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High Resistance to Cucumber Mosaic Virus Conferred by Satellite RNA and Coat Protein in Transgenic Commercial Tobacco Cultivar G-140

Y. Yie, F. Zhao, S. Z. Zhao, Y. Z. Liu, Y. L. Liu, and Po Tien

Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, P.R. China.
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A chimeric vector was constructed to express cucumber mosaic virus (CMV) satellite (Sat) RNA and coat protein (CP). Transgenic lines of tobacco cultivar G-140 expressing CP and Sat-RNA were obtained; these lines had high resistance to CMV. Fifty to 70% of the transgenic plants were symptomless 90 days after inoculation with 25–50 μg/ml of CMV. Resistance was about twice that conferred by the Sat-RNA or the CP gene alone in transformed plants.

Since the first demonstrations that a viral coat protein (CP) gene (Abel *et al.* 1986) or a viral satellite RNA (Sat-RNA) molecule with the ability to attenuate symptom expression (Tien and Zhang 1983, 1984; Baulcombe *et al.* 1986) could be useful for controlling plant virus disease, many labs have obtained transgenic plants in which viral CP or Sat-RNA confer resistance to viruses (Harrison *et al.* 1987; Tumer *et al.* 1987; Cuozzo *et al.* 1988; Hemenway *et al.* 1988; Nelson *et al.* 1988; Van Dun and Bol 1988; Wu *et al.* 1988; Stark and Beachy 1989; Nejidat and Beachy 1990; Lawson *et al.* 1990). Although some new approaches have been found (Golemboski *et al.* 1990; Haseloff and Gerlach 1988), viral CP and Sat-RNA are still effective in obtaining resistance to viruses in transgenic plants. However, the efficiency of these two approaches was demonstrated to have limitations.

In some cases, transgenic plants expressing viral CP showed a delay of symptom development for a period of time, after which the virus overcame the protection, and symptoms developed. The extent of CP-mediated protection can also be affected by the form and concentration of inoculum. When transgenic tobacco plants expressing tobacco mosaic virus CP (Abel *et al.* 1986) or alfalfa mosaic virus CP (Loesch-Fries *et al.* 1987; Van Dun *et al.* 1987) were inoculated with viral nucleic acids or virus at high concentrations, their resistance was reduced sharply or lost completely. On the other hand, the level of expression of Sat-RNA is rather low in transgenic plants. Transcripts of the Sat-cDNA are able to replicate to high levels by using the viral replicase but only after challenge virus infection; these transcripts then inhibit the replication of the viral genome. How can the limitations of these two approaches of resistance to viruses be overcome? One way is by the use of a much stronger promoter than the CaMV 35S RNA promoter for enhancing the level of expression of the resistance gene products. However, there are many examples of CP-mediated protection in which the concentration of CP and the extent of protection are not correlated. The other practical approach is the construction of a chimeric vector, including CP to interfere with early events in infection and Sat-RNA to interfere with replication of the viral genome in later stages; thus, transgenic plants with resistance to viruses during all stages of infection would be created. This paper reports the construction of a chimeric gene expression vector containing a Sat-RNA gene and a CP gene of cucumber mosaic virus (CMV) and the transformation of this chimeric gene into commercial tobacco cultivar G-140. Transgenic plants were tested for expression of both genes and for their level of protection against CMV. In transgenic plants, the chimeric vector conferred higher resistance to CMV than either CP or Sat-RNA alone.

Address correspondence to P. Tien.

MATERIALS AND METHODS

Virus and viral RNA purification. CMV-1, containing Sat-RNA-1, was obtained from J. M. Kaper (Microbiology and Plant Pathology Laboratory, Plant Sciences Institute, Agricultural Research Service, USDA, Beltsville, MD) and used for cloning the Sat-cDNA. CMV-B, a Chinese isolate that is free of Sat-RNA, was used as challenge inoculum. CMV-O strain was used for cloning the CP gene. These viruses were propagated in *Nicotiana tabacum* L. 'Samsun' and purified as described by Qiu *et al.* (1985). The viral RNA was purified from virions by phenol-sodium dodecyl sulfate (SDS) extraction as described by Chu *et al.* (1983).

