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A Gene Coding for a Monomeric Form of Cucumber Mosaic Virus Satellite RNA Confers Tolerance to CMV

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A gene whose transcript bears a monomeric form of cucumber mosaic virus (CMV) satellite RNA was introduced into tobacco (*Nicotiana tabacum* 'Xanthi' nc) plants by using an Ri plasmid-based vector system. On CMV infection, the transcript of the satellite RNA gene was used as a template to yield unit-length satellite RNA, which was efficiently amplified by the virus. Plants

bearing the satellite RNA gene displayed long-term tolerance to CMV infection and were also tolerant to CMV infection by aphids, the natural vector of CMV. Implications of these results concerning the mechanism of satellite RNA replication are discussed.

Additional keywords: transgenic plants, viral resistance.

The first experiments dealing with the introduction of foreign genes into plants used disarmed Ti plasmid vectors to transfer chimeric antibiotic resistance genes to plants (Bevan *et al.* 1983; Fraley *et al.* 1983; Herrera-Estrella *et al.* 1983). More recently, and particularly during the last three years, this system has been exploited for the transfer of traits of agronomic importance such as tolerance to herbicides (Comai *et al.* 1985; Shah *et al.* 1986; De Block *et al.* 1987), to insects (Fischhoff *et al.* 1987; Hilder *et al.* 1987; Vaeck *et al.* 1987), to bacteria (Jaynes *et al.* 1987), and also to viruses. Three different types of genes have been developed to confer virus tolerance in plants: ones coding for the viral coat protein (Powell-Abel *et al.* 1986; Loesch-Fries *et al.* 1987; Tumer *et al.* 1987; Van Dun *et al.* 1987; Cuzzo *et al.* 1988; Hemenway *et al.* 1988), ones coding for antisense RNA (Cuzzo *et al.* 1988; Hemenway *et al.* 1988), or ones whose transcript corresponds to a precursor of viral satellite RNA (Gerlach *et al.* 1987; Harrison *et al.* 1987).

These approaches differ greatly, both in terms of the mechanisms of resistance or tolerance involved, as well as in terms of how resistance is expressed. Genes encoding coat protein or antisense RNA confer resistance whose effectiveness depends on the level of expression of the gene introduced and the concentration of the viral inoculum; in many cases these types of resistance are overcome by high levels of inoculum. Except for the resistance conferred by the capsid of potato virus X (Hemenway *et al.* 1988), resistance due to the coat protein can also be overcome by an inoculum of purified viral RNA. When genes based on satellite RNA are used, tolerance is observed whatever the strain of virus, the form (RNA or virus particles), the concentration of the inoculum, and the level of satellite RNA gene transcription. Of course, this strategy is limited to the few viruses possessing a satellite RNA that limits their replication.

Cucumber mosaic virus (CMV) infects more than 800 higher plant species (Douine *et al.* 1979) and causes severe losses in many crop species worldwide. For most of them, no

natural disease resistance is available. CMV has a divided genome composed of three RNA species plus a subgenomic RNA. Certain strains exhibit a supernumerary RNA component that has been demonstrated to be a true satellite RNA (Diaz-Ruiz and Kaper 1977; Lot *et al.* 1977; Gould *et al.* 1978). This satellite RNA is of particular interest because it greatly reduces the synthesis of the viral genome in the infected plant (Kaper and Tousignant 1977; Mossop and Francki 1979; Takanami 1981; Jacquemond and Leroux 1982). For nearly all hosts tested so far, this reduction of virus replication correlates with an important attenuation of the symptoms induced by the virus alone. Satellite RNA worsening symptoms of CMV infection on tobacco (Takanami 1981; Palukaitis 1988) and tomato has been described. The biological response of tomato makes it possible to distinguish at least two groups of satellite RNA depending on whether the plant develops attenuated symptoms, as is true for other hosts, or lethal necrosis (Jacquemond and Lot 1981; Kaper *et al.* 1981). Study of interference between the two kinds of satellite RNA has shown that tomato plants infected with a strain containing a nonnecrogenic satellite RNA are protected against infection with one containing a necrogenic satellite RNA (Jacquemond 1982).

