A Cucumber Mosaic Virus (CMV) RNA 1 Transgene Mediates Suppression of the Homologous Viral RNA 1 Constitutively and Prevents CMV Entry into the Phloem

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Resistance to *Cucumber mosaic virus* (CMV) in tobacco lines transformed with CMV RNA 1 is characterized by reduced virus accumulation in the inoculated leaf, with specific suppression of accumulation of the homologous viral RNA 1, and by the absence of systemic infection. We show that the suppression of viral RNA 1 occurs in protoplasts from resistant transgenic plants and therefore is not due to a host response activated by the cell-to-cell spread of virus. In contrast, suppression of *Tobacco rattle virus* vectors carrying CMV RNA 1 sequences did not occur in protoplasts from resistant plants. Furthermore, steady-state levels of transgene mRNA 1 were higher in resistant than in susceptible lines. Thus, the data indicate that sequence homology is not sufficient to induce suppression. Grafting experiments using transgenic resistant or susceptible rootstocks and scions demonstrated that the resistance mechanism exhibited an additional barrier to phloem entry, preventing CMV from moving a long distance in resistant plants. On the other hand, virus from susceptible rootstocks could systemically infect grafted resistant scions via the phloem. Analysis of viral RNA accumulation in the infected scions showed that the mechanism that suppresses the accumulation of viral RNA 1 at the single-cell level was overcome. The data indicate that this transgene-mediated systemic resistance probably is not based on a posttranscriptional gene-silencing mechanism.

Pathogen-derived resistance to plant viruses in transgenic plants occurs by several mechanisms (2). Many reports on transgenic resistance to viruses are consistent with an RNA-mediated inhibition referred to as posttranscriptional gene silencing (PTGS). PTGS requires the existence of sequence homology between the transgene and the viral RNA. PTGS results in the degradation of both transgenic mRNA and any cytoplasmic, viral, or nonviral RNA that carries the targeted sequences and can be induced by viruses (12, 28, 29, 31, 39). The inhibition acts through the synthesis of short complementary RNAs involving host RNA-dependent RNA polymerase(s) and possibly also helicase activities. It has been proposed that these short RNAs bind to the targeted sequence, which is digested by host nucleases specific for double-stranded RNA (11, 17, 46). As might be expected, some viruses express proteins that neutralize PTGS (1, 6, 21, 22, 42). Some forms of pathogen-derived, transgene-expressed resistance are protein mediated. In these cases, the resistance acts through the interference of the transgene-encoded protein with some essential step in the virus infection cycle, for example, disassembly of the virus particle (3). In the case of replicase-mediated resistance to *Cucumber mosaic virus* (CMV), although specific RNA sequences are the targets of the resistance mechanism (19), a correlation has been found between resistance and expression of transgene-encoded protein (the CMV 2a protein) and also between resistance and accumulation of higher levels of transgene mRNA (43, 44). Pathogen-derived resistance associated

with protein synthesis from viral polymerase transgenes has been reported for two other viruses within the family *Bromoviridae* (5, 23). On the other hand, resistance conferred by replicase transgenes in other viruses seems to be caused by PTGS. For example the *Nlb* transgene of the potyvirus *Plum pox virus* induces a recovery phenotype with low transgenic mRNA levels (16). Within the family *Tobamoviridae*, the picture is more complex. Different types of PTGS-mediated resistances were associated with the 54-kDa transgenes of *Pepper mild mottle virus* and *Tobacco mosaic virus* (TMV), respectively (25, 36, 37). However, a protein-mediated component of resistance to tobacco mosaic virus was found after the transient expression in the plant of segments of the viral polymerase (10, 15) in addition to an RNA-mediated component of resistance (15). Thus, more than one mechanism appears to operate in different examples of resistance mediated by replicase genes (reviewed in reference 26).

