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Transgenic Tomato Plants Expressing Satellite RNA Are Tolerant to Some Strains of Cucumber Mosaic Virus

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Abstract. **Transgenic tomato plants (*Lycopersicon esculentum* Mill.) expressing cucumber mosaic virus (CMV) satellite RNA fused to a gene for β-glucuronidase were produced using *Agrobacterium*-mediated transformation. The R_1 progeny of self-crossed R_0 plants were challenge-inoculated with virion or RNA preparations of CMV or tomato aspermy virus (TAV). The transgenic plants challenged with CMV-1 showed mild disease symptoms in the first 2 weeks postchallenge followed by a decrease in symptoms, resulting in little difference between the transgenic and uninfected control group by the fourth week. Enzyme-linked immunosorbent assay results showed about a 10-fold decrease in virus accumulation in the transgenic plants compared to controls. Tolerance was evident only in plants that contained the recombinant insert and produced mature unit-length satellite RNA after CMV infection. Plants challenged with TAV showed no significant tolerance to virus-induced symptoms.**

Cucumber mosaic virus (CMV) has been identified as one of the most economically important plant viruses in field-grown vegetable crops worldwide (Tomlinson, 1987). Outbreaks of diseases incited by CMV have caused large losses in tomato and pepper crops in several Mediterranean (Gallitelli et al., 1988; Jorda et al., 1992; Marrou and Duteil, 1974) and Asian countries (Sayama et al., 1993; Tien and Wu, 1991). In the United States, high incidences of CMV have been reported in several states (Daniels and Campbell, 1992; Davis and Shifriss, 1983; Gonsalves et al., 1982; Kearney et al., 1990).

Some strains of CMV contain a small linear satellite RNA, which depends on its helper virus for replication and spread. CMV satellite RNAs show no significant sequence homology to the viral genomic RNAs and seem to be unnecessary for any known viral functions (Collmer and Howell, 1992; Kaper and Collmer, 1988; Roossinck et al., 1992). In most cases, satellite RNAs attenuate CMV symptoms and reduce the amount of infectious virus found in plant tissues. However, a few satellite variants can induce new disease symptoms when combined with CMV in an appropriate host plant and have been implicated as the main etiological agent in several CMV epidemics (Jorda et al., 1992; Kaper et al., 1990a).

The attenuating properties of some satellite RNAs can be exploited to control viral diseases by preinoculating crops with an attenuating satellite RNA variant combined with virus. This technique can effectively protect against CMV disease in the field (Gallitelli et al., 1991; Montasser et al., 1991; Sayama et al., 1993; Tien and Wu, 1991; Tien et al., 1987). Transgenic tobacco plants expressing an endogenous CMV satellite RNA transcript have also been shown to be tolerant to mechanical and aphid-borne CMV infections (Baulcombe et al., 1986; Harrison et al., 1987; Jacquemond et al., 1988; Masuta et al., 1989). We have previously shown that transgenic tomato plants expressing a necrosis-inducing satellite RNA variant do not develop lethal tomato necrosis unless challenged with CMV (McGarvey et al., 1990), while a recent report by Saito et al. (1992) suggests that a mild variant will induce tolerance to CMV.

In this paper we present a more comprehensive analysis of the protective effects of an attenuating CMV satellite variant in transgenic tomato plants against subsequent challenge infections with CMV and the related cucumovirus tomato aspermy virus (TAV).

Materials and Methods.

Plant transformation. Monomeric cDNA clones of the attenuating CMV satellite CMV associated RNA 5 from CMV strain S (S-CARNA 5) (Collmer and Kaper, 1986) were inserted into the binary vector RG-2 (Fig. 1). RG-2 is a Bin19 derivative similar to pBI121 (Jefferson, 1990). Recombinant plasmids containing the satellite insert and the RG-2 vector [containing β-glucuronidase (GUS) alone] were engineered into *Agrobacterium tumefaciens* and inserted into two varieties of tomato, UC82b (Petoseed, Saticoy, Calif.) and an Asian variety CL143 (provided by R.T. Opena, AVRDC, Taiwan) by *A. tumefaciens* infection of cotyledons, which were regenerated into plants using a modification of the methods of Fillatti et al. (1987) and McCormick (1991). Putative transgenic plants were screened for inserted DNA by the polymerase chain reaction (PCR) (McGarvey and Kaper, 1991) and by southern blot hybridization (Maniatis et al., 1982), and for RNA expression by northern blot hybridization of RNA (see below). GUS expression was tested by a histochemical assay (Jefferson, 1990).

