Delay of Disease Development in Transgenic Petunia Plants Expressing Cucumber Mosaic Virus I₁₇N-Satellite RNA

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Abstract. A cDNA clone of cucumber mosaic virus (CMV) I₁₇N-satellite RNA driven by the cauliflower mosaic virus (CaMV) 35S transcript promoter, was stably integrated into the genome of *Petunia hybrida* 'Bluepicotti' tissues by *Agrobacterium tumefaciens* Ti plasmid-mediated transformation. Transgenic plants producing CMV satellite RNA showed delayed disease development when inoculated with CMV-Y, a helper virus for the I₁₇N-satellite RNA. Furthermore, transgenic petunia plants showed delayed disease development against tobacco mosaic virus (TMV), a tobamovirus not related to CMV. Northern blot analysis revealed that large amounts of unit length satellite RNA (335 bp) were produced in CMV-infected transgenic petunia plants; whereas, mainly transcripts driven by the CaMV 35S promoter (approximately 1 kb) were produced in TMV-infected transgenic plants. SDS-PAGE and Western blotting showed that symptom reduction was correlated with a reduction in the amount of viral coat protein in transgenic plants.

Cucumber mosaic virus (CMV) is an economically important plant virus because of its large number of different strains, its worldwide distribution, and its numerous hosts, nearly 800 plant species (Collmer and Howell, 1992; Rizzo and Palukaitis, 1988). Symptoms induced by CMV infection include stunting, mottling, discoloration, and distortion of leaves, flowers, and fruit. Infected plants may be greatly reduced in size or killed. Crop yields are reduced along with reduced quality (Fraser, 1986). Agronomic practices, such as seed treatment, sanitation, or cross-protection with attenuated strains, have not been completely effective methods of control of CMV.

CMV belongs to the genus *Cucumovirus* in the family *Bromoviridae*. It has a tripartite genome consisting of single-stranded plus-sense RNAs (Peden and Symons, 1973) and four open reading frames (ORFs). RNAs 1 and 2 each contain one ORF encoding proteins of 111 and 97 kDa, respectively, that are required for replication (Hayes and Buck, 1990; Nitta et al., 1988). RNA 3 contains two ORFs: the 5' end of RNA 3 encodes a 30-kDa protein involved in viral movement and the 3' end of RNA 3 generates a subgenomic RNA, RNA 4, which acts as a messenger for the viral coat protein (Gould and Symons, 1977). In addition to genomic RNA, some CMV isolates contain extra RNA species of 330 to 380 nucleotides termed satellite RNAs (Francki, 1985; Fritsch and Mayo, 1989). Satellite RNAs are totally dependent on their helper viruses for replication and encapsidation (Kuwata et al., 1991; Masuta and Takanami, 1989; Murrant and Kumar, 1990;

Naidu et al., 1991; Palukaitis, 1988). The presence of a satellite RNA can alter symptoms caused by the helper virus (Jaegle et al., 1990; Kaper and Collmer, 1988). Generally, satellite RNAs cause attenuation of symptoms, but in some cases can induce severe and different symptoms. The type of symptom modification produced depends on the satellite RNA, the strain of the helper virus, and the host plant (Mossop and Francki, 1977; Wu et al., 1993).

The ability to transform plant genomes with foreign genetic material via the *Agrobacterium tumefaciens* Ti plasmid system has made it possible to enhance plant resistance to viruses by incorporating virus-related nucleotide sequences into the nuclear genome of the host (Baulcombe et al., 1986; Beachy et al., 1990; Buck, 1991; Harrison et al., 1987; Powell-Abel et al., 1986). Protection of transgenic plants against virus infection has been characterized by a decrease in the number of primary infection sites on inoculated leaves and by a delay or lack of systemic symptom development. We previously demonstrated resistance to CMV in transgenic tobacco plants that express a cDNA copy of CMV I₁₇N-satellite RNA (Kim et al., 1992; Paek and Hahn, 1991).