CMV is a tripartite RNA virus. RNA 3 codes for two proteins involved in viral movement and encapsidation (7). RNA 2 codes for the 2a protein, which is the viral RNA-dependent RNA polymerase subunit of the CMV replicase, whereas RNA 1 codes for the 1a protein, another viral component of the replicase complex (18). The 1a protein contains putative helicase and methyltransferase activities (18) and is also involved in viral movement (13). We previously reported the existence of systemic resistance to CMV in tobacco plants transgenic for the full-length RNA 1 (8). All of our RNA 1-transgenic lines expressed biologically functional 1a protein and could complement replication of RNAs 2 and 3. Some of these transgenic lines showed resistance to CMV, characterized by the specific suppression of the accumulation of RNA 1 in the inoculated leaves and by the absence of systemic infection (8). In the

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present work, we show that the suppression of RNA 1 operates constitutively at the single-cell level. However, at the whole-plant level, this suppression is overcome when virus enters resistant tissue grafted on infected susceptible tissue, via the phloem. The resistance at the single-cell level directed against RNA 1, combined with an additional barrier to CMV spread at the point of phloem entry, determines the phenotype of systemic resistance in these transgenic lines.

MATERIALS AND METHODS

Plants, viruses, and plant inoculation. Tobacco (*Nicotiana tabacum* cv. Sam-sun NN) plants either nontransformed or transgenic for the full-length RNA 1 of CMV were used. The transgenic plants displayed systemic susceptibility (line R₃-A1) or resistance (line R₃-B1C) to CMV (8). The plants were grown in the greenhouse or in a growth chamber at 25°C.

Two viruses were used for plant inoculation: CMV strain Fny (Fny-CMV) (30) and a pseudorecombinant virus made from Fny-CMV RNAs 1 and 2 and from CMV strain M (M-CMV) RNA, designated F1F2M3-CMV (45). In the case of Fny-CMV, the inoculum consisted of purified virus at a concentration of 50 µg/ml. In the case of the pseudorecombinant virus F1F2M3-CMV, extracts from virus-infected plants, inoculated 10 to 12 days earlier (diluted 1:10 in 50 mM sodium phosphate, pH 7), were used instead, because the virus particles did not store well after isolation. Plants were inoculated mechanically by gently rubbing the inocula on aluminium oxide-dusted leaves.

Plasmid constructs and transcript RNAs. CMV transcript RNAs 1, 2, and 3 were generated from the corresponding full-length cDNA clones pFny109, pFny209, and pFny309, as described previously (47). A modified CMV RNA 3 transcript, expressing the green fluorescent protein (GFP) in place of the viral coat protein (CP), was generated in a similar way from plasmid construct pL:3a/GFP (7).

Several constructs expressing full-length *Tobacco rattle virus* (TRV) RNA 2 carrying segments of the RNA 1 of Fny-CMV were generated from construct TRV-GFPc; the latter expresses a full-length TRV RNA 2 in which the 2b and 2c genes were replaced by the GFP gene (24). A fragment corresponding to the sequence of the 5' half of Fny-CMV RNA 1, from nucleotides 1 to 1680, was obtained by PCR amplification using a primer identical to nucleotides 1 to 16, containing an *NcoI* site, and a primer complementary to nucleotides 1669 to 1680 of the viral sequence, containing a *SacI* site. This fragment was cloned into TRV-GFPc, after digestion with *NcoI* and *SacI*, to generate construct TRV-F15'. A fragment corresponding to the 3' half of Fny-CMV RNA 1, nucleotides 1669 to 3357, was obtained by PCR using a primer identical to nucleotides 1669 to 1680, containing an *NcoI* site, and a primer complementary to nucleotides 3344 to 3357, containing a *SacI* site. This PCR fragment was cloned into TRV-GFPc, after digestion with *NcoI* and *SacI*, to generate construct TRV-F13'. A fragment corresponding to the central region of Fny-CMV RNA 1, specifically, to nucleotides 838 to 2532, was obtained by PCR amplification using a primer identical to nucleotides 838 to 851, containing an *NcoI* site, and a primer complementary to nucleotides 2521 to 2532, containing a *SacI* site. This PCR fragment was cloned into TRV-GFPc, after digestion with *NcoI* and *SacI*, to generate construct TRV-F11.