Challenge inoculations. Isolates of CMV-1 and TAV, free of satellite RNA, were used to infect transgenic tomato plants and control plants. CMV-1 (Kaper et al., 1981) produces symptoms of severe shoestring leaves, chlorosis, and stunting in tomato. TAV

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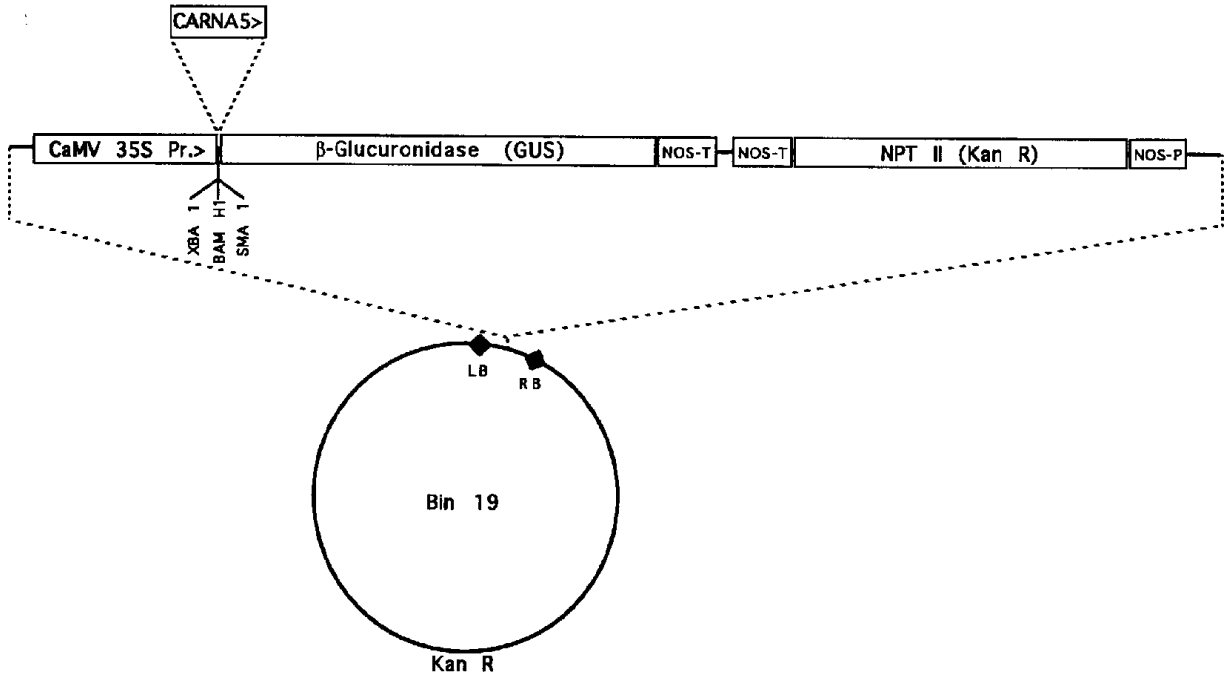

Fig. 1. Recombinant vector containing satellite cDNA. Monomeric cDNA clones of the CMV satellite S-CARNA 5 were inserted in the (+) orientation between the 35S promoter of cauliflower mosaic virus (CaMV pr.) and the gene for β-glucuronidase (GUS) followed by the nopaline synthase polyadenylation region (NOS-T) in the binary vector RG-2. RNA transcripts from this construct are expected to be ≈2500 nucleotides long and contain satellite RNA sequences and the coding region for GUS. The NPTII gene provides kanamycin resistance (KanR) in plants.