Cloning and sequencing of CMV Sat-cDNA. The double-stranded RNA of Sat-RNA-1 was isolated and purified from CMV-infected tobacco. The RNA was heated to 100° C for 5 min in 30% dimethyl sulfoxide and immediately put into an ice bath to denature the dsRNA. Synthesis of the first strand of cDNA was primed by a synthetic primer (5′-CCCGGGTCCTGTATAGG-3′) complementary to the 3′-end sequence of the positive strand and a primer (5′-GTTTTGTTTGATGG-3′) the same as the 5′-end sequence of the positive strand; avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI) was used. The RNA strands were then degraded with NaOH, and the synthesized positive and negative cDNA strands were annealed to form ds-cDNA. The ds-cDNA

was cloned into vector pUC12 (digested with *Sma*I). Selection and screening of full-length Sat-cDNA clones (termed pUI) were carried out with the 3′-end and 5′-end primers as probes as described by Maniatis *et al.* (1982). The cloned fragment was subcloned into M13, mp18, and mp19. DNA sequencing was by dideoxy-mediated chain termination (Sambrook *et al.* 1989).

Cloning and sequencing of the coat protein gene. To make the first strand of cDNA, we primed total viral RNA by using a synthetic oligonucleotide (5′-TCAGACTGGG-AGTACTCT-3′) that was complementary to the 3′-end sequence of the sense strand; the method of D'Alessio and Gerard (1988) was used. After the RNA strand was degraded with NaOH, the second strand was primed with a synthetic oligonucleotide (5′-ATGGACAAATCAGAA-3′). The cDNA was cloned into vector pUC19 (digested with *Sma*I). Selection and screening of full-length coat protein cDNA clones (termed pUCP) were by *in situ* hybridization with the 3′-end primer as probe (Sambrook *et al.* 1989). Sequencing of the insert was carried out by direct plasmid sequencing (Liszewski *et al.* 1989).

Construction of the chimeric gene expression vector pRCPI and tobacco transformation (Fig. 1). The Sat-cDNA insert was excised from pUI with *Xba*I and *Sac*I, and the CMV-O CP insert was excised from pUCP with *Bam*HI and *Sac*I. The Sat and CP fragments were then subcloned separately into plant expression vector pROK-II to make pRI and pRCP, respectively. The plasmid pROK-II contains five unique restriction sites (*Xba*I, *Bam*HI, *Sma*I, *Kpn*I, and *Sac*I) between the CaMV 35S promoter and *Nos* terminator. To construct the expression vector pRCPI, which contains expression cassettes for CP and Sat-RNA, pRI was digested with *Hin*dIII and made blunt-ended with the Klenow enzyme; the insert was then excised with *Sac*I. The resulting fragment containing the 35S promoter and satellite insert (35S-Sat) was recovered. pRCP was cut with *Eco*RI and made blunt-ended with the Klenow enzyme, and then the CP-*Nos* fragment was released with *Bam*HI. Finally, the 35S-Sat fragment and CP-*Nos* fragment were ligated into plant expression vector pROK-II (digested with *Bam*HI and *Sac*I) to make pRCPI. The pRCPI vector was mated into *Agrobacterium tumefaciens* (Smith and Townsend) Conn 'LBA 4404' by a triparental plasmid transfer method (Bevan 1984) and transformed into commercial tobacco cultivar G-140.

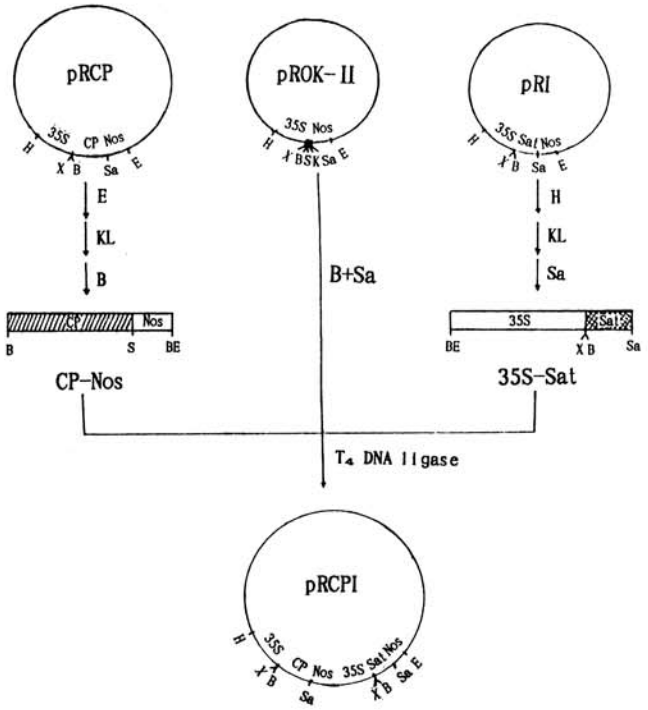

Fig. 1. Construction of the chimeric gene expression vector pRCPI. B, *Bam*HI; BE, blunt end; E, *Eco*RI; KL, Klenow enzyme; S, *Sma*I; Sa, *Sac*I; X, *Xba*I.

