two RNAs encode two proteins which are components of the RNA-dependent RNA polymerase (14). The third RNA encodes two proteins, the putative movement protein and the viral coat protein (CP); the latter is expressed by subgenomic RNA (7,24,31).

Several studies have shown that virus infection can be reduced by the expression of a viral CP gene in transgenic plants (1,9,19). This CP-mediated protection shares many characteristics with the well-known classical viral cross-protection using mild virus strains. For example, the transgenic plants were protected against virion infection but not viral RNA (22,29,39,40), although exceptions have been reported (15). Another characteristic is the transgenic plants expressing CP of a virus are not protected against the infection of all other strains of the same virus or against other unrelated viruses (29,40).

CMV is one of the viruses for which CP-mediated protection has been demonstrated (5,10,27,28,34,43). The CP gene of the strain CMV-D was first transferred into the genome of tobacco in sense or antisense directions as described by Cuozzo *et al.* (5). Some CP-expressed plant lines showed high resistance to CMV infection during the period of their experiment, and the resistance seemed superior to that of plant lines expressed antisense RNA. Over 60 strains of CMV have been reported, they appear to fall into two biologically and biochemically distinguishable groups (13). The subgroup I includes strains C, D, Fny, and Y, and

the subgroup II includes strains Q and WL. Within the subgroups, CP amino acid sequences share more than 95% sequence identity, but it is only approximately 80% between the subgroups (35). To answer the question of protection effect of CP transgenic plants between heterologous subgroups, Quemada *et al.* (34) and Namba *et al.* (28) constructed transgenic plants expressing the CPs of subgroup I CMV-C and subgroup II CMV-WL, respectively. Surprisingly, these transgenic lines showed a significant degree of protection when challenged with CMV strains of either subgroup. Field performance of CMV CP transgenic cucumber plants has also been shown by Gonsalves *et al.* (10), and the transgenic plants demonstrated a comparable level of resistance as a CMV-resistant cultivar did.

CMV causes a great loss in several crops in Taiwan, especially tobacco, tomato, pepper, and cucurbit (2,11,17,41). Lack of resistant source makes the problem difficult to solve (3). Thus, CP-mediated protection provides an innovative strategy to control CMV in these crops. The objective of this study is directed to construct transgenic tobacco plants expressing the CP of a local tomato-infecting CMV-T isolate. The expression of the CP gene and the resistance to CMV infection of the transgenic lines were analyzed.

MATERIALS AND METHODS

Cloning of the CMV-T CP gene

A genomic library was constructed from CMV-T total RNA, and a clone pCP10 covered the CP open reading frame (ORF) was selected previously (18). To construct an expressible clone reflected the native CMV RNA 4, polymerase chain reaction (PCR) was employed to amplify the RNA 4 cDNA from this selected clone. Two primers CMV-CP5, 5′-CTCTAGAGTTTCGTCTACTTATCT-3′, and CMV-CP3, 5′-CGAGCTCTGGTCTCCTTTGAGAGACCCC ATT-3′, located at 76th nucleotide upstream the CP initiation codon and the 3′ end of RNA 3, respectively, were used. Template DNA was denatured by boiling for 5 minutes and then subjected to PCR, which was engaged in Easy Cycler Series (Ericomp Co.) according to the following program: first cycle at 50 C for 3 min, 72 C 3 min; followed by 34 cycles at 94 C 1 min, 50 C 2 min, and 72 C 3 min; and the final cycle at 94 C 1 min, 55 C 2 min, and 72.5 C 10 min. The PCR product was digested with *Xba*I and *Sac*I. The restriction fragment was recovered from the agarose gel and then cloned in pBluescript KS(-) (Stratagene Co.). The resulting clone pCCPl was analyzed by restriction enzyme mapping and DNA sequencing to confirm the construction.

Four clones covered the entire CP gene with various leader sequences were obtained (Fig. 1). Two of them, pCP9 and pCP10, were selected from a cDNA library to CMV RNA 3 (18) and contained 1008 and 1207 nucleotides in length, respectively. These two clones had 51 and 250 nucleotides in front of the CP ORF, respectively. The clone pCCPl contained 76 nucleotides in front of the CMV CP ORF and the clone pGCP7 contained the leader sequence of the bacterial GUS gene in front of the CP ORF. To construct the pGCP7 clone, two primers CMV-NCO1, 5′-AGCCATGGACAAATCCGAAT-3′, and the previous described primer CMV-CP3 were used to amplify and clone a DNA fragment of 961 bp. The primer CMV-NCO1 included the initiation codon of the CMV CP gene and a *Nco*I site at the 5′ end. The PCR product was digested with *Nco*I/*Sac*I and cloned to the pGCP clone to replace the PRSV (papaya ringspot virus) CP gene and resulting a clone with GUS leader sequence in front of the CMV CP gene (4).

In vitro expression of the CMV-T CP gene

The four clones corresponding to the entire CP gene with various lengths of leader sequence were selected for *in vitro* transcription and translation analysis. RNA transcripts were synthesized from the clones in the transcription vector pBluescript SK(-) or KS(-) (Stratagene Co.) by run-off transcription using T3 or T7 RNA polymerase according to the manufacturer's directions (Stratagene Co.).

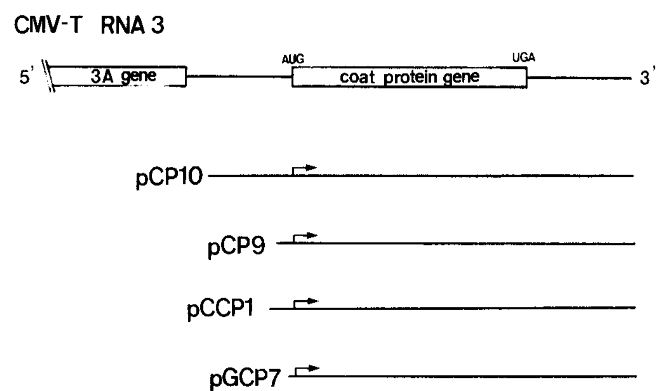

Fig. 1. Four CMV-T CP clones constructed with different leader sequences. These four clones covered the complete CP ORF, 3′ noncoding region, but with various lengths of the leader sequence. Clones pCP10 and pCP9 were selected from the cDNA library, containing 250 and 51 nucleotides in their leader sequences, respectively. Clones pCCP1 and pGCP7 were two PCR amplified clones. The pCCP1 contained 76 nucleotides leader sequence, yet the pGCP7 contained the leader sequence of the GUS gene. Arrows indicate the translation initiation codon of CMV-T CP gene.