The different TRV constructs containing CMV RNA 1 inserts, as well as construct TRV-GFPc carrying the GFP gene, were linearized with *SphI*, and transcripts were obtained using T7 RNA polymerase. For protoplast electroporation, transcripts were mixed with a TRV 1 transcript RNA obtained from a full-length cDNA clone of the TRV isolate PpK20 after linearization with *NcoI* and using T7 RNA polymerase (24).

Protoplast preparation and electroporation. Tobacco plants were kept for 5 days in a growth chamber at a constant temperature of 25°C and with 16 h of daylight prior to the isolation and electroporation of mesophyll protoplasts, as described previously (13). Approximately 5 µg of viral or transcript RNA was electroporated to 10⁶ protoplasts. The protoplast cultures were kept undisturbed in a growth chamber for 27 or 44 h prior to their harvest for nucleic acid or protein analysis, respectively.

Grafting procedure. Scions were grafted onto rootstocks using inverted saddle grafts constructed by making a V-shaped notch in the stem of the rootstock and a V-shaped wedge in the stem of the scion. The graft joint was wrapped tightly with surgical tape and covered with Parafilm, and the scions were protected from dehydration by covering them with cling film for 3 to 4 days. Nine days after a successful grafting, the leaves of the rootstock were inoculated as described above with sap from tobacco infected with the pseudorecombinant virus

F1F2M3-CMV, which induces a strong yellow chlorosis in tobacco. The scions were then regularly monitored for the appearance of symptoms until the plants were discarded 12 weeks later.

Nucleic acid extraction and analysis. Inoculated and systemic leaves were sampled by taking 10 small leaf disks (≈50 mg each), while protoplasts were harvested by centrifugation at low speed. To extract total nucleic acids, the leaf disks and protoplast pellets were ground or resuspended, respectively, in 300 µl of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 2% sodium dodecyl sulfate, and 0.5% 2-mercaptoethanol. The samples were then extracted with phenol, and RNA was precipitated with ethanol as described previously (27).

For Northern blot analysis, total nucleic acid samples were fractionated by electrophoresis in agarose gels (1.7% gels for CMV RNA analysis and 1% gels for TRV RNA analysis) under denaturing conditions (6% formaldehyde), blotted to nitrocellulose membranes, and hybridized to the appropriate digoxigenin-labeled RNA probe (see Fig. 1 and 5 for exceptions). CMV RNA-specific probes complementary to the 3' noncoding region of all Fny-CMV RNAs were obtained by linearization of the corresponding clones with *XhoI* and RNA synthesis using T3 RNA polymerase (13). RNA probes complementary to TRV RNAs 1 or 2, used in Northern blot detection, were provided by S. MacFarlane (Scottish Crop Research Institute). A fragment of the ubiquitin gene of *Anthirium majus*, corresponding to nucleotides 25 to 432 of the sequence contained in plasmid pSAM 293 (provided by A. J. Maule, John Innes Centre; GenBank accession number X67957), was used to generate a probe specific to ubiquitin mRNA. This DNA fragment was amplified by PCR and subcloned into pSK. After linearization with *XhoI*, an RNA probe complementary to the ubiquitin mRNA was generated with T7 RNA polymerase.

Nonradioactive blots were washed, incubated with antidigoxigenin antibody (Roche Diagnostics, Lewes, United Kingdom), and exposed following the manufacturer's instructions. In order to quantify relative amounts of viral RNA in the experiment shown in Fig. 1, the probe was ³²P labeled. The radioactive blots were washed and autoradiographed as described previously (33). After the blot analysis, the areas of nitrocellulose membranes to which each RNA had transferred were isolated, and the corresponding Cerenkov counts per minute were obtained in a liquid scintillation counter.

mRNA isolation and detection. Fresh leaf tissue from single plants (10 g) was frozen in liquid nitrogen and ground to a powder in a mortar. Total RNAs were then extracted as described previously (40). To isolate the mRNA fraction, a PolyAtract mRNA isolation kit (Promega, Madison, Wis.) was used. The isolated mRNA fraction was fractionated in denaturing agarose gels and analyzed by Northern blot hybridization, using the probe complementary to the 3' end of all Fny-CMV RNAs (13).