Western blot analysis of coat protein expression. Leaf tissue (200 mg) from transgenic or control tobacco plants was ground in 200 μl of extraction buffer (30 mM potassium phosphate, pH 7.5, 0.4 M sodium chloride, and 10 mM β-mercaptoethanol). Extracts were clarified at 10,000 rpm for 10 min at room temperature. Total protein was quantified by the method of Bradford (1976). Extracted proteins were fractionated electrophoretically on SDS-polyacrylamide gels, as described by Sambrook *et al.* (1989). The gel was blotted onto Zeta-probe membrane (BioRad, Richmond, CA). The blotted membrane was preincubated with phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) for 2 hr; then, anti-CMV immunoglobulin G (IgG) (1:1,000) was added and incubated for 2 hr. Blots were rinsed several times with PBS; alkaline-phosphatase-coupled goat anti-rabbit IgG (1:1,000) in PBS containing 4% BSA was added and incubated for 30 min. The blots were rinsed and developed in the BCIP-NBT system (Promega).

Northern blot analysis of coat protein gene transcripts. We extracted RNA from transgenic tobacco plants by powdering leaf tissue in liquid N_2 and homogenizing it in GPS buffer (0.2 M glycine, 0.2 M sodium phosphate, pH 7.5, 0.6 M sodium chloride) and phenol/chloroform/isoamyl alcohol (24:24:1), and by ethanol precipitation. Twenty micrograms of total RNA was loaded onto a 1.2% formaldehyde-agarose gel; 28S rRNA and 9S rabbit globin mRNA were used as markers. The separated RNA was electro-transferred to Zeta-probe membrane and hybridized with a ^{32}P-labeled CMV-CP cDNA probe.

Polymerase chain reaction (PCR) detection of Sat-cDNA in transgenic tobacco plants. Total DNA was extracted from transgenic tobacco plants by the CTAB method used by Draper and Scott (1988) and digested with *Eco*RI. To denature DNA, each sample was heated to 100° C for 5 min and was immediately put into an ice bath. Sat-cDNA was amplified by 35 cycles of PCR primed by the 3′-end and 5′-end primers for Sat-cDNA synthesis. Each cycle included 50 s at 95° C, 30 s at 50° C, and 2 min at 74° C. The amplified fragment was detected on a 1.5% agarose gel.

Detection of satellite RNA in transgenic tobacco plants. Transgenic tobacco plants were inoculated with CMV-B (25–50 μg/ml) that was free of Sat-RNA. After 2 wk, total RNA was isolated by the method described above and further fractionated with 2 M LiCl precipitation to purify dsRNA (Diaz-Ruiz and Kaper 1978). The dsRNAs were analyzed by running them on a 4% polyacrylamide gel and

were stained with silver.

Protection experiments with transgenic plants. Transgenic plants expressing CMV-O CP, CMV-1 Sat-RNA, or both CP and Sat-RNA at the four- to six-leaf stage were inoculated on Carborundum-dusted leaves with 25–50 μg/ml of purified CMV-B in 50 mM phosphate buffer (pH 7.0). After inoculation, the leaves were rinsed with water, and plants were placed in a greenhouse. Symptom development was scored visually at 10- or 20-day intervals up to 90 days after inoculation.

Quantitation of CMV levels in transformed plants. Control or transgenic tobacco leaves inoculated with 25–50 μg/ml of CMV-B were sampled 30 days after inoculation by grinding the leaves in phosphate buffer (50 mM, pH 7.0) at 1 g of leaves to 5 ml of buffer. The diluted sap was inoculated onto half-leaves of *Chenopodium quinoa* Willd. for determining the titer of biologically active virus in the infected plants (Wu *et al.* 1989).