In an attempt to establish virus-resistant transgenic petunia plants expressing the CMV I₁₇N-satellite RNA, we conducted virus-resistance assays using CMV-Y, a helper virus for the CMV I₁₇N-satellite RNA, and TMV, not a helper virus. In this study, we describe the delayed disease development ability of the CMV I₁₇N satellite RNA upon CMV-Y inoculation in transgenic petunia plants. In addition, transgenic petunia showed symptom attenuation upon TMV inoculation, which is not a helper virus for the CMV satellite RNA replication.

Materials and Methods

Materials Restriction endonucleases were purchased from Promega (Madison, Wis.) and used as instructed by the manufacturer. Other chemicals were from Sigma Chemical Co. (St. Louis). [³²P]dCTP was purchased from New England Nuclear (Boston) or Amersham International plc. (Seoul, Korea). *Petunia hybrida* 'Bluepicotti' was used as plant material. CMV-Y (provided by

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Eun-Kyung Park at Korea Ginseng and Tobacco Research Institute) and TMV (strain isolated from tobacco) were used for challenge inoculation. CMV-Y did not contain any detectable satellite RNA. The plasmid pROK1/105 containing the CMV I₁₇N satellite RNA used in this work was provided by Bryan D. Harrison (Baulcombe et al., 1986). pROK1/105 contains two complete cDNA satellite sequence units in a head-to-tail arrangement. The expression cassette comprises the cauliflower mosaic virus 35S RNA promoter and the termination fragment of the nopaline synthase gene of *Agrobacterium* T-DNA (Trios).

Plant transformation. The plasmid pROK1/105 containing the chimeric CMV I₁₇N satellite RNA was transformed into the disarmed *Agrobacterium tumefaciens* strain LBA4404 using the direct DNA uptake method (An, 1987). The structure of the plasmid transferred to *A. tumefaciens* was verified by purification and restriction digestion analyses. Transformation of petunia plants was based on the method of leaf disk cocultivation with *A. tumefaciens* (Horsch et al., 1985). Induction of callus and shoot

formation were conducted on solid MS medium (Murashige and Skoog, 1962) supplemented with 2.0 mg-liter⁻¹ indole acetic acid, 1.0 mg-liter⁻¹ benzyl-aminopurine, and 100 mg-liter⁻¹ kanamycin sulfate as a selection agent. For root induction, kanamycin-resistant regenerated shoots were transferred to hormone-free MS medium containing 50 mg-liter⁻¹ kanamycin. After root development (2 to 3 weeks), plantlets were transferred to potting soil.

DNA and RNA preparations. Plasmid DNA was prepared from *E. coli* and *A. tumefaciens* by the alkaline lysis method (Bimboim, 1983). Genomic DNA was extracted from plant tissues according to the method of Dellaporta et al. (1983). Total RNA was extracted from leaves of transgenic and control petunia plants by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987).

DNA blot hybridization. DNA samples were digested with *Sca*I, fractionated by electrophoresis on a 0.8% agarose gel, and blotted onto a nylon membrane (Hybond N). Hybridization was performed for 24 h at 43°C in 0.1% SDS, 5x Denhardt's solution,

Fig. 1. (A) Southern blot analysis of the cDNA of CMV I₁₇-satellite RNA from transformed petunia plants. Lane 1, *Sca*I digested pRok1/105; 2, *Sca*I digested genomic DNA from nontransformed petunia plant; 3, *Sca*I digested genomic DNA from transgenic plant line 1 transformed with pRok1/105; 4, transgenic plant line 2. Approximately 30 µg of genomic DNA was loaded on each lane. (B) The probe was a 32P labelled 335bp *Sca*I cDNA fragment of pRok1/105. The figure shows a region adjacent to the cDNA insert and the expression cassette of pRok1/105.

Fig. 2. Symptoms induced by CMV and TMV on nontransformed and transgenic petunia. (A and B) CMV inoculation only; (C and D), TMV inoculation only; (E and F), CMV and TMV coinoculation. Upper the pictures, nontransformed petunia plants lower three pictures, transformed typical petunia plants. Pictures were taken 20 days postinoculation.

5x SSPE, and 100 $\mu\text{g}\cdot\text{ml}^{-1}$ sheared, denatured salmon sperm DNA. DNA probe was prepared by labeling a 335-bp ScaI fragment of pROK1/105 with [$\alpha^{32}\text{P}$]dCTP by the random priming method (Feinberg and Vogelstein, 1983). The membrane was washed with constant agitation in 2x SSPE and 0.170 SDS for 10 rein; 0.2x SSPE and 0.170 SDS for 5 rein, and 0.1x SSPE and 0.1% SDS for 5 min at 43C.