was a Canadian isolate (Stace-Smith and Tremaine, 1973) and produces stunting, mild chlorosis, leaf puckering, and a reduction in apical dominance in tomato. Virions were purified from infected tomato plants and viral RNA was extracted as described (Kaper and West, 1972; Lot et al., 1972). Plants were mechanically inoculated at the cotyledon stage using RNA at 10 $\mu g \cdot ml^{-1}$ in 0.03 M Na_2HPO_4 or virions at 20 $\mu g \cdot ml^{-1}$ in water. Purified S-CARNA 5 at 2.5 $\mu g \cdot ml^{-1}$ was added in some positive control infections. Plants were kept in growth chambers at 24C with a 16-h photoperiod using fluorescent light at 150 $\mu E \cdot m^{-2} \cdot s^{-1}$. CMV-1. A total of 16 R_1 transgenic seedlings from a single UC82b-derived R_0 plant (designated UC47-2) were challenge-inoculated with CMV-1 RNA, and 10 similar seedlings were inoculated with CMV-1 virions. Six transgenic seedlings were mock-inoculated with buffer. Four R_1 seedlings derived from a separate R_0, UC82b-derived transgenic parent (designated UC47-6) were challenged with CMV-1 RNA, as were eight R_1 seedlings derived from a transgenic CL143 R_0 parent (designated 0110-1). Two each of the UC47-6 and 0110-1 progeny were mock-inoculated. As controls, nontransformed UC82b and CL143 seedlings were inoculated as follows: seven UC82b and three CL143 with CMV-1 RNA, four UC82b with CMV-1 virions, seven UC82b and three Cl143 with CMV-1 RNA plus S-CARNA 5, and eight UC82b and two Cl143 were mock-inoculated. As additional controls, eight R_1, UC82b-derived transgenic plants containing the RG-2 vector without the satellite RNA insert but with the GUS gene (designated UCRG2-1) were inoculated with CMV-1 RNA and four with CMV-1 virions as controls.

TAV. Four UC47-2-derived and four UC47-6-derived R_1 seedlings were inoculated with TAV RNA. As controls, eight nontransformed UC82b seedlings were inoculated with TAV RNA and six with TAV RNA plus S-CARNA 5. In addition, four UCRG2-1 transgenic seedlings were inoculated with TAV RNA as controls.

Nucleic acid extraction and northern blot hybridizations. Leaf RNA was extracted using a total RNA isolation kit (Promega, Madison, Wis.). Poly(A)-enriched RNA was prepared from total RNA using a Poly(A) Quik kit (Stratagene, La Jolla, Calif.). Poly(A)-enriched RNA was electrophoresed through 1.5% agarose–formaldehyde gels and transferred to nylon membranes (Maniatis et al. 1982). Total nucleic acids were prepared from all challenged plants and controls using 100 mg young leaf tissue, electrophoresed through a 9% semi-denaturing polyacrylamide gel, and electrotransferred to nylon membranes as described (White and Kaper, 1989). All membranes were hybridized, washed, and visualized using standard procedures. S-CARNA 5 and CMV RNA 4 sequences were detected using ^{32}P-labeled riboprobes from cDNA inserts in the plasmid pSP65 (Promega). TAV RNA was detected using a cDNA probe made from randomly primed TAV RNA (Maniatis et al., 1982).

Symptom scoring and analysis. Plants were evaluated for disease symptoms at 2, 3, and 4 weeks after challenge inoculations and assigned a score of 0 = symptomless; 1 = mild chlorosis; 2 = full chlorosis or mild chlorosis plus mild shoestring leaves; 3 = shoestring leaves or other leaf or stem malformations; and 4 = severe stunting plus any combination of other symptoms. Evaluation was made without knowledge of the inoculum used or the plants' status as transgenic or control. Disease intensity for a particular treatment was calculated by the formula $DI = [\sum(s \times n)/S \times N] \times 100\%$, where s = score of 0 to 4, n = number of plants with a particular numerical score, S = highest possible score, and N = total number of plants. Challenged plants that showed no viral infection by northern blot analysis or enzyme-linked immunosorbent assay (ELISA) were omitted from the final analysis as were R_1 plants that did not show the presence of the recombinant DNA insert as determined by PCR.