About 1 μg of *Kpn*I or *Sac*I-linearized DNA templates were diluted in 25 μl transcription mixture [40 mM Tris-HCl, pH7.5, 50 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 1 mM rNTPs, 0.5 mM cap analog (5'7meGppp5'G), 30 mM DTT, and 10 units of RNA polymerase], and incubated at 37 C for 30 min. After the synthesis of the capped RNA transcripts the DNA templates were removed by treatment with 10 units RNase-free DNase at 37 C for 5 min. The capped RNA transcripts were dissolved in 25 μl RNase-free TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA) after phenol/chloroform extraction and ethanol precipitation. The molecular size and quantity of the capped RNA transcripts were estimated by 1% formaldehyde agarose gel electrophoresis.

In vitro translation was performed using the rabbit reticulocyte lysate system (Stratagene Co.). Five micrograms of RNA transcripts were used as templates and CMV-T total genomic RNA was used for positive control. The translation mixtures containing [^{35}S] methionine (>1000 Ci/mmole, Amersham) were incubated at 30 C for 60 min. Protein products were subsequently immunoprecipitated with the antiserum against CMV-T CP (18). Translation products were diluted with 4 volumes of immunoprecipitation buffer (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH7.5, 1% Nonidet P-40) containing 10 mM methionine. The solution were then mixed with 1/2 volume of antiserum against CMV-T CP. After incubation at room temperature for 2 hr, 10 μl protein A Sepharose CL-4B (2 mg/ml, Sigma Co.), which was prepared in immunoprecipitation buffer, was added to the mixture and incubated at room temperature for 1 hr. Antibody-precipitated proteins were collected by centrifugation at 6,000 g for 5 min. The pellets were resuspended thoroughly in immunoprecipitation buffer and the suspension was centrifuged again. This washing process was repeated three times. The final pellets were dissolved in an original volume of SDS-sample buffer (2% SDS, 2% 2-mercaptoethanol, 10% sucrose, 0.005% bromophenol blue, 0.01 M EDTA, and 0.1 M Tris-HCl, pH7.2) followed by heating at 100 C for 3 min. One fourth volume of the immunoprecipitated products were analyzed by 12% SDS-polyacrylamide gel electrophoresis and autoradiography.

Engineering of the CMV-T CP gene for expression in plants

DNA of pCCP1 was isolated by alkaline lysis method as described by Davis (6). The CMV-T CP gene was excised from the plasmid as an *Xba*I-*Sac*I fragment. This fragment contains the complete sequence corresponding to RNA 4 of CMV-T and the terminal residues of enzyme cutting sites. DNA was recovered from agarose gel and subcloned in the binary vector pBI121 (Clontech Co.). This vector contained the CaMV 35S promoter, GUS gene from *Escherichia coli*, T-DNA border fragments (B_R and B_L), the promoter and the terminator of nopaline synthase, and the kanamycin selection marker NPT II (neomycin phosphotransferase II). The excised 1.0 kb DNA fragment was ligated into the *Xba*I/*Sac*I site of pBI121 (Clontech, Co.) to replace the GUS (β-glucuronidase) gene, and thus ensured the orientation of the insert downstream from the CaMV (cauliflower mosaic virus) 35S promoter. The insert of clone pBICBI2 was confirmed by restriction enzyme digestion, and thus chosen for further experiments.

Transfer of the CMV-T CP gene into *Agrobacterium*

The pBICBI2 construct was transferred to the disarmed strain LBA 4404 of *Agrobacterium tumefaciens* by triparental mating as described by Rogers *et al.* (36). Bacterial cells of *Agrobacterium* was cultured at 28 C in LB medium (tryptone 10 g, yeast extract 5 g, and NaCl 10 g per liter) containing 100 mg/1 streptomycin, and the cells of *E. coli* JM109/pBI121 and *E. coli* HB101/pRK2013 were cultured at 37 C in LB containing 50 mg/1 kanamycin. When the OD_{600} of the cultures reached 0.8, aliquots of 1 ml bacteria were taken from each species. The cells were mixed and centrifuged, and the pellet was then resuspended in 2 ml of 10 mM $MgSO_4$. The suspension was filtered through a 0.2 μm filter membrane, which was then placed onto a freshly prepared LA medium (LB contained 15 g agar per liter) and incubated at 28 C overnight. Subsequently, the membrane was washed with 2 ml of 10 mM $MgSO_4$, and one twentieth volume of this suspension was spread on the minimal medium (Na_2HPO_4 6g, KH_2PO_4 3g, NaCl 0.5 g, NH_4Cl 1g, agar 20 g, 1 M $MgSO_4$ 2 ml, 20% glucose 10 ml, and 1 M $CaCl_2$ 0.1 ml per liter, pH 7.4) containing 50 mg/1 kanamycin. The plates were incubated at 28 C for three days, the transconjugants growing on this kanamycin-containing minimal medium were selected. The transformed *Agrobacterium containing* pBICBI2 was used for plant transformation.