RNA blot hybridization. RNA samples (20 μg) from leaves collected 20 days postinoculation, were treated with formaldehyde and formamide according to Sambrook et al. (1989). Samples were electrophoresed on a 1.0% agarose gel containing 0.67 M formaldehyde and then transferred to a nylon membrane. Hybridization conditions were similar to those used for DNA blotting described above, except temperature was 52C. Membrane filters were exposed to X-ray films with an intensifying screen at -70C.

Protein analysis. Twenty days after inoculation, leaves of transgenic and nontransformed petunia plants were collected, ground in liquid nitrogen, and homogenized with a mortar and pestle in 2-ml buffer (10 mM sodium phosphate buffer, pH 7.0) per gram fresh weight. The homogenate was clarified by centrifugation at 12,000 rpm for 10 min in an Eppendorf centrifuge, and the protein concentration was determined according to the method of Lowry et al. (1951).

Electrophoresis of proteins and immunoblot analysis. Protein extracts were fractionated on 10% SDS-polyacrylamide gels and either stained with Coomassie brilliant blue or transferred onto a nitrocellulose membrane (Hoefer, San Fernando, Calif.) as de-

scribed by Towbin et al. (1979). Antisera to CMV-Y and TMV virions were used at a 1:250 dilution. Immunoblotting was conducted using goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma). Calorimetric reactions were performed by using dichloronaphthol as a substrate.

Symptom assays of transgenic plants. Seeds from self-fertilized transgenic plants were collected and 100 seeds from each transgenic line were aseptically germinated on an MS medium (Murashige and Skoog, 1962) supplemented with 200 $\text{mg}\cdot\text{liter}^{-1}$ kanamycin sulfate, and then kanamycin-resistant and kanamycin-sensitive seedlings were counted. None of the 100 nontransformed seedlings survived on this selection medium. Progenies of self-fertilized transgenic line 1 (100% germination) survived at an approximate ratio of 15:1 on kanamycin-containing medium, suggesting that two copies of T-DNA were inserted in the nuclear genome. Germination ratio could not be accurately estimated for transgenic line 2 ($\approx 60\%$ of germination) because of a poor germination rate. About 3 weeks after transfer to potting soil, 10 nontransformed plants and 10 of each transgenic line for each inoculation were used for assays of virus infection. Two or three leaves from nontransformed and R1 transgenic plants expressing the cDNA of CMV satellite RNA were dusted with Carborundum (500 mesh) and mechanically inoculated with a crude sap from systemically CMV-Y infected *N. tabacum* 'w38' leaves prepared in 10 mM sodium phosphate buffer at a 1 to 1 ratio. Purified TMV was inoculated at a concentration of 3 $\mu\text{g}\cdot\text{liter}^{-1}$ in 50m of 0.01 M phosphate buffer, pH 7.2. Two plants of each transgenic petunia line and one

Fig. 3. Immunoblot analysis of nontransformed and transgenic petunia plants inoculated with TMV. Upper panel: purified TMV virions (Vp), total plant proteins from control (C) and transgenic (T1 and T2) plants stained with Coomassie brilliant blue. Lower panel: Immunoblot analysis with polyclonal TMV antiserum. M designates low molecular weight markers from BRL (top to bottom 97.4, 66.2, 45.6, 31, 21.5, and 14.4 kDa). Lanes 1,2 and 3 correspond to non-inoculated plant, inoculated primary leaves, and non-inoculated apical leaves. Bar indicates the position of the TMV coat protein.

nontransformed control plant were mock-inoculated. After rinsing the inoculated leaf surface with tap water, the plants were grown in the greenhouse at 25°C. Symptom development was monitored on a daily basis for 2 months after inoculation.

Results

Integration of CMV satellite RNA cDNA in petunia plants.