Protein analysis. Protoplast pellets were resuspended in denaturing buffer, and the total protein content was analyzed by protein blotting. The samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% acrylamide-bisacrylamide gels), blotted to nitrocellulose membranes (38), and probed with a polyclonal antiserum against GFP (Molecular Probes, Eugene, Oreg.).

RESULTS

The accumulation of CMV RNA 1 is suppressed in protoplasts from resistant plants. Previously, we showed that resistance in transgenic plants expressing CMV RNA 1 resulted in accumulation of CMV RNAs 2 and 3 in the inoculated leaf but the accumulation of CMV RNA 1 was reduced to subliminal levels (8). However, it was not clear whether this effect was due to a suppression of CMV RNA 1 replication or cell-to-cell movement. Therefore, the effects on CMV RNA 1 replication were analyzed directly. In protoplasts isolated from resistant RNA 1-transgenic tobacco and electroporated with CMV RNA, the accumulation of viral RNA 1 was suppressed, in contrast to the situation in protoplasts from either nontransformed tobacco plants or RNA 1-transgenic susceptible plants (Fig. 1A, lane R versus lanes NT and S, respectively). In protoplasts from transgenic susceptible plants, the accumulation of RNA 1 also decreased, but to a lesser extent (Fig. 1A, lane S versus lane NT). This pattern of accumulation of viral RNA is similar to those observed in CMV-inoculated leaves of both nontransformed tobacco (Fig. 1B, lanes NT) and some suscep-

FIG. 1. Detection of CMV RNA by Northern blot hybridization analysis of total RNA extracted from protoplasts electroporated with viral RNA (A) and inoculated leaves of four different nontransformed tobacco plants (B, lanes NT), four different plants from the transgenic susceptible line R_3 -A1 (B, lanes S), or four different plants from the transgenic resistant line R_3 -B1C (B, lanes R). (A) Protoplasts were isolated from nontransformed tobacco (lane NT), RNA 1-transgenic susceptible line R_3 -A1 (lane S), and RNA 1-transgenic resistant line R_3 -B1C (lane R). Lanes M show noninfected protoplasts isolated from nontransformed tobacco. (B) A 32 P-labeled probe complementary to the common 3'-end noncoding region of all CMV RNAs was used for hybridization analysis. Quantitation of the RNA 1 levels in panel B by Cerenkov counting gave the following counts per minute (minus background; left to right): 4,016 (NT), 3,505 (NT), 6,434 (S), 6,042 (S), 187 (R), 8 (R), 5,813 (NT), 4,701 (NT), 3,310 (S), 1,071 (S), 11 (R), and 1 (R).

tible transgenic tobacco plants (Fig. 1B, lanes S) versus resistant transgenic tobacco plants (Fig. 1B, lanes R). The patterns of accumulation of CMV RNAs in the inoculated leaves of four different plants of the transgenic resistant line were found to be virtually identical, whereas more variability was observed in the accumulation of CMV RNAs between transgenic susceptible plants (Fig. 1B). Quantification of RNA 1 accumulation in the experiments shown in Fig. 1B indicated a level of suppression of RNA 1 accumulation in resistant plants of between 1 and more than 2 orders of magnitude (Fig. 1B legend). In comparison, RNA 1 accumulation in protoplasts from transgenic resistant versus nontransformed plants was reduced about 20-fold. Therefore, the protoplast data demonstrated that the selective suppression of viral RNA 1 operates constitutively at the single-cell level and was not due to a host response activated by viral movement.