All these results suggest that introducing a chimeric gene whose transcript corresponds to satellite RNA into plants could be a means for conferring tolerance to CMV. This approach has already been tested; Baulcombe and his colleagues (Harrison *et al.* 1987) have shown that tobacco plants expressing genes whose transcript corresponds to 1.3 or 2.3 units of satellite RNA are partially protected against infection by CMV. Protection has also been obtained with transgenic plants expressing tobacco ringspot virus satellite RNA genes corresponding to oligomeric forms of satellite RNA (Gerlach *et al.* 1987). Here we report that similar protection is obtained after introduction into host plants, with an Ri-plasmid-based vector, of a chimeric gene containing a monomeric copy of CMV satellite RNA. The presence of nonviral sequences, corresponding to the homopolymeric tails added for the synthesis and the cloning

of the cDNA as well as a fragment of the cloning vector (Jacquemond and Lauquin 1988), does not interfere with the biological activity of the transcripts. Moreover, these plants are equally tolerant when infected either by mechanical inoculation or with viruliferous aphids, the natural vector of CMV.

MATERIALS AND METHODS

Plasmids and bacterial strains. Restriction endonuclease digestions, ligations, transformation of *Escherichia coli*, maxi- and mini-preps of plasmid DNA, and other usual molecular biology techniques were as described in Maniatis *et al.* (1982).

Transformation of *Nicotiana tabacum*. The techniques used to transfer the intermediate vector pMarsat36 to *Agrobacterium rhizogenes* A4, to inoculate stem segments of tobacco (*N. tabacum* 'Xanthi' nc, strain XHFD8), and to regenerate transformed plants from the resultant transformed roots were as described by Tepfer and Casse-Delbart (1987). Briefly, pMarsat36 was introduced into *A. rhizogenes* A4 by triparental matings by using pRK2013 as helper plasmid. The resulting *A. rhizogenes* strain bearing the cointegrate between pMarsat36 and pRiA4 was used to inoculate surface-sterilized tobacco stem segments *in vitro*. Transformed roots were decontaminated, maintained as organ clones, and induced to regenerate plantlets.

Isolation of total RNA and northern analysis. Total RNA was isolated from axenic root cultures and transformed tobacco plants before and after inoculation by using the technique of Chirgwin *et al.* (1979), followed by a CsCl gradient according to Glisin *et al.* (1974). Samples of RNA were analyzed by electrophoresis in formaldehyde denaturing gels as described by Lehrach *et al.* (1977) and transferred to Amersham Hybond-N nylon membranes, according to the manufacturer's protocol. ³²P-labeled probes were prepared from isolated fragments of satellite RNA cDNA by using random oligonucleotide priming as described by Feinberg and Vogelstein (1983).

Infection of transgenic tobacco plants with CMV. The satellite-RNA-free strain of CMV, I17F, was maintained on tomato (cv. Monalbo) either to keep it satellite-RNA-free or to detect satellite RNA if it accumulates during virus replication. Leaves, 10–15 days postinoculation, were dried over CaCl₂ and further used as inoculum after grinding in 10 ml of 0.03 M Na₂-phosphate, 0.2% DIECA (sodium diethyldithiocarbamate) per gram of fresh tissue. Young tobacco plants (five developed leaves) were mechanically inoculated (the whole plant was rubbed with inoculum) and placed in an insect-proof greenhouse compartment for at least 1 mo.

Aphid transmission was performed as described by Lecoq *et al.* (1979) with *Myzus persicae* or *Aphis gossypii*. The virus source was melon (*Cucumis melo* 'Cantaloup charentais') infected with I17F CMV strain 7 days previously. At least 10 viruliferous aphids were deposited on each tobacco plant to ensure infection. Two hr later, aphids were killed by fumigation and the plants kept in an insect-free greenhouse.