Transformed petunia plants were regenerated from *A. tumefaciens*-infected leaf disks, and 24 primary transformants were selected. Transformed plantlets were analyzed for the presence of the CMV

Fig. 4. Northern blot analysis of the CMV satellite RNA accumulation in 20 days post-inoculated petunia. (A) Inoculated with CMV only (B) inoculated with TMV only (C) co-inoculated with CMV and TMV. RNA was extracted from nontransformed (C) and transgenic (T1 and T2) petunia plants. Lane illustration is as same as in Fig. 3. Probe was a 32P-labeled 335 bp *ScaI* cDNA fragment of CMV satellite RNA. Upper bar indicates the position of 1000-bp transcripts and lower bar, 335-bp unit length transcripts.

satellite RNA sequence by Southern blot hybridization (Fig. 1). Using a 32P-labeled cDNA insert (335-bp *ScaI* fragment, Fig. 1B) as a probe, fragments of 0.3,0.8, and 1.3 kb were detected in *ScaI* digests of genomic DNA isolated from transgenic plants, lines 1 and 2 (Fig. 1A). In addition, transgenic lines 1 and 2 showed two and three additional hybridizing fragments, respectively, suggesting that two or more copies of the T-DNA were inserted in these transgenic plants. No hybridization signals were observed in genomic DNA isolated from nontransformed plants. Thus DNA blot hybridization data confirmed the presence of the cDNA copy of CMV satellite RNA in the genome of transgenic petuniaplants.

Symptom suppression in transgenic petunia plants upon virus

Fig. 5. Immunoblot analysis of the viral protein accumulation in nontransformed and transgenic petunia plants 20 days postcoinoculation with CMV and TMV. Upper panel, total proteins stained with Coomassie brilliant blue. Vp, purified CMV virion; Lower panel (A): immunoblot analysis with TMV antiserum. Vp-purified TMV virion Lower panel (B): immunoblot analysis with CMV antiserum. Vp, purified CMV virion. Lane illustration is same as in Fig. 3.

Fig. 6. Symptoms induced in nontransformed (left) and transgenic petunia (right) 2 months after coinoculation with CMV and TMV.

inoculation. Experiments were initiated to determine whether transgenic petunia plants containing the cDNA copy of the CMV satellite RNA could suppress symptoms caused by CMV infection. To minimize the bias due to plant growth differences, plants of similar height (≈ 3 weeks after transfer to potting soil) were selected; 10 plants from each transgenic line and 10 nontransformed plants were inoculated with CMV-Y. Control plants developed typical yellow mosaic and leaf-curling symptoms within 1 week after inoculation followed by systemic infection leading to severe necrosis. Control and transgenic plants developed chlorotic lesions in inoculated leaves. However, only two or three apical leaves developed mild mosaic and mottling symptoms after 10 to 14 days in transgenic plants. New leaves produced subsequently showed no mosaic symptoms and plants grew well (Fig. 2 a and b).

We conducted immunoblot analysis to determine if symptom suppression reflected a reduction in the amount of coat protein. Protein extracts were prepared from leaves of selected transgenic and nontransformed plants 20 days after inoculation with CMV-Y, run on 10% SDS-polyacrylamide gels, and either stained with Coomassie brilliant blue or processed for immunoblot analysis by using antiserum to CMV virions. Coomassie brilliant blue stained gels showed that approximately equal amounts of soluble protein extracts were loaded onto gels for each sample except for T1 lane 1 (noninoculated control), which had less protein. The immunoblot analysis revealed that a protein migrating at the same position as the purified CMV and reacting with the CMV antiserum accumulated in inoculated leaves of transgenic lines 1 and 2, but at a lower level than in the control. No viral protein was detected in noninoculated apical leaves of transgenic lines 1 and 2; whereas, CMV coat protein was detected in inoculated and noninoculated apical leaves of control plants. These results show that the symptom delay and perhaps suppression observed upon CMV infection in transgenic plants was accompanied by some reduction in viral protein content, at least in apical leaves of transgenic petunia.