Plant transformation

The leaf-disk transformation method (16) was used to introduce the T-DNA region of pBICBI2 into plant cells. *Agrobacterium tumefaciens* was cultured in LB containing 50 mg/1 kanamycin and 100 mg/1 streptomycin at 28 C overnight. Tobacco plants of *Nicotiana tabacum* cv. Havana were grown in greenhouse and used for transformation. Leaves were allowed to air dry in a laminar flow hood for 20 minutes, sterilized for 10 minutes in 0.5% sodium hypochlorite (diluted from 10% Clorox commercial bleach) containing 0.1% Tween 20, and rinsed three times with sterile distilled water. The leaves were cut with a sterile scalpel into squares approximately 0.5 ×

0.5 cm in size, and then submerged in the overnight culture of *Agrobacterium tumefaciens* for 5 minutes. They were removed from the *Agrobacterium* culture and blotted dry on sterile filter papers. The leaf pieces were then transferred to the MS104 medium (16), which contained MS salts (Gibco Co.), B5 vitamins, 3% sucrose, 1 mg/1 BA (6-benzylamino purine), 0.1 mg/1 NAA (naphthaleneacetic acid) and 0.8% agar, for a two-day co-cultivation period. After co-cultivation, the leaf pieces were transferred to the selection medium (MS104 medium containing 500 mg/1 carbenicillin and 200 mg/1 kanamycin). Leaf pieces with developing callus were transferred once every three weeks to the fresh selection medium until shoots developed. Shoots were excised and cultured on the hormone-free medium for rooting (MS medium containing 200 mg/1 kanamycin). Shoots that rooted in the presence of kanamycin were transplanted to soil for further analyses.

DNA and protein analysis of the CMV-T CP gene in transgenic plants

The existence of CMV-T CP gene in the chromosome of the putative transgenic plants was checked by PCR using two primers CMV-CP5 and CMV-CP3 specific to the CMV-T CP gene as described above. The total DNAs of transgenic plants were prepared following the procedure described by Mettler (26). Plant tissue of 100 mg was ground in a mortar with two volumes (w/v) of homogenization buffer which contained 1% Sarkosyl, 0.25 M sucrose, 50 mM NaCl, 20 mM EDTA, and 50 mM Tris-HCl, pH 8.0. The homogenate was transferred to microfuge tubes and an additional two volumes of buffer was used to rinse the mortar and recover residual homogenate. The homogenate was incubated at room temperature for 30 minutes. An equal volume of phenol was added and vortexed to mix well. The mixture was then centrifuged to separate the layers. The aqueous phase was carefully removed and extracted with phenol once more. The second aqueous phase was collected and 0.1 volume ethanol was added. The solution was placed on ice for 5 minutes and then centrifuged for 5 min to remove polysaccharide. The supernatant was removed for ethanol precipitation, and the subsequent pellet was resuspended in a desire volume of TE buffer. Total DNA of 1 μg was used for PCR under the same program described previously.

The expression of the CMV-T CP was analyzed by Western blotting using antiserum specific to the CP (18). Total proteins of putative transformed or untransformed plants were prepared by homogenizing leaf tissue in four volumes (w/v) of sample degrading buffer (0.1 M Tris-HCl, pH 7.2, 2% SDS, 2% 2-mercaptoethanol, 10% sucrose, 0.005% bromophenol blue, and 0.01 M EDTA). The samples were boiled for 3 minutes and 20 μl of each was subjected to electrophoresis in 12% polyacrylamide gel (20). The separated proteins were transblotted to Immobilon PVDF membrane (polyvinylidene difluoride membrane, Millipore Co.), and immunostaining was performed following the procedure described in GUS Gene Fusion System user's manual (Clontech Co.). The membrane was washed in 30 ml TSW buffer (10 mM Tris-HCl pH 7.4, 0.9% NaCl, 0.25% gelatin, 0.1% Triton X-100, 0.02% SDS) for one hour, and then repeated once for 30 minutes. The membrane was then incubated with the antiserum against the CMV-T CP at 1:1000 dilution in TSW buffer, shaking gently for one hour. After decanting the solution, the membrane was washed with 20 ml TSW buffer for 30 minutes and further reacted with the secondary antibody goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma or Jackson Co.) at 1:2000 dilution in TSW buffer, and incubated with gentle shaking for 30 minutes. After the reaction with the secondary antibody, the membrane was washed with TSW buffer for 10 minutes, and then the buffer of 100 mM Tris-HCl pH 9.5, 10 mM NaCl, and 5 mM $MgCl_2$ was added, shaking for 5 minutes at room temperature. Subsequently, color developing solution was prepared by adding 33 μl NBT (nitro-blue tetrazolium, 50 mg/ml in 70% dimethylformamide) and 16.5 μl BCIP (5-bromo-4-chloro-3-indolyl-phosphate 50 mg/ml in dimethylformamide) to 5 ml of the buffer in last step. The membrane was incubated in this solution for color development at room temperature for 30 min.

The expression of the CP gene in putative transgenic tobacco plants was also analyzed by indirect ELISA, following the procedure described by Yeh and Gonsalves (42). Leaves of 0.1 g was collected from transgenic plants grown in greenhouse and ground in 2 ml coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3 per liter, pH 9.6). Each sample of 200 μl was loaded to ELISA plates (Corning Co.), and incubated at 37 C for 2.5 hr. The plates were washed with PBST (phosphate buffered saline, pH 7.4, containing 0.05% Tween 20) three times, each for five minutes. CMV-T CP antiserum (18) diluted at 1:1000 was added to each well, and the plates were incubated at 37 C for 2.5 hr and then washed three times with PBST. The secondary antibody, goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma or Jackson Co.), was diluted 1:2000 in conjugate buffer (PBST containing 2% PVP-40, 0.2% ovalbumin), and aliquots of 200 μl were loaded to each well. The plates were incubated at 37 C for 2 hr, and then washed with PBST three times. Color developing solution was prepared by dissolving the alkaline phosphatase substrate tablets (Sigma Co.) in substrate buffer (97 ml diethanolamine, and 0.2 g NaN_3 per liter, pH 9.8) to a final concentration of 1 mg/ml and 200 μl of the solution

was added to each well. The expression level of the CP gene was then determined based on the OD_{405} reading by a Bio-Tek microplate reader.

Mechanical inoculation tests

To test the resistance of transgenic plants against CMV infection, clones of the R_0 plants were micropropagated to increase the number of plants available for evaluation. The CP-detectable or non-detectable transgenic lines, each with six to ten plants, were subjected to the mechanical challenge inoculation. Inoculum was prepared from leaves of CMV-T-infected *N. tabacum* cv. Havana 7 days after inoculation. The leaves were ground in 0.01 M potassium phosphate buffer (pH 7.0), and the extract was rubbed on leaves of the test plants dusted with Carborundum abrasive (two leaves per plant). The infectivity of the inoculum was measured by inoculating *Chenopodium quinoa* with the extracts and the number of local lesions was counted. The progress of symptom was investigated by visual inspection each day after inoculation or by indirect ELISA with an interval of two days as described previously.