Accumulation of TRV vectors expressing CMV RNA 1 sequences was not suppressed in protoplasts from resistant plants. To address whether the mechanism of suppression at the single-cell level may also suppress any viral RNA sharing sequence homology with CMV RNA 1, the accumulation of TRV vectors containing the different CMV RNA 1 sequences shown in Fig. 2 was analyzed in protoplasts from transgenic resistant and nontransgenic tobacco. Accumulations of TRV RNA containing sequences of CMV RNA 1 in plus-sense orientation, corresponding to the 5' half, the 3' half, or an overlapping intermediate region, were found to be comparable in protoplasts derived from resistant versus nontransformed plants (Fig. 2, constructs TRV-F15', TRV-F1I, and TRV-F13'). The slightly lower accumulation of TRV-F15' in the transgenic protoplasts shown in the blot labeled TRV 2 in Fig. 2 was not observed in other experiments (bottom blot, labeled TRV 2*). Variation in accumulation of viral RNA vectors of the magnitude observed in Fig. 2 is not unusual (20, 26). By

contrast, PTGS of viral vectors usually shows a reduction of accumulation of 1 to 2 orders of magnitude (20, 34, 41). Thus, these results indicate that the presence of CMV RNA 1 sequences alone is not sufficient to suppress the accumulation of an RNA containing such sequences.

Steady-state levels of the transgene mRNA are higher in resistant than in susceptible plants. Steady-state levels of the transgene mRNA were found to be higher in individual transgenic resistant plants (Fig. 3, lanes R) than in transgenic susceptible plants, in which the amount of transgene mRNA was below the threshold of detection (Fig. 3, lanes S). In similar analyses of mRNA isolated from pools of 10 transgenic susceptible or 10 transgenic resistant plants, the results were similar (data not shown). This result suggests that the mechanism of resistance may not be acting on the homologous transgene mRNA in the same way as on the replicating viral RNA.

In addition to the full-length transcript RNA, four other RNA bands of positive polarity and smaller size (ranging from 2.2 to 1.2 kb or less) were also detected by the probe in samples from transgenic resistant plants. These smaller RNAs could not be detected in samples from transgenic susceptible or nontransgenic plants (Fig. 3, lanes R versus lanes S and NT).

Levels of activity of transgenically expressed 1a protein are similar in protoplasts from susceptible and resistant plants. Does the difference in the levels of transgenic mRNA correlate with a similar difference in the levels of transgenically expressed 1a protein? Efforts to detect the transgenic 1a protein directly by serological means in resistant or susceptible plants were unsuccessful (8). However, the relative biological activity of the transgenically expressed protein could be measured in leaves inoculated with transcript RNAs 2 and 3 of either transgenic susceptible or transgenic resistant plants. Similar levels of viral RNA accumulation were found in both types of plants (8). To exclude possible effects due to movement, a similar

FIG. 2. Detection of TRV RNA by Northern blot hybridization. Total RNA was extracted from protoplasts 48 h after electroporation with the different TRV constructs represented on the left, which harbor the GFP gene or segments of CMV RNA 1 designated 5', 3', or intermediate (I). Probes complementary to TRV RNA 1, TRV RNA 2, and ubiquitin were used for hybridization analysis. The detection of TRV RNA 2 (TRV 2*) from a different experiment is also shown. The positions of inserts of either a CMV RNA 1 fragment or the gene encoding GFP contained in TRV 2 are indicated by dashes on the left of the blots.

assay was done at the single-cell level. Protoplasts prepared from transgenic susceptible or transgenic resistant plants were electroporated with Fny-CMV transcript RNA 2 plus transcript RNA 3 derived from construct pL:3a/GFP, in which the gene encoding GFP substitutes for the gene encoding the viral CP (7). Accumulations of GFP were also found to be similar in protoplasts from both transgenic resistant and susceptible plants (Fig. 4, lanes 5 and 6 versus lanes 7 and 8). The level of GFP accumulation was between 25 and 100 times lower than was observed when the 1a protein was expressed from a replicating viral RNA 1 in protoplasts from nontransformed tobacco (Fig. 4, lanes 5, 6, 7, and 8 versus lanes 1 to 4). Therefore, the functional levels of the transgene product seem to be similar in resistant and susceptible plants, despite the differences observed in steady-state levels of transgene mRNAs.