To test the susceptibility of transgenic petunia expressing the CMV satellite RNA cDNA to an unrelated virus, 10 plants from each transgenic line and 10 nontransformed plants were inoculated with TMV. Upon TMV inoculation, nontransformed plants showed veinyellowing and mild leaf distortion (milder symptoms than those observed with CMV inoculation), while transgenic lines showed less severe veinyellowing and distortion (Fig. 2 C and D). We conducted immunoblot experiments using a polyclonal TMV antiserum to confirm the visual observation and found that TMV CP production was also reduced in apical leaves of transgenic plants (Fig. 3). Since TMV is not a helper virus for the CMV satellite RNA, the production level of CMV satellite RNA transcripts was examined by Northern blot analyses in these plants upon TMV inoculation. At the RNA level, as expected, large amounts of unit length transcripts (335 bp) accumulated in CMV-infected transgenic plants (Fig. 4A). However, transcripts $\approx 1,000$ bp in size accumulated in TMV-infected transgenic plants (Fig. 4 B). In this case, overexposure was necessary to show 1 kb transcripts. Since pRok1/105 contains 2.3 copies of the satellite RNA cDNA, a 1000-bp size transcript corresponds to transcripts driven by the CaMV 35S promoter.

When nontransformed control petunia plants were co-infected with CMV and TMV, symptoms were much more severe compared to single viral infections. Severe symptoms including necrosis, severe leafcurling, and severe growth retardation were observed (Fig. 2 E and F). In transgenic plants, symptom development was less severe and was accompanied by reduced accumulation of CMV and TMV coat protein in Western blot analysis (Fig. 5). At the RNA level, large amounts of unit length satellite RNA

were produced upon CMV and TMV co-inoculation in transgenic plants (Fig. 4C). Especially after flowering, the difference of symptom development was much more evident. Inoculated control plants produced no flowers, whereas inoculated transgenic plants showed flowers with normal shape and color (Fig. 6).

Discussion

Recently, engineered resistance to virus infection has made it possible to increase plant resistance against otherwise devastating viruses by introducing a single pathogen-derived or virus-targeted sequence into the nuclear genome of the host (Baulcombe et al., 1986; Powell-Abel et al., 1986).

In this study, we report that petunia plants transformed with a cDNA clone of CMV I₁N satellite RNA previously used successfully in tobacco (Baulcombe et al., 1986; Harrison et al., 1987; Kim et al., 1992; Pack and Hahn, 1991) have exhibited delayed disease symptoms upon infection with CMV and TMV, an unrelated virus. CMV satellite RNAs are small RNAs that are not infectious by themselves and require helper viruses for their replication and encapsidation (Simon, 1988). In some cases, satellite RNAs enhance the severity of symptoms in conjunction with the helper virus infection, and in other cases the symptoms are ameliorated (Jaegle et al., 1990; Kaper and Collmer, 1988). The expression of satellite RNAs in transgenic plants has decreased virus symptoms and/or titers in a manner that appears to mimic a natural system (Baulcombe et al., 1986; Harrison et al., 1987).

In the case of satellite RNA-mediated protection, CMV satRNA also protects against symptoms of tomato aspermy virus (TAV) but without causing any reduction in virus replication (Harrison et al., 1987). Thus, reduced replication may not be the sole mechanism of satRNA-mediated protection. Satellite tobacco ring spot virus (sTRSV) also interferes with the replication and disease spread of yet another nepovirus, cherry leafroll virus, even though cherry leafroll virus is not a helper virus for sTRSV (Ponz et al., 1987). In this study, the production of CMV satRNA in transgenic petunia enhanced disease protection against not only CMV but also TMV. In TMV-infected plants, transcripts of ≈ 1 kb (Fig. 5B) accumulated instead of single unit-length transcripts (335 bp) in CMV-infected plants. Western blot analysis also confirmed that TMV coat protein production was reduced in transgenic plants upon TMV infection. However, it remains to be determined whether ELISA assays will confirm the quantities of coat protein levels expressed in these plants and whether protection was achieved by interference of viral multiplication at the RNA level. As the number of plants used in the virus protection assay was rather small (10 of each transgenic lines), further experiments will be required to assess the CMV satellite RNA-mediated protection against the TMV infection. Furthermore, the mechanism of protection needs to be investigated particularly to ascertain whether it is a simple tolerance or a true resistance. However, our results clearly indicate that transgenic petunia plants expressing the CMV satellite RNA cDNA exhibited delayed symptom expression and possibly protection from infection with CMV and TMV, an unrelated virus.

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