RESULTS

Cloning of CMV-T CP gene

A cDNA library was previously constructed in the Lambda ZAP II vector, and two clones pCP9 (1.0 kb) and pCP10 (1.2 kb) corresponding to the 3′ terminus of CMV-T RNA 3 were selected by immunoscreening and plaque hybridization (18). These two clones with various lengths of leader sequence have been transferred into tobacco *N. tabacum* cv. Havana. Unfortunately, the CP was undetectable from these transgenic plants. Therefore, attempt was shifted to construct a CMV-T CP clone reflecting the exact native CMV RNA 4. Two primers CMV-CP5 and CMV-CP3 were designed to amplify a DNA fragment reflecting RNA 4 by PCR. The PCR DAN product was then cloned into *XbaI/SacI* sites of the pBluescript KS(-) vector. The clone pCCPl was selected from PCR amplification and its correct orientation was confirmed by enzyme-digestion pattern and DNA sequencing at both ends of the insert. This clone had 1033 nucleotides in length and reflected the full-length RNA 4. The pCP9 and pCP10 selected from cDNA library were 1008 and 1207 nucleotide in lengty with a leader sequence of 51 or 250 nucleotides, respectively. The pGCP7 contained the leader sequence of the GUS gene upstream the CP ORF. The four clones covered the CP ORF with different lengths of the leader sequence were subjected to the *in vitro* expression assay. *In vitro* transcripts from these four clones were produced and their lengths appeared as expected (Fig. 2). *In vitro* translation of

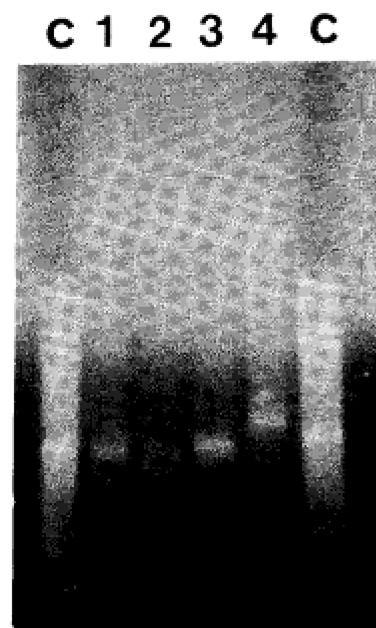

Fig. 2. *In vitro* transcription of four CMV-T CP clones. Transcripts were analyzed in a 0.8% agarose gel followed by ethidium bromide staining. Lane C, genomic RNA of CMV-T; lanes 1 to 4, *in vitro* transcripts from clone pCCP1, pGCP7, pCP9, and pCP10, respectively.

the transcripts of these four clones seemed expressing at a similar level in the rabbit reticulocyte lysate system. The major translation products reacted with the CMV-T CP antiserum and had the same mobility as the CMV-T CP (Fig. 3). Since the expression levels of these four clones are similar, the putative CMV RNA 4 clone pCCPl was chosen for further experiments.

Production of the CMV-T CP transgenic tobacco plants

The CMV-T CP gene of pCCPl was cloned in the binary vector pBI121 at the *XbaI/SacI* site to replace the GUS gene (Fig. 4), and then transferred to *Agrobacterium* by triparental mating. Leaf pieces of tobacco *N. tabacum* cv. Havana were used for transformation and subsequently placed on the MS104 medium for regeneration. Twenty eight kanamycin-resistant tobacco plant lines were obtained after six weeks cultivation. These transgenic lines were micropropagated by tissue culture, transplanted in pots and placed in the greenhouse for further evaluation.

PCR detection of the CP gene in transgenic plants

The 28 putative transgenic lines obtained were subjected to PCR detection for presence of the CP gene. Total DNAs were extracted from the putative transgenic tobacco plants and amplified with two CMV-T CP gene specific primers by PCR under the conditions described as above. The PCR products were

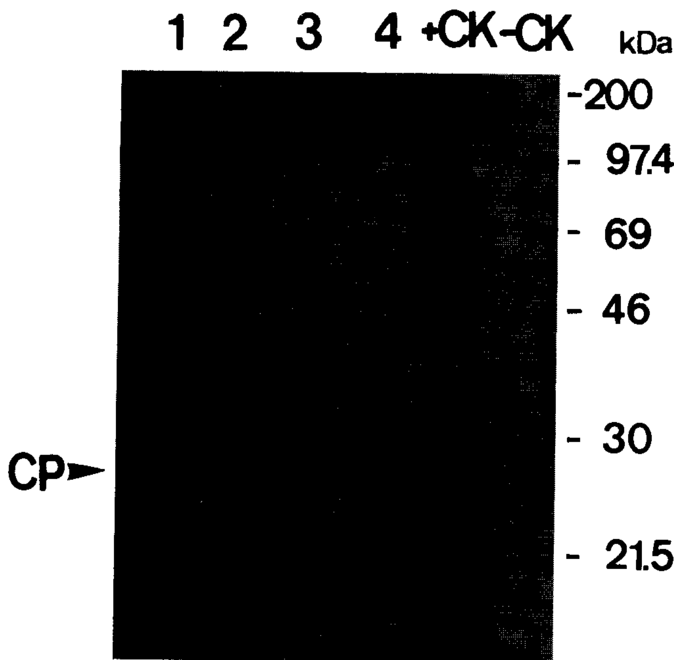

Fig. 3. *In vitro* translation of four CMV-T CP clones. Transcripts of pCCP1 (lane 1), pGCP7 (lane 2), pCP9 (lane 3), and pCP10 (lane 4), or CMV genomic RNA (lane+CK), or without RNA(-CK) were translated in rabbit reticulocyte lysate system and subsequently immunoprecipitated with the antiserum against CMV-T CP. Translation products were analyzed in a 12% polyacrylamide gel and detected by autoradiography.

analyzed in a 0.8% agarose gel and stained with ethidium bromide. Fig. 5 shows that the expected 1.0 kb DNA products were obtained from lines 1-3 and 1-11. Similar results were also observed from the other 26 lines (data not shown).