Serological detection of virus. ELISA was used to test for the presence of CMV in challenged plants. Tissue was collected 4 weeks after challenge and frozen at –70C. Tissue was later ex-

tracted and ELISA was performed as described (Clark and Adams, 1977; Montasser et al., 1991), except that multiple dilutions of each sample were used and a dilution series of the purified CMV-1 virions was included as a standard. Statistical analysis was performed using Student's *t* test. Challenged plants that showed no sign of viral infection by northern blot hybridization or ELISA were omitted from the final analysis.

Results

Plant transformation. After transformation, two UC82b-derived (designated UC47-2 and UC47-6) and six Cl143-derived (designated 0110-1 to 6) primary (R_0) transgenic plants were obtained. In morphology and fruiting behavior, the plants were essentially identical to nontransformed plants regenerated from tissue culture. The R_1 generation seeds germinated in soil and developed like the control seedlings. Expression of the cDNA insert in the R_0 transformed plants was determined by northern blot analysis, which showed an RNA transcript of ≈2.5 kb (Fig. 2), and by assaying for GUS activity, which was present in the transgenic plants and absent in controls (not shown). PCR screening of the R_1 progeny showed that 41 of 51 UC47-2 progeny contained the gene, consistent with the 3:1 segregation ratio expected from a single insert in the primary transgenic plant ($\chi^2 = 0.79$). The segregation ratio of the UC47-6 and 0110-1 offspring also suggested a single insert, but the numbers screened were insufficient for a final determination.

CMV-1 challenge. All 26 of the UC47-2 progeny challenged with either CMV-1 RNA or virions became infected with CMV-1. Twelve of the plants challenged with CMV-1 RNA and six plants challenged with virions showed the accumulation of single-stranded (ss) and double-stranded (ds) S-CARNA 5 (Fig. 2, lane 3; Fig. 3 a and b). S-CARNA 5 was observed only in plants that contained the transgenic satellite insert. Of the eight remaining offspring challenged with CMV-1, but in which no ss or ds S-CARNA 5 accumulated, five did not contain the recombinant insert, while