Determination of virus propagation in experimental tobacco plants. Virus was purified according to Lot *et al.* (1972) 7 days after inoculation. RNA was extracted from the virions by the phenol-sodium dodecyl sulfate method and

analyzed on a 2.4% polyacrylamide gel. Crude extracts from a young leaf of identical position from each infected plant were prepared in phosphate buffer (4 ml/1 g of leaf). Part of the extract was assayed for biological properties on young tomato plants. Development of necrosis by these plants is characteristic of the presence of a necrogenic satellite RNA in the inoculum. The remainder of the extract was kept at –20° C for quantitation of capsid protein.

The amount of capsid protein was quantified by double-antibody sandwich ELISA (Clark and Adams 1977), with reagents prepared against the LQ strain of CMV (a gift from H. Lot), which belongs to the same group as I17F. Crude extracts were further diluted 1,000- to 16,000-fold, depending on whether they contained satellite RNA. Controls were based on purified I17F virions diluted to 1–200 µg/ml in a crude extract of noninfected tobacco; control samples were further diluted, as were the experimental treatments.

RESULTS

Construction of a satellite RNA gene. A gene whose transcript corresponds to a monomeric precursor form of CMV satellite RNA was constructed by using the intermediate expression vector, pMarcel19 (Robaglia *et al.* 1987), which has a unique *Bam*HI cloning site between the cauliflower mosaic virus (CaMV) 19S promoter and a fragment bearing the polyadenylation signals of the nopaline synthase gene. The pMarcel family of vectors can be inserted by cointegration into either a wild-type *A. rhizogenes* strain A4 Ri plasmid or into certain disarmed Ti plasmids, such as pGV3850 (Zambryski *et al.* 1983).

The cDNA clone used corresponds to a single copy of a 335-nucleotide necrogenic isolate of satellite RNA, I17N, cloned and sequenced by Jacquemond and Lauquin (1988). The *Bam*HI fragment of this clone, with the following structure (oligo dC/satellite/oligo dA/oligo dG/vector fragment), was cloned in the intermediate plant expression vector pMarcel19 to create pMarsat36 (Fig. 1). We have used the Ri plasmid system described in detail elsewhere (Tepfer and Casse-Delbart 1987) to introduce pMarsat36 into tobacco plants. Transformants bearing this gene are referred to as T362(n), in which (n) is the clone number.

Expression of the satellite RNA gene in plants before and after CMV infection. Several clones of genetically transformed tobacco roots bearing the satellite RNA gene were regenerated and the resulting plantlets propagated *in vitro*. Southern blots of transformants confirmed the correct restriction map of the transferred genes (data not shown). Northern blots of total RNA of these plants (Fig. 2) when probed with the satellite RNA cDNA showed a single transcript of the expected size, approximately 0.95 kb (insert of 0.59 kb plus 3' noncoding and polyA of 0.35 kb). The level of expression was quite variable among clones; for instance, clones T362(17) and T362(25) had consistently higher levels of transcript than clone T362(18). As can be seen in Figure 2, a band visible in ethidium bromide staining appeared at 0.33 kb in RNA extracted from T362 plants after inoculation with CMV. Densitometry of several RNA gels of different CMV-infected T362 plants showed that this band consistently represents 10–12% of the total plant RNA. Northern blots of these gels probed with the satellite RNA cDNA showed that the new band corresponds to satellite

RNA. The satellite RNA was produced from the precursor transcript to yield unit-length monomer satellite RNA that is strongly amplified by the virus. Dimeric forms of 0.67 kb were also visible. The level of satellite RNA was not correlated with the level of the precursor transcript, as much