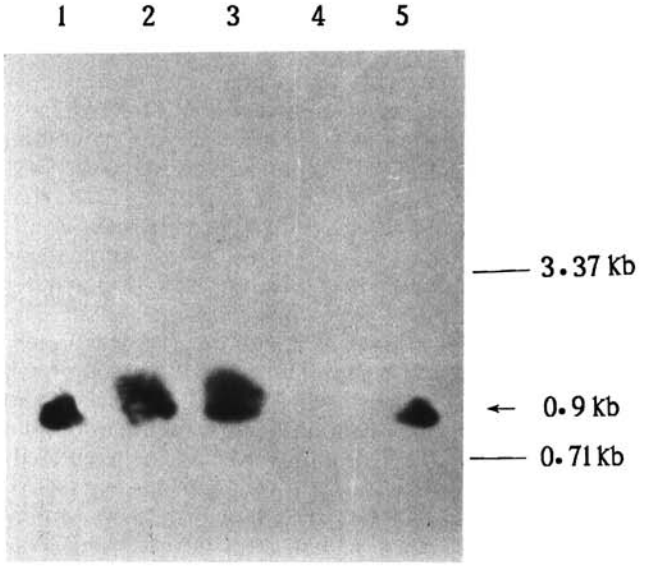

Fig. 2. Analysis of cucumber mosaic virus O (CMV-O) coat protein (CP) gene transcripts in transgenic tobacco plants expressing CP and satellite (Sat) RNA. Total RNA (20 μg) isolated from leaves of transgenic plants was fractionated on a 1.2% agarose gel containing formaldehyde, transferred to Zeta-probe membrane, and hybridized with a synthesized CP cDNA probe. Lanes 1, 2, 3, 5, RNAs from plants transformed with CP and Sat-RNA; Lane 4, RNAs of plant transformed with vector pROK-II (control).

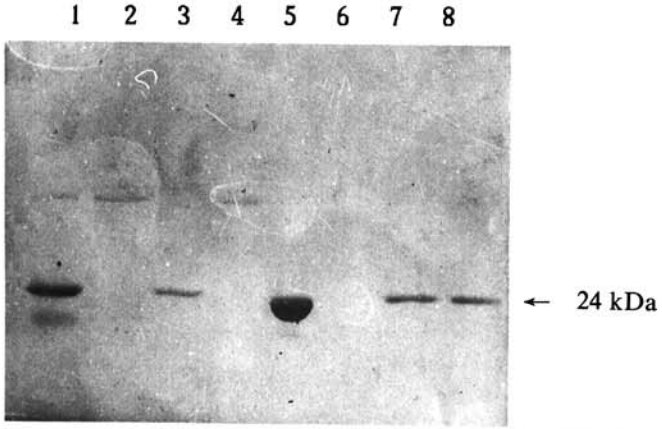

Fig. 3. Western blot analysis of cucumber mosaic virus O (CMV-O) coat protein (CP) expression in transgenic tobacco plants expressing CP and satellite (Sat) RNA. Total protein (40 μg) extracted from leaves was separated on a 12% sodium dodecyl sulfate polyacrylamide gel, and CP was identified by immunoblotting with anti-CMV IgG. Lane 5, purified CMV CP (10 μg). Lane 1, proteins of transgenic plant expressing CP and Sat-RNA inoculated with CMV. Lanes 3, 7, and 8, proteins of transgenic tobacco without inoculation. Lanes 2, 4, and 6, proteins of pROK-II-transformed control plants.

RESULTS

Cloning and sequencing of CMV Sat-RNA and CP genes. Sat-cDNA was made from Sat-dsRNA by use of two synthetic oligodeoxynucleotides as primers. A full-length cDNA clone was selected with these two primers as probes, and the sequence was determined to be identical to the original sequence of 334 nucleotides (Collmer *et al.* 1983). Total CMV-O RNA was used as a template for CP gene synthesis. The CP sequence of 657 nucleotides encodes a protein of 218 amino acids with a predicted molecular weight of 24 kDa (Yie *et al.* 1991).

Construction of the chimeric gene expression vector pRCPI. As described above, the 35S-Sat fragment and the CP-*Nos* fragment were recovered and ligated into the plant expression vector pROK-II to make the chimeric vector pRCPI (Fig. 1). Transcripts from each cassette in pRCPI contain 10 nucleotides from the promoter and 200 nucleotides from the terminator, in addition to the sequence of the insert. The resulting *Agrobacterium* co-integration was shown by *in situ* hybridization. We obtained transgenic tobacco plants (cultivar G-140) separately by *A. tumefaciens*-mediated transformation of leaf disks (Wu *et al.* 1989) with pRCPI (CP + Sat), pRCP (CP), or pRI (Sat), as well as pROK-II, as control.

Expression of CP and Sat-RNA in transgenic tobacco plants. Transgenic tobacco plants were analyzed for expres-