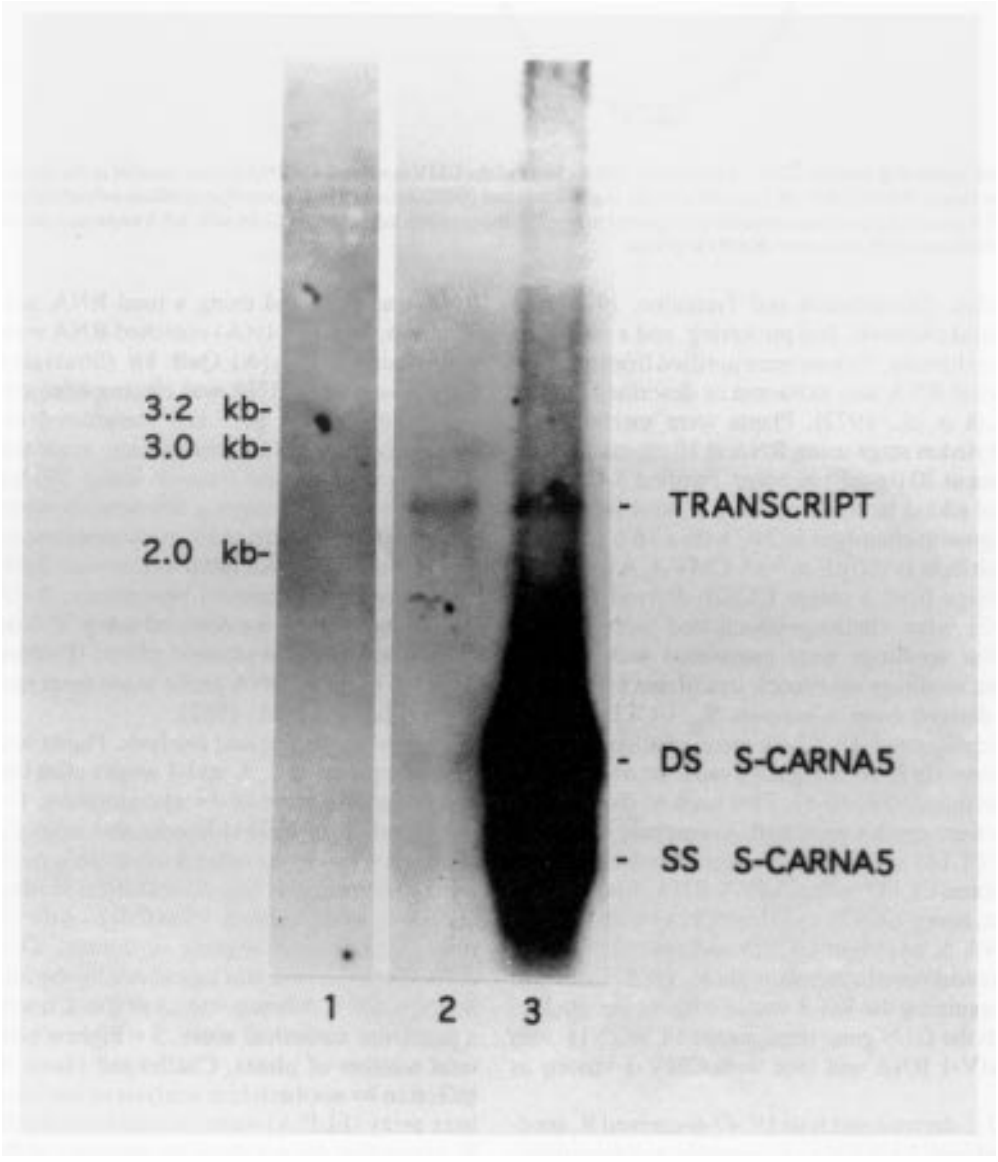

Fig. 2. Northern blot hybridization of transgenic plant RNA. Poly(A)-enriched RNA was electrophoresed through a 1.5% agarose–formaldehyde gel, transferred to a nylon membrane, hybridized with a ^{32}P riboprobe for S-CARNA 5, and visualized by autoradiography. Size standards were CMV-1 genomic RNAs (not shown) and the sizes are shown at left. Lanes contain 1) RNA (5 µg) from a healthy nontransgenic UC82b control tomato plant; 2) RNA (5 µg) from a healthy UC47-2 transgenic tomato plant; and, 3) RNA (5 µg) from a CMV-1 infected UC47-2 transgenic tomato plant containing the single and double stranded S-CARNA 5 produced after virus infection. Note that poly(A)-enriched RNA is ≈90% poly(A) RNA and, thus, still contains some mature satellite RNA.