Expression of the CP in transgenic plants

Expression of the CP gene in the putative transgenic plants were assayed by Western blotting and indirect ELISA. In the initial trials, nitrocellulose membranes (Bio-Rad Co.) were used, but there were no positive signals observed. Therefore, the PVDF membrane (polyvinylidene difluoride membrane, Millipore Co.) was introduced to this study. Three plant lines 1-3, 1-11, and 1-26 expressed the CP at relatively higher levels than others as judged from the distinct protein bands in immunostaining membrane (Fig. 6). These transgenic lines assayed by Western blotting were also subjected to indirect ELISA. The results obtained from indirect ELISA (Fig. 7) also showed that the transgenic lines 1-3, 1-11, 1-26 expressed CMV-T CP higher than the other lines. The OD_{405} of these three lines were over 0.5, while the others showed weak positive signals.

Inoculation tests of the R_0 plants

The verified transgenic lines, whether their

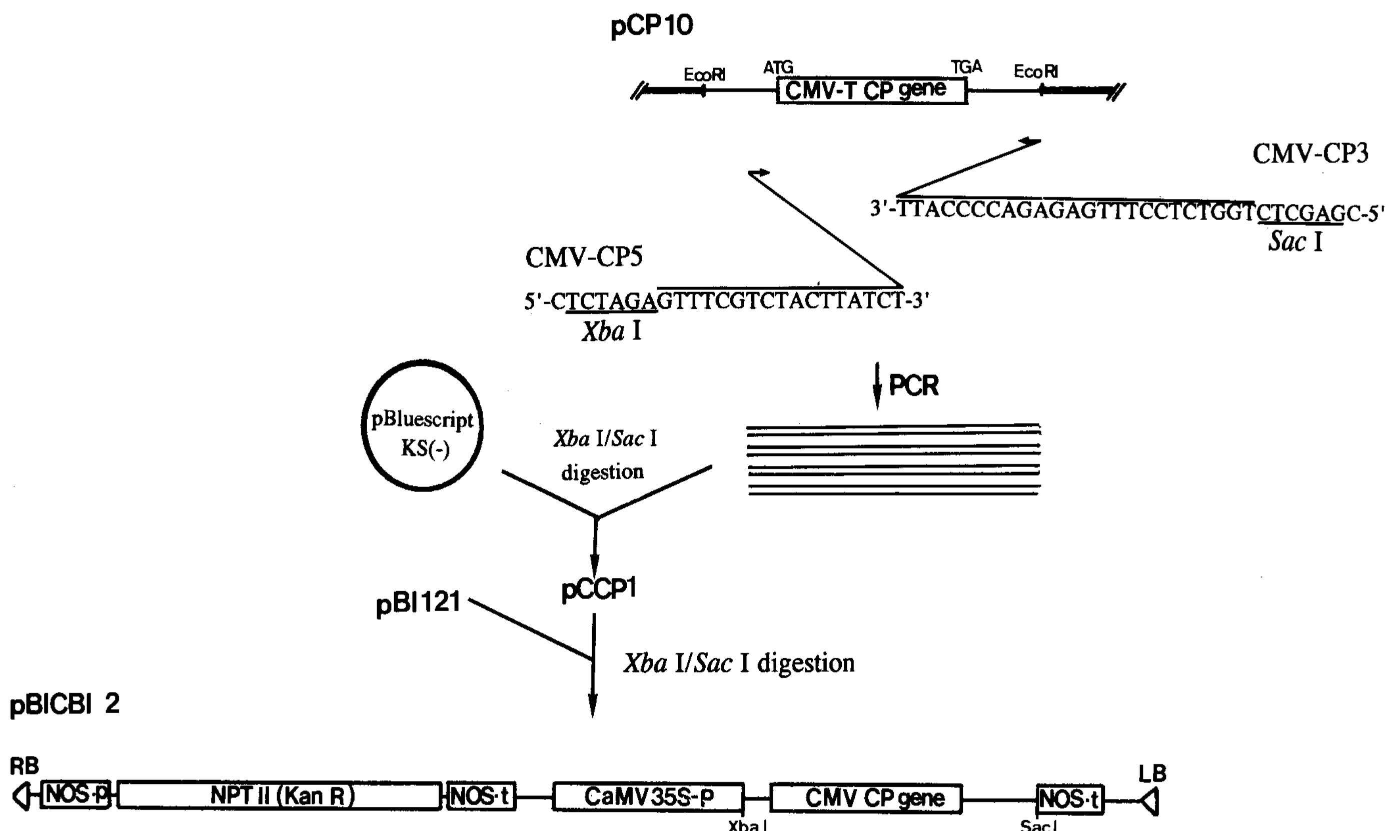

Fig. 4. Engineering the CP gene of CMV-T for expression in plants. The CMV-T CP gene was amplified by PCR from the pCP10 plasmid DNA with specific primers flanking an 1.0 kb dsDNA. This DNA fragment was cloned into pBluescript KS(-), and then subcloned into the *XbaI/SacI* site of the binary vector pBI121 to replace the GUS gene.

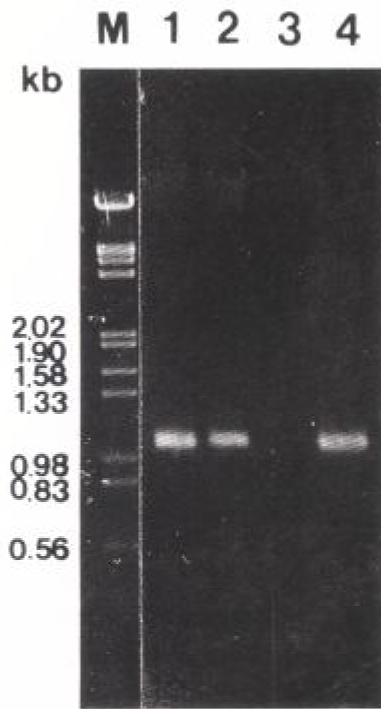

Fig. 5. PCR detection of the CMV-T CP gene in putative transgenic tobacco lines. Total DNAs were extracted from the test plants and two CP gene specific primers were used. Lane M, DNA markers, lambda DNA digested with *Eco*RI and *Hind*III; lanes 1 and 2, transgenic lines 1-3, and 1-11, respectively; lane 3, DNA template from a nontransformed plant; lane 4, DNA template from pBICBI2. PCR products were analyzed in a 0.8% agarose gel.