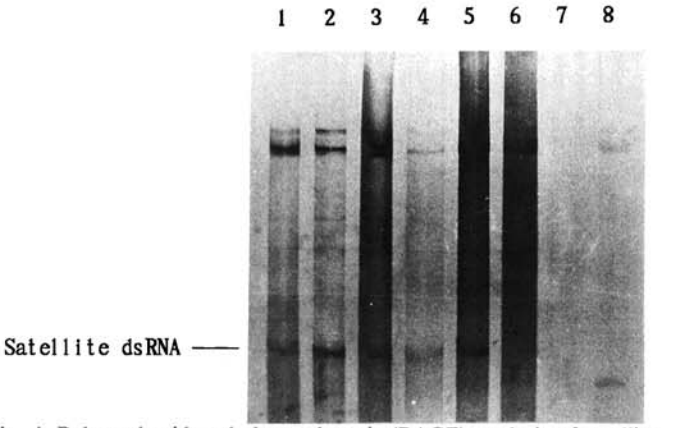

Fig. 4. Polyacrylamide gel electrophoresis (PAGE) analysis of satellite (Sat) dsRNA in tobacco plants transformed with cucumber mosaic virus (CMV-B) coat protein (CP) and Sat-RNA. Total RNA was isolated from leaves, and the dsRNA was purified by LiCl fractionation. The dsRNA was fractionated in a 4% polyacrylamide gel and silver-stained. Lanes 1–6 and 8, dsRNAs from transgenic plants expressing CP and Sat-RNA inoculated with CMV-B (25 μg/ml). Lane 7, dsRNA from pROK-II transformed plant as control.

Table 1. Comparison of relative cucumber mosaic virus B (CMV-B) concentrations in transformed tobacco plants expressing coat protein (CP) and satellite RNA (Sat-RNA) and Sat-RNA alone with concentrations in nontransformed control plants

Transformed genotype	Transgenic plant line	Extent of infection (average number of lesions on 10 half-leaves of *Chenopodium quinoa*) Control (non-transformed)	Transformed plant	Relative CMV concentration (transformed/control [%])
CP + Sat-RNA	2G-14	28	1	4
	2G-22	35	0	0
	2G-25	54	0	0
	2G-26	37	0	0
	4G-2	37	2	5
Sat-RNA only	T-2	141	15	10.6
	T-9	80	19	23.7
	T-10	61	13	21.3

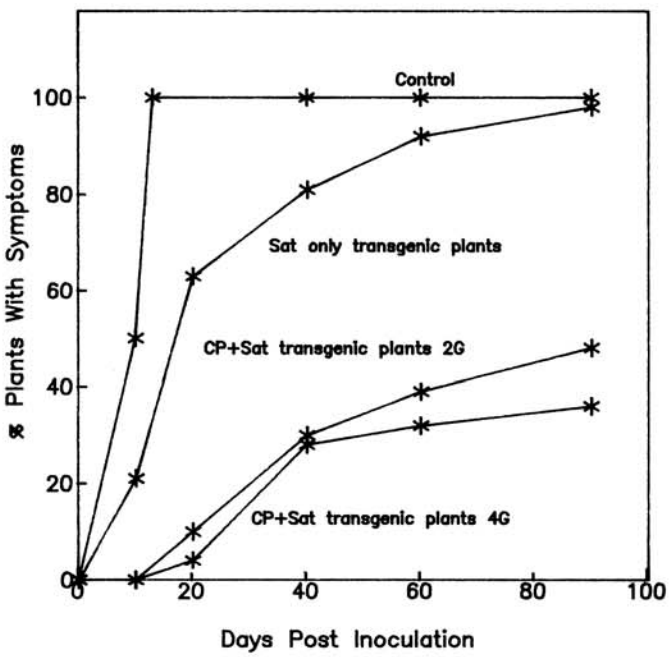

Fig. 5. Development of systemic symptoms in control satellite (Sat) RNA only and transgenic plants expressing coat protein (CP) and Sat-RNA after inoculation with 50 μg/ml of cucumber mosaic virus (CMV-B). 2G and 4G are two separate independent lines of transgenic plants expressing CP and Sat-RNA tested at different times.

sion of CP and/or Sat-RNA genes. CP mRNA was detected by Northern blotting, as shown in Figure 2. A single transcript of the expected size (0.9 kb) was detected in all but one line of the transgenic plants. Expression of CP was analyzed by Western immunoblotting. As expected, plants expressing the CMV CP message produced a 24-kDa protein that cross-reacted with CMV antisera and comigrated with the CMV CP standard (Fig. 3). Transgenic plants expressing both CP and Sat-RNA and those expressing only CP produced comparable levels of CP, estimated between 0.1 and 0.2% of total extractable leaf protein. The presence of Sat-cDNA in transgenic plants was confirmed by PCR. Expression of Sat-RNA was detected after transgenic tobacco plants were infected with CMV-B that was Sat-RNA-free. Because of the high concentration of Sat-dsRNA and the low concentration of CMV genomic dsRNA, expression of Sat-dsRNA can be detected directly by polyacrylamide gel electrophoresis (PAGE) analysis (Fig. 4), as described previously (Wu *et al.* 1989). Of 12 double-transgenic plants analyzed, 12 contained Sat-cDNA and 11 expressed CP, but Sat-dsRNA was detected in only nine of them.