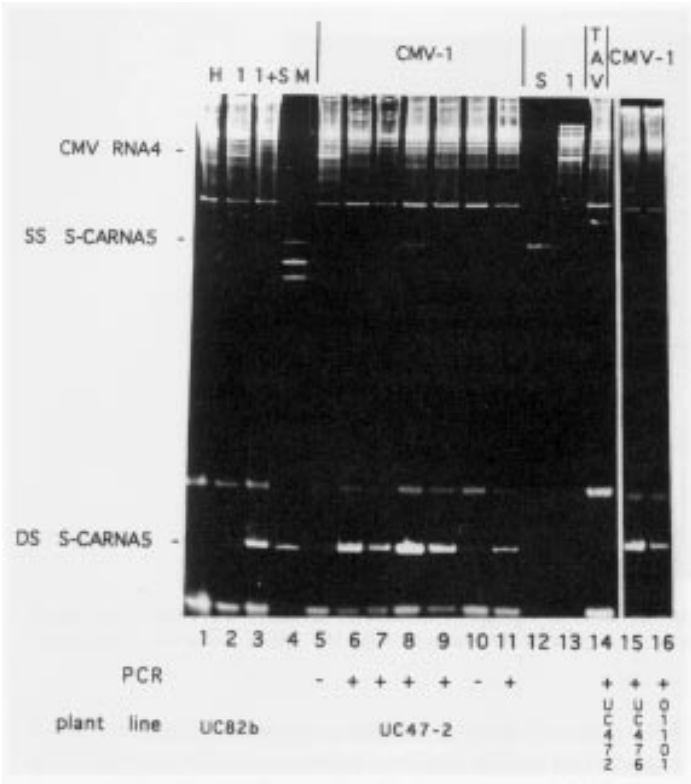

Fig. 3**a.** Semidenaturing electrophoresis of RNA from challenge-inoculated plants. Lanes contain RNA from 1) healthy nontransgenic UC82b tomato; 2) nontransgenic UC82b tomato infected with CMV-1 RNA; 3) same as lane 2 infected with CMV-1 and S-CARNA 5; 4) satellite RNA markers, (top to bottom) single-stranded PARNA 5, 1-CARNA 5, n-CARNA 5, and double stranded D-CARNA 5; 5–11) individual tomato plants from the R_1 generation of the UC47-2 transgenic plant that were all challenge-inoculated with CMV-1 RNA; 12) gel-purified S-CARNA 5; 13) CMV-1 RNA (1 µg) used as inoculum; 14) transgenic tomato plant (UC47-2 line) challenged with TAV RNA; 15) transgenic tomato plant (UC47-6 line) challenged with CMV-1 RNA; and 16) transgenic tomato plant (0110-1 line) challenged with CMV-1 RNA. The presence or absence of the recombinant DNA insert in the individual offspring was determined by polymerase chain reaction and is indicated by a (+) or (–) at the bottom.

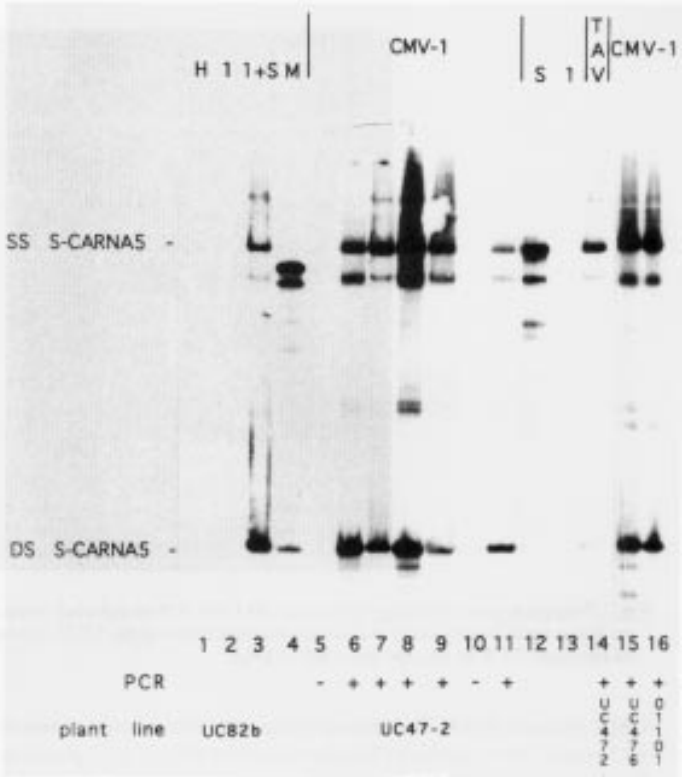

Fig. 3**b.** Northern blot hybridization of RNA from challenge-inoculated plants. Lanes are the same as (**a**). Note that PARNA 5 does not hybridize with S-CARNA 5 and that single-stranded S-CARNA 5 often migrates as two conformational isomers in semidenaturing gels and a second band immediately below the main band in the single-stranded region is observed in lane 13 and lanes 5–11 and 14–16. The two bands have been gel-purified, sequenced, and are both S-CARNA 5 (H. Sayama, personal communication).