The inability of CMV to enter the vasculature prevents viral long-distance movement. To address whether the inhibition of long-distance movement resulting in systemic resistance was due solely to the suppression of viral RNA 1 accumulation at the single-cell level, a series of grafting experiments was performed, using transgenic resistant or transgenic susceptible, as well as nontransgenic, rootstocks and scions (Table 1). When susceptible or nontransgenic scions were grafted onto resistant rootstocks and the rootstocks were inoculated, the experiments showed that CMV was unable to move into and infect either scion (Table 1, S/R and NT/R, respectively). On the other hand, when resistant scions were grafted onto susceptible or nontransformed rootstocks and the rootstocks were inoculated, CMV from the rootstock entered the scion via the graft, and the virus was able to systemically infect the resistant scions (Table 1, R/S and R/NT, respectively). This indicates that in resistant plants, there is a barrier to viral entry into the phloem but not to viral release from the phloem. In addition, susceptible scions grafted

FIG. 3. Detection of the transgenic mRNA by Northern blot hybridization analysis. Total mRNAs were extracted from three different transgenic susceptible (line R₃-A1) plants (lanes S) and three different transgenic resistant (line R₃-B1C) plants (lanes R). Lane M, CMV RNA as a size marker. Lane NT, mRNA extracted from a noninfected, nontransformed tobacco leaf. A probe complementary to the common 3'-end noncoding region of all CMV RNAs was used for hybridization analysis in the upper blot, and a probe complementary to ubiquitin mRNA was used in the lower blot. In the upper blot, note the presence of additional small RNA fragments of discrete sizes (ranging from 2.2 to 1.2 kb), indicated by short dashes on the right. mRNA 1 indicates the position of the polyadenylated RNA 1 transgene transcript.

FIG. 4. Immunoblot analysis of the accumulation of GFP. Tobacco protoplasts were electroporated either with Fny-CMV transcript RNAs 1 and 2 plus transcript RNA 3 derived from construct pL:3a/GFP, in which the GFP gene substitutes for the CP gene (lanes 1 to 4), or with only Fny-CMV transcript RNA 2 plus transcript RNA 3 derived from construct pL:3a/GFP (lanes 5 to 8). Lanes 1 to 4 contain protoplast extracts from nontransformed tobacco. Lanes 5 and 6 contain protoplast extracts from transgenic susceptible (line R₃-A1) plants. Lanes 7 and 8 contain protoplast extracts from transgenic resistant (line R₃-B1C) plants. Lanes 4, 6, and 8 each contain 1/30 of the total protein content of 10⁶ protoplasts, and this amount has been arbitrarily indicated as a protein content of 1 below these lanes. Lanes 1 and 2 respectively contain 100 (0.01) and 25 (0.04) times less protein than lane 4. Lanes 3, 5, and 7 contain 5 (0.2) times less protein, respectively, than lanes 4, 6, and 8. M, molecular mass markers.

onto resistant rootstocks subsequently inoculated with virus remained susceptible to virus inoculation (Table 1, experiment 3). That is, resistance was not induced in the susceptible scion by a signal emanating from the infected, inoculated leaf of the resistant rootstock.

The differential suppression of viral RNA 1 that operates at the single-cell level in resistant plants is overcome by virus released from the phloem. Symptoms of infection after resistance breakage by graft inoculation (Table 1) were surprisingly severe, similar to those induced by the virus in nontransgenic scions (data not shown). Therefore, the accumulation of viral RNA in the infected scions was analyzed further. The accumulation of CMV RNA in the transgenic resistant scions grafted onto transgenic susceptible rootstocks subsequently infected with CMV was comparable to

that found in susceptible plants (Fig. 5, lanes 3 and 4 versus lanes 1 and 2). Furthermore, the accumulation of RNA 1 was no longer suppressed in the transgenic resistant scion (Fig. 5, lanes 3 and 4 versus lanes 1 and 2). Thus, in resistant plants, the mechanism that constitutively suppresses the accumulation of RNA 1 at the single-cell level is overcome when a continuous supply of virus enters the tissue via the phloem.