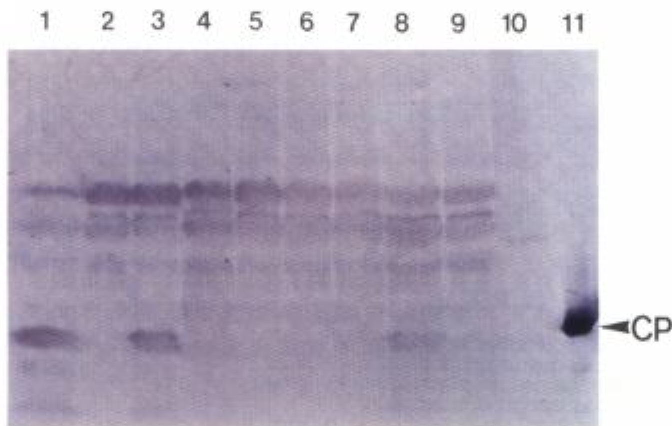

Fig. 6. Western blotting analysis of the putative CMV-T CP gene transgenic tobacco lines. Total proteins were extracted from transgenic plants, and subjected to electrophoresis in a 12% polyacrylamide gel. After transblotting the protein to the PVDF membrane (Millipore Co.), the membrane was immunostained with CMV-T CP specific antiserum. Lanes 1 to 9, transgenic lines 1-3, 1-7, 1-11, 1-14, 1-15, 1-19, 1-21, 1-26, and 1-27, respectively; lane 10, total protein from nontransformed plant; lane 11, purified CMV-T CP.

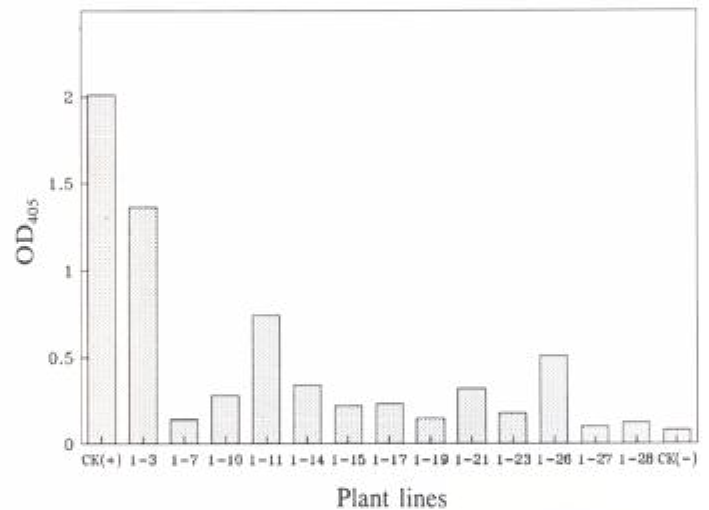

Fig. 7. Accumulation of the CMV-T CP in transgenic tobacco lines detected by ELISA. The accumulation levels of CP were presented as absorbency at wavelength of 405 nm. CK(+) represented the total leaf extract of a CMV infected tobacco, and the CK(-) represented the total extract of a nontransformed plant.

expression of CP were readily detectable or barely detectable, were challenge inoculated with CMV-T. In two individual experiments held in winter (12–28 C) and summer (22–38 C), four (1-10, 1-17, 1-23, and 1-28) and five (1-3, 1-11, 1-15, 1-21, and 1-26) plant lines were chosen for inoculation, respectively (Table 1 and 2). It was shown that the symptom development of the transgenic lines tested in winter was delayed for 4-20 days when compared to the untransformed plants which were also propagated from similar tissue culture procedure. Moreover, some transgenic plants of lines 1-10, 1-23, and 1-28 and all plants of line 1-17 were symptomless during the period of the experiments (Table 1 and Fig. 8). In both experiments, inocula were prepared by grounding the CMV-infected leaves of tobacco in inoculation buffer with a dilution ratio of 1:30 (w/v). In the first inoculation experiment which held in winter, the inoculum yielded about 30 to 50 local lesions per leaf on the host *Chenopodium quinoa*, while that in the second experiment in summer was 160 to 180 local lesions per leaf. In the second inoculation experiment, the delay of symptom expression in different lines tested was only 1-6 days, much shorter than the winter test (Table 2). Besides, the accumulation of CMV in transgenic plants tested in summer was also measured by indirect ELISA every two days after inoculation. Owing to the high temperature and high inoculum pressure, the accumulation of virus increased sharply 2-3 days after

TABLE 1. Symptom development of the CMV-T CP transgenic tobacco lines following mechanical inoculation with CMV-T under greenhouse conditions in winter (12-28 C)

Transgenic lines	No. of plants showing symptoms at different days post inoculation										No. of plants inoculated[2]
	6	10	14	18	22	26	30	34	36	40[1]	
Normal	0	8	10	10	10	10	10	10	10	10	10
1-10	0	0	5	6	7	7	7	7	7	7	10
1-17	0	0	0	0	0	0	0	0	0	0	4
1-23	0	0	2	4	4	4	4	4	4	4	8
1-28	0	0	3	4	5	5	6	6	6	6	10

[1] Days after inoculation with the virus.

[2] Plants of lines 1-10, 1-17, 1-23, and 1-28 were subjected to mechanical inoculation with CMV-T prepared from a virus-infected tobacco and diluted at 1:30 in inoculation buffer. The infectivity of the inoculum was 30-50 lesions per leaf of *C. quinoa*.