three of the plants challenged with virions did. The disease symptoms were scored, expressed as disease intensity, and are shown in Fig. 4. During the first 2 weeks, there were only small differences between the transgenic and control groups with most plants showing some mild chlorosis. However, by the third and fourth weeks, the systemic spread of viral disease seemed to stop and disease symptoms receded dramatically in the transgenic group until there was little obvious difference between challenged transgenic plants and the mock-inoculated transgenic and control plants, except for some mild chlorosis on the older leaves of some transgenic plants (Fig. 5). The transgenic offspring that lacked the recombinant insert showed no symptom attenuation and were similar in appearance to the CMV-1-infected UC82b controls and the CMV-1-infected transgenic controls containing the RG-2 vector alone. ELISA showed a significant difference in virus titer between the transgenic group challenged with CMV-1 RNA and the control group, with averages of 31.6 ($\sigma \pm 24.5$) $\mu g \cdot g^{-1}$ and 355.0 ($\sigma \pm 15.8$) $\mu g \cdot g^{-1}$ ($P = 0.01$) tissue, respectively. A similar result was obtained in the groups challenged with virions: transgenic = 31.5 ($\sigma \pm 14.1$) $\mu g \cdot g^{-1}$; control = 500.0 ($\sigma \pm 178.0$) $\mu g \cdot g^{-1}$ ($P = 0.005$). The five transgenic offspring that did not contain the recombinant insert had a average virus titer of 370.0 ($\sigma \pm 95.0$) $\mu g \cdot g^{-1}$ tissue and the RG-2-containing transgenic control plants had an average virus titer of 398.0 ($\sigma \pm 97.1$) $\mu g \cdot g^{-1}$ tissue.

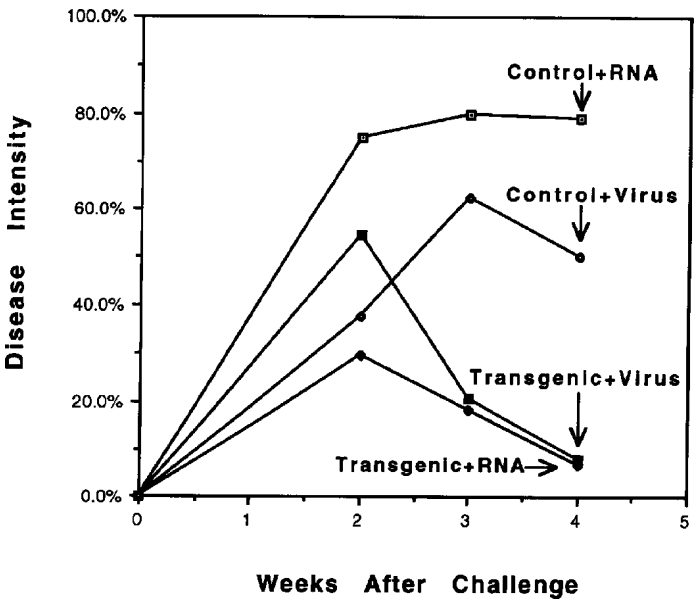

Fig. 4. Disease intensity of challenged plants. Transgenic (UC47-2) and normal (UC82b) tomato seedlings were inoculated with CMV-1 RNA or CMV-1 virions. Plants were evaluated and disease index was calculated as described in Materials and Methods.

All of the UC47-6 and 0110-1 progeny were infected with CMV-1 RNA. Two of the UC47-6 and six of the 0110-1 plants contained the satellite insert and showed accumulation of ss and ds S-CARNA 5 (Fig. 3 a and b, lanes 15 and 16) and a reduction in

Fig. 5. Transgenic plants challenge-inoculated with CMV-1. Four and a half-week-old tomato plants photographed 3.5 weeks after inoculation with CMV-1 RNA. Plants are (left to right) healthy mock-inoculated transgenic tomato plant (UC47-2), transgenic tomato plant (UC47-2) infected with CMV-1 RNA, and nontransgenic control tomato plant (UC82b) infected with CMV-1 RNA.