TABLE 1. Spread of CMV infection on grafted nontransformed susceptible and resistant scions^a

Expt	No. infected ^b						
	NT/NT ^c	NT/R	R/NT	R/R	S/S	R/S	S/R
1	3/3	0/3	3/3	0/3	3/3	3/3	0/3
2		0/4	4/4	0/3	4/4	4/4	0/4
3 ^d		3/3		0/2			2/3

^a Three types of plants were used as scions or rootstocks in the grafting experiments: NT, nontransformed tobacco; S, transgenic susceptible tobacco (line R₃-A1); and R, transgenic resistant tobacco (line R₃-B1C). After grafting, the rootstocks were inoculated with F1F2M3-CMV and the scions were monitored for the presence of viral infection.

^b Number of grafted scions that became infected with CMV over the number inoculated.

^c Type of scion/type of rootstock.

^d Leaves in scions grafted onto resistant rootstocks that remained uninfected in experiment 2 were inoculated with F1F2M3-CMV to assess whether they had acquired systemic resistance to the virus via a signal originating from the rootstock.

FIG. 5. Detection of CMV RNA by Northern blot hybridization analysis. Total RNA was extracted from two transgenic resistant (line R₃-B1C) scions grafted onto two transgenic susceptible (line R₃-A1) rootstocks (diagram) and showing symptoms of viral infection after inoculation of the rootstock with the pseudorecombinant virus F1F2M3-CMV. Lanes 1 and 2, show the respective CMV RNA accumulation in the inoculated leaves of the two transgenic susceptible rootstocks. Lanes 3 and 4 show the respective CMV RNA accumulation in the corresponding transgenic resistant scions displaying symptoms of systemic infection. A probe complementary to the common 3'-end noncoding region of all CMV RNAs was used for hybridization analysis.

DISCUSSION

In this analysis of RNA 1-mediated resistance to CMV infection, we demonstrate that a preestablished mechanism of suppression operates at the single-cell level in RNA 1-transgenic plants. The resistance operates in inoculated protoplasts. Its constitutive nature implies that viral cell-to-cell movement is not required for its activation. The suppression targets the viral RNA 1 (Fig. 1). The systemic resistance to CMV in these transgenic plants is also strain specific and maps to the viral RNA 1: pseudorecombinants formed between Fny-CMV and LS-CMV that contain LS-CMV RNA 1 (with ca. 75% sequence identity to Fny-CMV RNA 1) could infect the plants systemically with no effect on LS-CMV RNA 1 accumulation (reference 8 and data not shown). Therefore, the presence of a high degree of sequence homology between the transgene and the viral RNA seems to be required for the suppression to take place. However, CMV was capable of replication and local movement when inoculated onto the upper leaves of transgenic plants that previously had been inoculated with the same virus but still displayed systemic resistance (8). This suggested that the suppression of RNA 1 might not be based on PTGS. This conclusion is supported by other observations in this study. (i) There was no systemic signaling of resistance from resistant rootstocks to susceptible scions (Table 1). (ii) TRV vectors expressing fragments of CMV RNA 1 were not prevented from accumulating in inoculated protoplasts from resistant plants (Fig. 2). (iii) The levels of transgene mRNA 1 were consistently higher in resistant than in susceptible plants (Fig. 3). (iv), although the susceptible and resistant plants belong to different transgenic lines, and therefore some differences in steady-state levels of transgene mRNA might be expected due to intrinsic levels of transgene expression, the data in Fig. 3 and 4 suggest that the transgene mRNA in resistant plants is not suppressed, as is the homologous viral RNA 1.