TABLE 2. Symptom development of the CMV-T CP transgenic tobacco lines following mechanical inoculation with CMV-T under greenhouse conditions in summer (22-38 C)

Transgenic lines	No. of plants showing symptoms at different days post inoculation										No. of plants inoculated[2]
	2	3	4	5	6	7	8	9	10	20[1]	
Normal	0	9	10	10	10	10	10	10	10	10	10
1-3	0	0	1	5	5	7	9	10	10	10	10
1-11	0	0	1	3	6	7	7	10	10	10	10
1-15	0	0	5	8	8	10	10	10	10	10	10
1-21	0	0	7	8	8	10	10	10	10	10	10
1-26	0	0	3	3	5	8	8	10	10	10	10

[1] Days after inoculation with the virus.

[2] Plants of lines 1-3, 1-11, 1-15, 1-21, and 1-26 were subjected to mechanical inoculation with CMV-T prepared from systemic leaves of CMV-T infected tobacco and ground in inoculation buffer with 1:30 dilution. The infectivity of inoculum was 160-180 lesions per leaf of *C. quinoa*.

inoculation. The ELISA data were closely correlated with the observation of symptom development (data not shown). The three lines 1-3, 1-11, and 1-26 that expressed the CP gene at higher levels, as detected by indirect ELISA and Western blotting previously, showed a better resistance to CMV-T than the lower expressors of lines 1-15 and 1-21, as reflected in a longer delay in symptom expression (Table 2).

DISCUSSION

Since the first report that transgenic plants expressing the CP of tobacco mosaic virus (TMV) showed a delay in disease development caused by TMV infection (33), this strategy has been widely adopted for other viruses (19). The CP-mediated resistance is manifested by several features. First, there are fewer sites where infection occurs on inoculated leaves. Nelson *et al.* (29) reported 95-98% fewer necrotic local lesions caused by TMV infection on CP(+) than on CP(-) Xanthi-nc tobacco plants. Similar observations in AlMV (alfalfa mosaic virus) or PVX (potato virus X) infection events were also reported (15,22). Second, manifestation of resistance is a reduced rate of systemic disease development throughout the CP(+) plants. Reports of CP-mediated resistance showed that CP(+) plants were less likely to develop systemic disease symptoms than CP(-) plants (1). Third, this form of protection has varying effects on the spread of systemic infection, ranging from preventing it altogether to slowing it down. There is plant-to-plant variation in systemic spread even within a batch of a single line of transgenic plants (1). The fourth is that the protection can be overcome by high levels of inoculum and does not operate against inoculation with RNA, except the cases of PVX (15) and PVS (potato virus S)(23). In addition, the degree of protection depends on the similarity between the amino acid sequence of the CP of the challenging virus and that expressed in the transgenic plants. The protection is usually more effective against closely related strains than the distantly related strains (22,40).

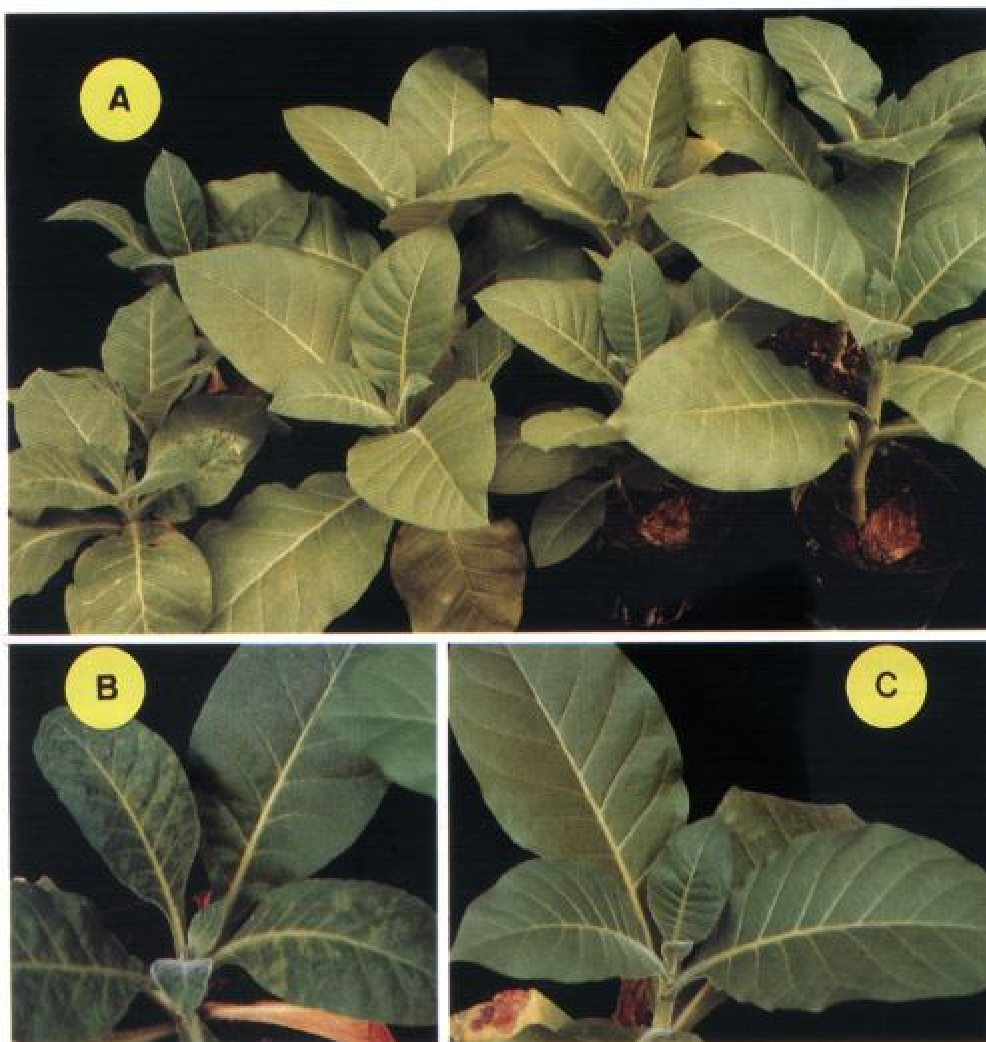

Fig. 8. Comparison of symptom development between transgenic and nontransformed plants in the first challenge inoculation experiment. A, symptoms of nontransformed plants (first left row) and transgenic plants (the three right rows, 1-23, 1-10, and 1-17, from left to right, respectively); B and C, the close vision of symptoms of a nontransformed plant and a plant of transgenic line 1-17, respectively. Photographs were taken 40 days after inoculation.