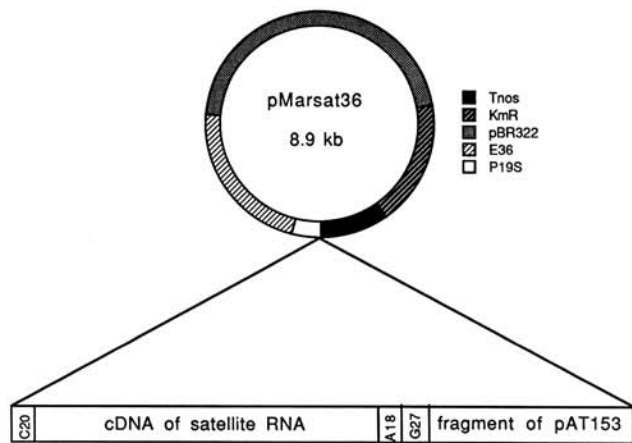


Fig. 1. Map of pMarsat36, intermediate vector bearing the satellite RNA gene. The 0.59-kb *Bam*HI fragment bearing a single-copy cDNA of satellite RNA isolate I17N (inset) was inserted in the unique *Bam*HI cloning site of the expression vector pMarcel19. The latter bears *Eco*RI fragment 36 of the pRiA4 TL region, as well as pBR322 sequences, allowing recombination with either pRiA4 or with certain disarmed Ti plasmids such as pGV3850. The oligo(dA), oligo(dC), and oligo(dG) tracts and fragment of pAT153 are the result of cDNA priming and cloning in pAT153 (Jacquemon and Lauquin 1988). Tnos = 3' region of nopaline synthase, Km^R = kanamycin-resistance gene from Tn5, E36 = *Eco*RI fragment 36 of the pRiA4 Ti region, P19S = CaMV 19S promoter.

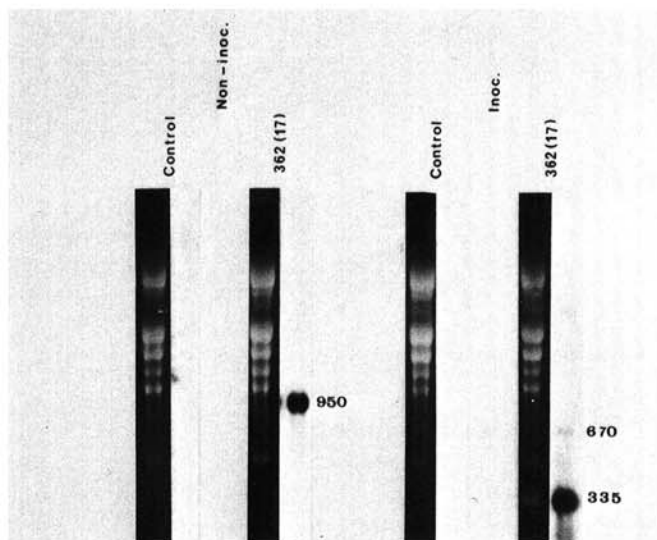


Fig. 2. Analysis of the transcript from pMarsat36 in transgenic T362(17) plants and amplification of the resulting satellite RNA on CMV infection. Total RNAs were extracted from leaves of either noninoculated (left) or CMV-inoculated (right) plants. Control plants were transformed with the wild-type Ri plasmid. Lanes from a single ethidium bromide stained gel and the corresponding northern blot with the satellite RNA cDNA as probe are shown in pairs. Fifteen micrograms of RNA was applied per lane. The autoradiogram of RNA from noninoculated plants and control CMV-inoculated plants was exposed for 48 hr at -80° C with intensifying screens, whereas the one after inoculation was exposed for 20 min at room temperature without screens. RNA size, indicated in nucleotides, was estimated by interpolation from the migration of standard marker RNAs (Boehringer Mannheim RNA ladder).

satellite RNA was present in infected T362(18) plants as in T362(17) and T362(25) plants.