Comparison of CMV levels in nontransformed plants and plants transformed with CP and Sat-RNA or Sat-RNA only. In one experiment, nontransformed tobacco or transgenic tobacco plants expressing either CMV Sat-RNA alone or both CMV CP and Sat-RNA were inoculated with CMV-B. The extent of CMV replication in transformed and control plants was determined by passage onto *C. quinoa* (Table 1). The results showed that the CMV concentration in plants transformed with Sat-RNA was about 10–20% of that in untransformed plants, whereas in plants transformed with CP and Sat-RNA it was only 4–5%.

Comparison of resistance to CMV of plants transformed with CP and Sat-RNA, CP alone, or Sat-RNA alone. Two separate experiments were done. In the first experiment (CP + Sat-RNA), gene-transformed plants (independent transgenic lines 2G [50 plants] and 4G [10 plants]) and plants transformed with Sat-RNA alone (30 plants) were challenged by inoculation with 50 μg/ml of CMV-B. Disease development was scored at 10, 20, 40, 60, and 90 days after inoculation (Fig. 5), and the "disease index" was determined at 90 days (2G and Sat-RNA only) or at 50 days (4G) after inoculation (Table 2). For comparison,

Table 2. Resistance of tobacco plants expressing coat protein (CP) and satellite RNA (Sat-RNA) and Sat-RNA alone to infection by cucumber mosaic virus B[a]

Transformed genotype	Number of plants	Days post inoculation	Severity of symptoms[b] (number of plants) 0	1	2	3	Disease index (%)
CP + Sat-RNA	50	90	24	15	8	3	26.7
	10	50	7	3	0	0	10.0
Sat-RNA alone	30	90	0	21	8	1	44.4
Control	10	90	0	0	0	10	100.0
Control	10	...[d]	10	0	0	0	0

[a] Concentration of cucumber mosaic virus B was 50 μg/ml.

[b] Severity of symptoms was rated on a scale of 0–3, in which 0 = no symptoms and 3 = severe symptoms.

[c] Disease index $= \frac{\Sigma \text{ (No. of plants of each grade)} \times \text{(severity)}}{\text{(Total no. of plants)} \times \text{(the highest severity)}} \times 100.$

[d] Not inoculated.

Table 3. Comparison of disease incidence and disease index[a] of transgenic tobacco plants expressing coat protein (CP) and satellite RNA (Sat-RNA), CP alone, or Sat-RNA alone after inoculation with cucumber mosaic virus B (CMV-B)[b]

Transformed genotype	Number of plants analyzed	Days after CMV inoculation					
		30 days		60 days		90 days	
		Disease incidence (%)	Disease index (%)	Disease incidence (%)	Disease index (%)	Disease incidence (%)	Disease index (%)
Sat-RNA	20	80	23.7	95	30.0	100	45.0
CP	20	50	37.5	60	40.0	60	46.3
CP + Sat-RNA	20	25	7.5	30	10.0	30	20.0
Inoculated control	20	100	63.7	100	85.0	100	100.0
Control without inoculation	20	0	0	0	0	0	0

[a] Disease index $= \frac{\Sigma \text{ (No. of plants of each grade)} \times \text{(severity)}}{\text{(Total no. of plants)} \times \text{(the highest severity)}} \times 100.$

[b] CMV-B concentration was 25 μg/ml.

Fig. 6. Comparison of symptom development of tobacco plants transformed with coat protein and satellite RNA (left) and control tobacco plants (right). Each plant was inoculated with 50 μg/ml of cucumber mosaic virus (CMV-B) and photographed 60 days later.

a second experiment including plants transformed with CP was carried out. A comparison of disease incidence and disease index of transformed tobacco plants expressing either CP and Sat-RNA, CP alone, or Sat-RNA alone (20 of each) is shown in Table 3. From Tables 2 and 3, about 50% of transgenic plants expressing CP and Sat-RNA were symptomless 90 days post-inoculation with 25–50 μg/ml of CMV. Table 3 indicates that 30 days after inoculation, the disease incidence of transgenic plants expressing CP was lower than those expressing Sat-RNA, but their disease index was higher. We suggest that this is because replication of Sat-RNA inhibited the replication of CMV, and therefore they showed milder symptoms. Meanwhile, the disease incidence and disease index of transgenic plants expressing both CP and Sat-RNA were lower than the above two. With increasing time, plants (CP + Sat-RNA) showed protection in disease incidence and disease index. The resistance was about twice that of transgenic plants expressing either Sat-RNA or CP alone. Symptom development is shown in Figure 6. Control tobacco plants infected by CMV developed severe mosaic symptoms, whereas the transgenic plants (CP + Sat-RNA) were symptomless or developed only mild mosaic symptoms.

