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Resistance in Transgenic Tobacco Plants Expressing a Nonstructural Gene Sequence of Tobacco Mosaic Virus Is a Consequence of Markedly Reduced Virus Replication

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Recently, tobacco plants transformed with the sequence encoding the putative nonstructural 54-kDa protein of tobacco mosaic virus (TMV) were found to be resistant to the virus (D. B. Golemboski, G. P. Lomonossoff, and M. Zaitlin, Proc. Natl. Acad. Sci. USA 87:6311-6315, 1990). As a first step in the dissection of the mechanism(s) underlying this novel form of genetically engineered resistance to TMV, studies with protoplasts were initiated. Resistance to strain U1 TMV was expressed in protoplasts derived from 54-kDa transgenic tobacco, showing that resistance was not a direct result of restricted virus movement. However, low levels of both (+) and (−) sense TMV-specific RNAs were detected in these protoplasts. Similar results were obtained with inoculated leaves of 54-kDa transgenic tobacco plants to which extremely high concentrations (0.5–1.0 mg/ml) of strain U1 TMV had been applied. These results indicated that expression of the 54-kDa protein gene sequence in transgenic tobacco plants does not confer absolute immunity to TMV but rather it protects the plant from subsequent spread of the virus by strongly inhibiting viral replication at the infection site.

The genome of strain U1 tobacco mosaic virus (TMV), a 6,395 nucleotide (Goelet *et al.* 1982), (+) sense, capped, single-stranded (ss) RNA encodes at least four proteins (Palukaitis and Zaitlin 1986). Reading from the 5′ towards the 3′ end of TMV RNA, open reading frames (ORFs) code for: the 126- and 183-kDa proteins, the 30-kDa movement protein (Deom *et al.* 1987), and the 17.5-kDa coat protein (CP). The 126- and 183-kDa proteins are translated from the genomic RNA, the 183-kDa protein being synthesized as the result of occasional readthrough of the termination codon of the sequence coding for the 126-kDa protein (Pelham 1978). Although the 126-kDa protein is synthesized in larger quantities than the 183-kDa protein, both are enriched in partially purified preparations of the viral replicase complex from TMV-infected tobacco leaves suggesting that these proteins are components of the viral-coded replicase (Young *et al.* 1987). In addition, the 126-kDa protein may have an RNA capping function (Dunigan and Zaitlin, 1990).

Our laboratory has contended that a fifth (54 kDa) protein is also encoded by TMV (Palukaitis and Zaitlin 1986). Synthesis of this protein would be directed by an ORF within the 183-kDa readthrough region. The 54-kDa protein sequence would be in-frame with the 183-kDa protein sequence and would share with the 183-kDa protein a gly-asp-asp (GDD) motif characteristic of several viral RNA-dependent RNA polymerases (Argos 1988; Habili and Symons 1989; Inokuchi and Hirashima 1987). This implies a role for the 54-kDa protein as a third viral-coded replicase component. However, despite the fact that ss and double-stranded (ds) forms of a subgenomic RNA (I_1 RNA) corresponding to the ORF for the 54-kDa protein occur in TMV-infected cells (Zelcer *et al.* 1981; Sulzinski *et al.* 1985), that the I_1 RNA is found on polyribosomes (Sulzinski *et al.* 1985), and that the protein can be synthesized by *in vitro* translation systems (Golemboski *et al.* 1990), the 54-kDa protein has not been detected *in vivo* (our own unpublished data, and Saito *et al.* 1986).

Over the past few years it has been possible to express viral CP genes in transgenic plants, rendering them less susceptible to the virus from which the CP gene was derived. Initially this was achieved with tobacco and TMV (Powell-Abel *et al.* 1986), but the approach has since been applied successfully to many other plant-virus combinations (Beachy *et al.* 1990). Recently, another form of genetically engineered resistance was described in transgenic tobacco plants harboring the putative 54-kDa protein gene of TMV (Golemboski *et al.* 1990). The resistance exhibited by the 54-kDa transgenic plants differed in several important respects from TMV CP-mediated resistance: resistance was exhibited against both TMV RNA and TMV virions; it did not appear to break down over time or with increasing concentrations of inoculum; and it was effective against the TMV strain from which the 54-kDa protein gene was derived (strain U1) or a closely related mutant, but not against other TMV strains such as U2, or against other viruses (Golemboski *et al.* 1990). Curiously, although the transcript corresponding to the 54-kDa protein gene was present in these transgenic plants, they contained no detectable 54-kDa protein (Golemboski *et al.* 1990). Therefore, it remains unclear whether the 54-kDa protein or its corresponding RNA mediates this resistance phenomenon.