Susceptibility of transgenic plants to virus infection by mechanical inoculation. Transgenic tobacco plants regenerated from three clones of transformed roots expressing the satellite RNA gene, as well as control plants (nontransformed tobacco or tobacco bearing Ri T-DNA without a satellite RNA gene), were infected with the satellite RNA-free CMV strain I17F. Behavior of both types of control plants toward CMV (Fig. 3) was as follows: inoculated leaves and the first noninoculated leaf developed weak symptoms (vein clearing and a diffuse mosaic) during the first week after infection. Then the next two leaves exhibited severe mosaic; the leaf area was also reduced. The symptoms developed by the following two leaves were slightly milder, but those of the next ones were again very severe. Transformed plants expressing the satellite RNA gene showed weak symptoms similar to controls during the week following infection. After this, symptoms exhibited by the upper leaves were extremely attenuated; often the plants showed essentially no symptoms (Fig. 3). Similar protection was observed for the transformants of the three clones.

Virus replication, as judged by yields of viral nucleoproteins purified 7 days after infection and serological quantitation of the capsid protein, is described in Table 1. Viral replication was greatly reduced in plants expressing the satellite RNA gene, because three- to fourfold less of the virus could be recovered from these plants, and the amount of capsid was approximately 10% of that isolated from the two types of control plants. This decrease was correlated with an important accumulation of satellite RNA in the viral nucleoproteins (more than 50% of the encapsidated RNA). The same results were obtained for the three clones of transformants expressing the satellite RNA gene. No satellite RNA was detected in the preparations of virions recovered from infected control plants (results not shown). These results confirm those obtained by northern analysis of total plant RNA shown above (Fig. 2). Biological properties of this RNA were determined by inoculating young tomato plants with a crude extract from infected tobacco plants. All the plants infected with an inoculum prepared from transformants expressing

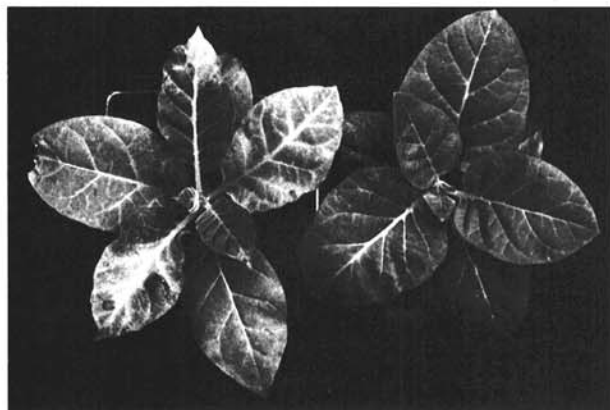


Fig. 3. Effects of expression of the satellite RNA gene on CMV symptom development 10 days after infection. Nontransformed (left) and transgenic tobacco expressing the satellite RNA gene (right) inoculated with satellite RNA-free CMV strain I17F. Note the mild leaf wrinkling due to the presence of Ri T-DNA genes (Tepfer 1984).

the satellite RNA gene displayed lethal necrosis, whereas those infected with an inoculum prepared from control plants developed the severe fernleaf symptoms characteristic of the absence of satellite RNA in this inoculum (not shown).

Inoculation by aphids. Transformants from clones T362(17) and T362(25), as well as three types of control plants—nontransformed (Xanthi/Xanthi), transformed with the wild-type Ri plasmid (A4[4]/A4[4]), and transformed with the Ri plasmid bearing a chimeric gene unrelated to viruses (314[1]/Xanthi)—were selfed, and progeny plants from each were tested for susceptibility to infection by aphids, the natural vector for CMV. Among the progeny, transformants could be distinguished by their modified phenotype (Tepfer 1984). The modification was relatively mild (T) in transformed progeny of T362(17)/Xanthi plants and quite pronounced (T') in transformed progeny of control A4(4)/A4(4), 314(1)/Xanthi, and of T362(25)/Xanthi ones. To see if this modification could interfere with virus transmission, control plants alone were analyzed in a first set of experiments. Results presented in Table 2, experiment 1, show that two of the three types of control plants shared similar response to CMV infection, whatever their phenotype. In subsequent experiments, control, T362(17), and T362(25) plants were tested. As shown in Table 2, experiment 2, all of the various types of control plants exhibited severe mosaic symptoms on infection and did not produce satellite RNA as judged by the symptoms developed by the tomato plants inoculated with crude sap from these plants. On the other hand, all the plants bearing the satellite RNA gene (these plants have the modified phenotype) developed only attenuated symptoms

and produced a necrogenic satellite RNA. As had been found with mechanically inoculated plants (Table 1), virus replication, as measured by capsid quantitation, was also considerably reduced in aphid-inoculated plants as compared with nontransformed sibling plants. No differences were observed in experiments with two genera of aphids, *A. gossypii* and *M. persicae*.