DISCUSSION

It has been suggested that CP-mediated resistance is due to CP accumulation, which interferes with the uncoating of virions, an early event of infection, and the later spread of virus from cell to cell (Wisniewski *et al.* 1990; Beachy *et al.* 1990) and that Sat-RNA-mediated resistance is due to interference with the replication of the viral genome. Therefore, we combined Sat-RNA and CP genes. Combining different resistance genes to block or interfere with the expression of virus functions is a promising method for improving genetically engineered resistance to virus infection.

There are two approaches to obtaining transgenic plants with multiple traits. One approach is to clone each gene into a plant expression vector and transform plants separately. Transgenic plants stably inheriting and expressing multiple genes are then obtained by crossing. The other approach is to clone multiple genes into one or more plant expression vectors and transform them into a plant simultaneously. In this approach, the multiple genes are linked together and inserted into the chromosome at the same locus. Lawson *et al.* (1990) employed the second strategy to construct a chimeric vector containing the CP genes of potato viruses X and Y (PVX and PVY). Potato plants transformed with this vector were protected against infection by both PVX and PVY. We have developed tobacco plants that express CMV Sat-RNA, CP, or both to determine if the resistance to virus infection can be improved by combining resistance genes with different operative mechanisms.

Analysis of transgenic tobacco plants demonstrated that CMV CP and Sat-RNA genes could be stably expressed together in plants. The results showed that all 12 transgenic plants transformed with a vector containing both CMV CP and Sat-cDNA expressed Sat-cDNA; 11 expressed CP; but Sat-dsRNA was detected in only nine plants after inoculation with CMV-B that was free of Sat-RNA. This may be due to the CP gene conferring good early stage resistance to CMV infection and, thus, significantly reduc-

ing the replication of Sat-RNA, which depends on expression of CMV replicase in the transgenic plants. The CP level (0.1–0.2% soluble leaf protein) in transgenic plants expressing CP and Sat-RNA was the same as in CP-only transformants. Thus, the twofold increase in resistance of transgenic plants (CP + Sat-RNA) to CMV-B is not simply due to higher levels of CP expression than those in the CP-only line. The results of the resistance experiments indicated that about 50% of the plants were symptomless 90 days after inoculation with 25–50 μg/ml of CMV. In addition, virus levels in transgenic plants (CP + Sat-RNA) were significantly lower than in Sat-cDNA-transformed plants. In summary, resistance to CMV in gene-transformed plants (CP + Sat-RNA) is quite superior to that in plants transformed with only CMV CP or Sat-RNA.

LITERATURE CITED

Abel, P. P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T., and Beachy, R. N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743.

Baulcombe, D. C., Saunders, G. R., Bevan, M. W., Mayo, M. A., and Harrison, B. D. 1986. Expression of biologically active viral satellite RNA from the nuclear genome of transformed plants. Nature (London) 321:446-449.

Beachy, R. N., Loesch-Fries, S., and Tumer, N. E. 1990. Coat protein-mediated resistance against virus infection. Annu. Rev. Phytopathol. 28:451-474.

Bevan, M. W. 1984. Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. 12:8711-8721.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 112:195-203.

Chu, P. W. G., Francki, R. I. B., and Randles, J. W. 1983. Detection, isolation and characterization of high molecular weight double-stranded RNAs in plants infected with velvet tobacco mottle virus. Virology 126:480-492.

Collmer, C. W., Tousignant, M. E., and Kaper, J. M. 1983. Cucumber mosaic virus-associated RNA-5. X. The complete nucleotide sequence of CARNA5 incapable of inducing tomato necrosis. Virology 127:230-234.

Cuozzo, M., O'Connell, K. M., Kaniewski, W., Fang, R. X., Chua, N. H., and Tumer, N. E. 1988. Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. Bio/Technology 6:549-557.

D'Alessio, J. M., and Gerard, G. F. 1988. Second-strand cDNA synthesis with *E. coli* DNA polymerase I and RNase H: The fate of information at the mRNA 5'-terminus and the effect of *E. coli* DNA ligase. Nucleic Acids Res. 16:1999-2014.

Diaz-Ruiz, J. R., and Kaper, J. M. 1978. Isolation of viral double-stranded RNAs using a LiCl fractionation procedure. Prepar. Biochem. 8:1-17.

Draper, J., and Scott, B. 1988. Page 205 in: Plant Genetic Transformation and Gene Expression. The Alden, Oxford, England.

Golemboski, D. B., Lomonossoff, G. P., and Zaitlin, M. 1990. Plants transformed with a tobacco mosaic virus nonstructural gene sequence are resistant to the virus. Proc. Natl. Acad. Sci. USA 87:6311-6315.