The transgenic mRNA found in resistant plants coexisted with several smaller RNAs with sizes ranging from 2.2 to 1 kb or less detected by the probe complementary to the 3' end of CMV RNA (Fig. 3). Their discrete sizes and lack of heterogeneity indicate that they were not the products of nonspecific RNA degradation during extraction. These smaller RNAs of positive-sense polarity could be the 3'-coterminal products of the degradation of the intact transgene by some host nuclease, as has been proposed for some small RNAs observed in tobacco transformed with the CP gene of the potyvirus *Tobacco etch virus* (35). Alternatively, they could result from the presence of cryptic internal transcription initiation sites or cryptic introns within the open reading frame of CMV RNA 1. Whatever their origin, the possibility that they play a role in the mechanism of suppression of CMV RNA 1 cannot be ruled out. Transgene RNAs with aberrant sizes have been described in cases of resistance associated with PTGS, and it was found that degradation fragments of such RNAs only 25 nucleotides in size are implicated as components of the homology-dependent degradation mechanism proposed for PTGS (11, 17, 25). However, the much larger transgene RNA fragments observed here could play a role in resistance by binding to the viral replication complex and interfering with the replication cycle of the homologous viral RNA 1.

Grafting experiments showed a second feature of this resis-

tance to CMV: a barrier to CMV entry into the phloem (Table 1). In tobacco, CMV replicase is most readily detected in phloem parenchyma (F. Cillo, I. M. Roberts, and P. Palukaitis, unpublished results). However, CMV particles accumulate poorly in phloem parenchyma and companion cells of tobacco plants (32), suggesting that the replicating RNA is rapidly mobilized. Thus, high levels of replication may be a requirement for the successful spread of the virus through the phloem. On the other hand, expression of genes driven by the 35S promoter is enhanced in phloem (4), and this could lead to an increase in the steady-state levels of transgene mRNA. If there was a correlation between higher transgene mRNA levels and the mechanism of suppression of RNA 1, then the inhibition of systemic spread of CMV could be due to an enhancement of suppression in the cells that constitute the point of entry into the vasculature.

There was no apparent barrier to the exit of CMV from the phloem in resistant plants (Table 1). Surprisingly, phloem-released virus was able to infect resistant tissue and accumulate to wild-type levels (Fig. 5). Furthermore, there was no suppression of RNA 1 in these infected tissues. This indicates that the mechanism of suppression was overcome (Table 1, experiments 1 and 2; Fig. 5, lanes 3 and 4). This would be unexpected if the suppression of RNA 1 accumulation was based on PTGS. Instead, this restoration of the levels of RNA 1 accumulation could be explained more easily if the resistance was based on a mechanism of suppression that blocks replication of viral RNA 1 or the formation of replication complexes containing viral RNA 1. The high levels of viral RNA entering sink mesophyll tissue could potentially saturate and overcome this hypothetical mechanism.

The resistance described here is similar to resistance observed in transgenic plants expressing a truncated version of CMV RNA 2, where two resistance mechanisms also operate, one at the single-cell level and another one restricting virus movement (9, 43), both of which target the viral RNA (19). Furthermore, it was shown that the level of resistance to CMV in tobacco plants transgenic for the RNA 2 of CMV correlated with higher relative levels of the transgene mRNA and also with the expression of the 2a protein (44). This resistance could also be overcome by graft inoculation (44). However, in a different species, transgenic tomato expressing the same truncated CMV RNA 2, resistance could not be overcome by grafting (14). In another example of replicase-mediated resistance, PTGS was ruled out as the mechanism inhibiting virus infection. A member of the family *Bromoviridae*, *Brome mosaic virus*, showed resistance in *N. benthamiana* plants transformed with brome mosaic virus RNA 2, along with suppression of RNA 2 accumulation in protoplasts (23). The authors provided evidence for the absence of any degradation process affecting RNA 2. The suppression was sequence specific but also context specific, was correlated with the expression of 2a protein, and, interestingly, was overcome in systemically infected tissue (23). These examples of pathogen-derived resistance could share a common mechanism that targets RNA but would require the expression of transgene-encoded protein to suppress the accumulation of the homologous viral RNA.

In conclusion, CMV RNA 1-transgenic plants possess a mechanism of resistance operating at the single-cell level that specifically suppresses the accumulation of the homologous

viral RNA. An additional barrier, perhaps also based on the same inhibition of viral RNA 1 accumulation, prevents the long-distance movement of CMV through the phloem. The combination of both would explain the phenotype of systemic resistance to CMV.

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