Four clones with different leader sequences were obtained from the cloning of the CP gene of CMV-T. Clones pCP9 and pCP10 were selected from cDNA library, and subsequently transferred to the genome of tobacco *N. tabacum* cv. Havana. However, expression of CP could not be detected in our condition (18). Therefore, two other clones pCCP1 and pGCP7 were constructed by PCR, using CMV-T CP specific primers, to improve the expression. All four clones pCP10, pCP9, pCCPl, and pGCP7 contained the entire CMV-T CP gene with the 3'-noncoding region. They also contained 250, 51, and 76 nucleotides of the CMV CP gene leader sequence, and the GUS leader sequence, respectively. To evaluate the influence of the different leader sequences in CP expression, these four clones were subjected to *in vitro* transcription and *in vitro* translation. The results indicated that these clones expressed the CP at a similar level in the rabbit reticulocyte lysate system. Thus, the differences in the leader sequence seemed having no dramatic influence in the expression of CMV-T CP *in vitro*.

It is still a question that the expression of the CMV-T CP in pCP9 and pCP10 transgenic tobacco were undetectable. Some possibilities were considered. Firstly, the long leader sequence of pCP10 generated a secondary structure to interrupt the translation of CP in plant cells, or the translation stop codons in the long leader inhibit the scanning of ribosome. Secondly, the leader sequence of pCP9 is too short for ribosome binding and scanning. Thirdly, the membrane used in Western blotting previously may affect the sensitivity of detection. The new PVDF membrane seems a better supporting material for immunoblotting. Fourthly, the stability of CMV CP may influence the detection. Although we have detected the CP in pCCP1 transgenic lines, the positive lines is still in a low

proportion. Fifthly, the biosynthesis condition in plant cells is complex, a little variation in leader sequence may result in a significant change in translation which cannot be observed *in vitro*.

In this study, the clone pCCP1 reflected the whole RNA 4, which is the CMV CP mRNA, was used to produce transgenic tobacco plants. Most of the transgenic plant lines expressed CMV-T CP at an hardly detectable level in our condition, yet three lines (1-3, 1-11, and 1-26) expressed at a higher level as detected by Western blotting and indirect ELISA. The integration of the CMV-T CP gene in the transgenic plants was confirmed by PCR. Four and five transgenic plant lines were subjected to challenge inoculation in the first and second inoculation experiments, respectively. The first experiment was held in late winter, and the observation of symptom development was lasted for 40 days. Slow development of symptom might due to the lower inoculum pressure, the age of plants, and the low temperature after inoculation. However, the erratic numbers of the plants, for example the line 1-17 had only 4 plants, made the result difficult to interpret statistically. Nevertheless, transgenic plants showed a clear protection effect against CMV infection in this experiment.

In the second inoculation experiment five plant lines, including the three higher expression lines (1-3, 1-11, and 1-26), were employed to determine the resistance against CMV infection. The much higher temperature (about 30–35 C in the day time) coupled with the much higher the inoculum pressure (160–180 local lesions per leaf of *C. quinoa*) made the symptoms induced by CMV develop quickly. All of normal tobacco plants became infected on the fourth day after inoculation and all the plants tested developed symptom within 9 days after challenge inoculation (Table 2). Although the condition was not ideal, this experiment showed that the CP-expressed lines significantly delayed the symptom expression, but the high inoculum pressure and high temperature conditions masked the protection effect of the CP transgenic plants. Thus, it is necessary to design more stringent experiments under which the plant lines are subjected to challenge inoculation with a lower inoculum pressure and the inoculated transgenic plants are placed under a temperature-controlled greenhouse condition.

In general, we have transferred the CP gene of a local CMV-T into *N. tabacum* var. Havana. The transgenic tobacco plants expressed the CP gene well and showed a significant delay in symptom development when challenged with CMV-T. Since CMV causes severe damages on important crops such as tomato and pepper in Taiwan, our success in the production of CP transgenic tobacco resistant to CMV infection provides a model system for control of the disease caused by CMV in these crops. Since the transformation and regeneration processes for tomato and pepper have been established (12,21,25,32,37), the application of this CP-mediated protection in these crops are being attempted.

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摘　　要

陳富永、葉錫東．1994．本省分離之胡瓜嵌紋病毒鞘蛋白基因轉型菸草特性之研究．植物病理學會刊 3:186-197．(台中市　國立中興大學植物病理學系)

胡瓜嵌紋病毒 (cucumber mosaic virus, CMV) 爲本省茄科、豆科、葫蘆科等作物栽培上的重要問題，成爲菸草、番茄、甜椒產量上的主要限制因子之一，在無抗病材料可利用下，問題難解決。爲解決此難纏的病毒病害，本實驗即藉由遺傳工程方式，嘗試構築胡瓜嵌紋病毒鞘蛋白 (coat protein, CP) 的轉型菸草，以期對此病毒產生抗性。自本省分離之胡瓜嵌紋病毒番茄分離株 CMV-T 之鞘蛋白基因先經聚合鏈鎖反應 (PCR) 放大後加以選殖，再轉移至二位元農桿菌載體 *Agrobacterium* 經其媒介轉殖於菸草中。在所得之二十八個轉型株系 (transgenic lines) 中，轉型植株復經聚合鏈鎖反應 (PCR) 確定其染色體中確含有 CMV 之 CP 基因。復以西方轉漬法 (Western blotting) 及間接聯血清吸附法 (indirect ELISA) 偵測得三株系表現較强，其它則表現較弱不易測得。所得轉型植株中分別以四個株系及五個株系在冬夏兩季進行兩次接種 CMV-T 試驗，結果顯示這些轉型植株均可明顯地延遲病徵表現，甚至在整個冬季試驗過程中，有些轉型植株並不產生病徵。綜合上述 CMV CP 轉型菸草構築成功之結果，可預期此遺傳工程保護方式將可應用到其他作物上，構築鞘蛋白轉型植株，以期對 CMV 的感染產生抗性。

關鍵詞：胡瓜嵌紋病毒、鞘蛋白、轉型植物、遺傳工程。