Harrison, B. D., Mayo, M. A., and Baulcombe, D. C. 1987. Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. Nature (London) 328:799-802.

Haseloff, J., and Gerlach, W. L. 1988. Simple RNA enzymes with new and highly specific endoribonuclease activities. Nature (London) 334:585-591.

Hemenway, C., Fang, R. X., Kaniewski, J. J., Chua, N. H., and Tumer, N. E. 1988. Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. EMBO J. 7:1273-1280.

Lawson, C., Kaniewski, W., Haley, L., Rozman, R., Newell, C., Sanders, P., and Tumer, N. E. 1990. Engineering resistance to mixed virus infection in a commercial potato cultivar: Resistance to potato virus X and potato virus Y in transgenic Russet Burbank. Bio/Technology 8:127-134.

Liszewski, M. K., Kumar, V., and Atkinson, J. P. 1989. "Mini-prep" isolation of plasmid DNA in less than two hours for sequencing, subcloning and hybridization. Bio/Technology 7:1079-1081.

Loesch-Fries, L. S., Merlo, D., Zinnen, T., Burhop, L., Hill, K., Krahn, K., Jarvis, N., Nelson, S., and Halk, E. 1987. Expression of alfalfa mosaic virus RNA 4 in transgenic plants confers virus resistance. EMBO J. 6:1845-1851.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Nejidat, A., and Beachy, R. N. 1990. Transgenic tobacco plants expressing a coat protein gene of tobacco mosaic virus are resistant to some tobamoviruses. Mol. Plant-Microbe Interact. 3:247-251.

Nelson, R. S., McCormick, S. M., Delannay, X., Dube, P., Layton, J., Anderson, E. J., Kaniewska, M., Proksch, R. K., Horsch, R. B., Rogers, S. G., Fraley, R. T., and Beachy, R. N. 1988. Virus tolerance, plant growth, and field performance of transgenic tomato plants expressing coat protein from tobacco mosaic virus. Bio/Technology 6:403-409.

Qiu, B. S., Tien, P., and Zhang, X. H. 1985. Satellite RNA as a biological control agent of diseases caused by cucumber mosaic virus. 1. Construction of agent by adding Sat-RNA to genomic RNAs of CMV. Acta Microbiol. Sin. 25:87-88.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Stark, D. M., and Beachy, R. N. 1989. Protection against potyvirus infection in transgenic plants: Evidence for broad spectrum resistance. Bio/Technology 7:1257-1262.

Tien, P., and Zhang, X. H. 1983. Control of two plant viruses by protection inoculation in China. Seed Sci. Technol. 11:969-972.

Tien, P., and Zhang, X. H. 1984. Vaccination of pepper with cucumber mosaic virus isolates attenuated with satellite RNA. Abstr. Int. Congr. Virology, 6th. W46-7.

Tumer, N. E., O'Connell, K. M., Nelson, R. S., Sanders, P. R., Beachy, R. N., Fraley, R. T., and Shah, D. M. 1987. Expression of alfalfa mosaic virus coat protein gene confers cross protection in transgenic tobacco and tomato plants. EMBO J. 6:1181-1188.

Van Dun, C. M. P., and Bol, J. F. 1988. Transgenic tobacco plants accumulating tobacco rattle virus and pea early-browning virus coat protein resist infection with tobacco rattle virus. Virology 161:649-652.

Van Dun, C. M. P., Bol, J. F., and Van Vloten-Doting, L. 1987. Expression of alfalfa mosaic virus and tobacco rattle virus coat protein genes in transgenic plants. Virology 159:299-311.

Wisniewski, L. A., Powell, P. A., Nelson, R. S., and Beachy, R. N. 1990. Local and systemic spread of tobacco mosaic virus in transgenic tobacco. Plant Cell 2:559-567.

Wu, S. X., Zhao, S. A., Wang, G. J., Yang, X. C., Zhang, C. X., Wang, X., and Tien, P. 1988. Transgenic tobacco plants with resistance to cucumber mosaic virus by expressing its monomer satellite cDNA. Chinese Sci. Bull. 33:480.

Wu, S. X., Zhao, S. A., Wang, G. J., Yang, X. C., Zhang, C. X., Wang, X., and Tien, P. 1989. Transgenic tobacco plants with resistance to cucumber virus by expressing satellite cDNA. Sci. Sin. (B) 9:948-956.

Yie, Y., Xu, L. X., and Tien, P. 1991. Synthesis, cloning and sequencing of coat protein gene of cucumber mosaic virus O strain (Chinese isolate). Chinese Sci. Bull. 37:1340-1344.