CMV genomic RNA. The disease symptoms were scored as above with results very similar to that described for the UC47-2 plants in Fig. 4, with mild CMV-1 symptoms appearing during the first 2 weeks but receding dramatically during the next 2 weeks (data not shown). ELISA showed the presence of virus but was not quantified.

TAV RNA challenge. Three of four 47-2 and three of four 47-6 transgenic offspring became infected with TAV. All six of the infected plants contained the recombinant insert but only five of the six showed the presence of ss and ds S-CARNA 5. In each case, ds S-CARNA 5 did not accumulate to the amounts typical of the CMV infections (Fig. 3 a and b, lane 14). Little to no reduction in the accumulation of TAV RNA was observed in plants in which satellite RNA was found (not shown). Equally strong TAV symptoms were observed in the transgenic and control plants, regardless of whether satellite RNA accumulated or not.

Discussion

The results of the challenge infections with CMV-1 clearly show that transgenic tomato plants expressing an attenuating satellite RNA sequence have improved tolerance to CMV-induced disease (Figs. 4 and 5), probably as a result of about a 10-fold reduction in virus titer. This result had been predicted after our previous tomato transformation with a necrogenic satellite (McGarvey et al., 1990), and was recently also reported by Saito et al. (1992), who described their transformation of tomato plants expressing an attenuating satellite and showed preliminary data on R_0 plant tolerance to CMV-O infection. In the more comprehensive analysis presented here, this tolerance was quantified with measurements of disease intensity and virus titer in R_1 plants of two tomato varieties. Further, it was shown that tolerance holds for virion and RNA challenges, and the analysis was extended to TAV and other virus strains (see below). The practical importance of this result is that CMV satellite-mediated tolerance can be engineered into tomato for which, to date, no naturally resistant varieties have been found (Phills et al., 1977; Stamova et al., 1990). In addition, since the inserted satellite sequences lie embedded in a much larger RNA transcript that also encodes an expressed protein (GUS), it suggests that satellite-mediated tolerance could be combined in a single RNA transcript with other forms of protection that require protein expression. However, any quantitative effects on protein expression remain to be determined. In tobacco, a combination of satellite and coat protein protection using separate promoters for the individual genes has recently been reported to provide increased levels of tolerance to CMV infection (Yie et al., 1992).

The lack of symptom attenuation in TAV challenge infections is different from the reduction in symptoms reported for satellite transgenic tobacco (Harrison et al., 1987). In addition, when transgenic tomato plants expressing the necrogenic satellite D-CARNA 5 (McGarvey et al., 1990) were challenged with TAV, no necrosis or other symptom modulation was observed (unpublished data), although unit-length satellite accumulated in a manner similar to that seen for S-CARNA 5 (Fig. 3 a and b, lane 14) with a proportionately lower amount of ds D-CARNA 5. Similarly, control inoculations of nontransgenic UC82b tomato plants with S-CARNA 5 or D-CARNA 5 with TAV as a helper virus failed to modulate the symptoms of the TAV isolate used in our experiments. Moriones et al. (1992) have also reported differences in symptom modulation and CMV satellite accumulation between two different isolates of TAV and noticed a proportional decrease in the accumulation of the ds satellite with TAV infections compared to CMV infections. That lower ds/ss CARNA 5 proportions may relate to a failure in eliciting tomato necrosis has been shown for the Ixora strain of CMV (Kaper et al., 1990b). All this suggests that differences in the response of satellite-transgenic plants to infections by TAV and CMV strains may depend on qualitative and quantitative differences in satellite vs. virus replication, which in turn determines the ultimate biological effect.

Challenges of UC47-2-, UC47-6-, and 0110-1-derived transgenic plants using CMV-S and CMV-D also showed the accumulation of ss and ds S-CARNA 5 and a reduction in symptoms by the fourth week postchallenge (unpublished results). However, the engineered tolerance should not be assumed to extend to all CMV isolates until further testing of a wide variety of CMV isolates, including isolates containing their own satellite RNAs, is completed.

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