DISCUSSION

We have introduced a gene into tobacco whose transcript of 0.95 kb bears a single full-length copy of CMV satellite RNA. Northern experiments have shown that this transcript does not undergo autolytic cleavage, which has been observed for oligomeric precursors of the satellite RNA of tobacco ringspot virus (Gerlach *et al.* 1987). On infection with CMV however, the 0.95-kb transcript is used as a template to yield RNA of the length of authentic satellite RNA (335 nucleotides), which is amplified to high levels (10–12% of total RNA). Such plants display long-lasting tolerance to CMV. We observed no difference in tolerance among several clones of transformants that transcribe the satellite RNA precursor to different levels. This suggests that even lower levels of expression would be sufficient to confer CMV tolerance, and reflects the very high biological activity of satellite RNA (Jacquemond and Leroux 1982). This is in contrast to what is observed in virus-resistant plants expressing viral coat protein or antisense RNA, in which high levels of expression must be achieved to confer effective resistance.

Our observation that a precursor RNA transcript bearing

Table 1. Behavior toward CMV of plants expressing the CMV satellite RNA gene

	Control plants		Plants expressing satellite RNA gene		
	T ^a	NT ^a	T362(17)	T362(18)	T362(25)
Number of plants	4	13	12	2	4
Average yield of virus (μg of virus/g of leaf) ^b	1,887(455)	2,329(227)	687(148)	566(27)	614(99)
Capsid quantitation ^c					
Day 7 postinoc.	760(165)	755(171)	108(58)	97(21)	77(24)
Day 14 postinoc.	n.a.	775(249)	53(11)	45(7)	60(20)

^aT = transformed control plants; NT = nontransformed control plants.

^bVirus yield calculated as in Lot *et al.* (1972).

^cCapsid concentration, measured serologically, is expressed as μg of capsid protein/g of leaf tissue; numbers in parentheses are standard deviations. Average values of all the plants expressing the satellite RNA gene are significantly different (0.1%) from those of both types of control plants. n.a. = not analyzed.

Table 2. Results of the infection by aphids of progeny from tobacco plants transformed or not with the satellite RNA gene

Genotype of parent plants ^a	Experiment 1			Experiment 2					
	Xanthi/Xanthi	314(1)/Xanthi		Xanthi/Xanthi	A4(4)/A4(4)	T362(17)/Xanthi	T362(25)/Xanthi		
Phenotype of progeny before inoculation ^b	N	N	T'	N	T'	N	T	N	T'
Number of plants tested	21	17	5	11	16	14	4	6	11
Capsid quantitation ^c	548(106)	681(122)	620(89)	790(249)	790(102)	325(30)	47(8)	275(21)	18(5)
Symptom gravity ^d	+	+	+	+	+	+	–	+	–
Necrosis among tomato plants inoculated with crude extract of infected tobacco (%) ^e	0	0	0	0	0	0	100	0	100

^aXanthi = nontransformed controls; 314(1)/Xanthi = hemizygous plants transformed with a control gene; A4(4)/A4(4) = homozygous pRi-transformed plants; T362(17)/Xanthi and T362(25)/Xanthi = hemizygous plants transformed with the satellite RNA gene.

^bN = wild-type phenotype; T = mild pRi-transformed phenotype; T' = strong pRi-transformed phenotype.

^c μg of capsid/g of leaf tissue 2 wk after infection; numbers in parentheses are standard deviations.

^d+ = severe mosaic; – = extremely attenuated or no mosaic.

^eTwo or three tomato plants were infected in the first and second experiment, respectively, with a crude extract of each tobacco plant.

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