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As a first step in an effort to unravel the mechanism underlying this novel form of resistance, we initiated studies aimed at discovering the stage(s) at which the virus life cycle is disrupted. Subsequently we determined that resistance appears to be due to a marked, general inhibition of virus replication rather than a block in a specific step in replication.

MATERIALS AND METHODS

Plants and virus strains. Tobacco plants (*Nicotiana tabacum* L.), cv. Xanthi nn, as well as the 54-kDa transgenic Xanthi nn (Golemboski *et al.* 1990), and the TMV local lesion indicator host Xanthi NN, were maintained under greenhouse conditions. Plants used for protoplast preparation were transferred to a growth chamber on a 14-hr light/10-hr dark cycle at 24° C for at least one week before use. The light intensity was reduced to 125–150 $\mu E \cdot m^{-2} \cdot s^{-1}$ by shading with cheese cloth. TMV strains U1 and U2 (Siegel and Wildman 1954) were purified as previously described (Asselin and Zaitlin 1978). The TMV strain U1 used for some experiments was derived from transcripts generated from a full-length cDNA clone of the virus (gift of R. N. Beachy and C. Holt, Washington University). Virus infection of whole plants was achieved by inoculation of both upper and lower surfaces of fully expanded Xanthi nn or 54-kDa transgenic tobacco leaves with 0.05, 0.5, or 1.0 mg/ml of TMV strain U1 in 0.05 M potassium phosphate, pH 7.0, with Celite as an abrasive. Viral RNA was prepared from TMV strains U1 and U2 by phenol extraction and ethanol precipitation.

Protoplast preparation and infection. Protoplasts were obtained (Hills *et al.* 1987) from leaves of 54-kDa transgenic plants and control, nontransgenic tobacco plants. The protoplasts ($0.5–1.0 \times 10^6$ cells per milliliter) were infected by electroporation with viral RNA (Hills *et al.* 1987) extracted from TMV strains U1 or U2. Electroporation was performed in a final volume of 2 ml of sterile 0.7 M mannitol, using a single ring electrode (2.5 mm high, 1 cm gap) connected to a ProGenetor 1 electroporation apparatus (Hoefer Scientific Instruments, San Francisco, CA), by applying two 5 msec pulses of 300 V. The viral RNA concentrations ranged from 10 to 100 μg/ml although routinely 10 μg/ml was used. In addition, all experiments included a set of mock-inoculated protoplasts electroporated in buffer alone. After electroporation, protoplasts were resuspended in incubation medium modified from Takebe *et al.* (1968): 0.7 M mannitol containing 1 mM KNO_3, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 μM KI, 0.01 μM $CuSO_4$, 10 μg/ml rimocidin (Pfizer Inc., New York, NY), and 100 μg/ml carbenicillin (Sigma, St. Louis, MO) buffered with 50 mM citrate, pH 5.5. The protoplasts (3 ml) were transferred to agar plates (1% noble agar in incubation medium prepared in 60- × 15-mm petri dishes) and incubated in low light at 25° C (Hills *et al.* 1987).

Analysis of protoplast proteins. Accumulation of TMV CP in protoplasts was detected by western blotting. Protoplasts were harvested by low speed centrifugation and disrupted in 50–100 μl of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (Laemmli 1970). The released proteins were separated by SDS-PAGE, electroblotted to nitrocellulose, and probed using a rabbit polyclonal antiserum (diluted 1:1,000) to strain U1 TMV CP and [^{125}I] protein A (Berry *et al.* 1985). To monitor the synthesis of virus-encoded proteins in protoplasts, L-[^{35}S] methionine (New England Nuclear, Boston, MA) was added to the incubation medium at a concentration of 10 μCi/ml. After continuous labeling, protoplasts were washed in 0.7 M mannitol and disrupted in Bradley buffer (Bradley and Zaitlin 1971). [^{35}S]-labeled proteins were analyzed by SDS-PAGE (Laemmli 1970) and autoradiography.

Analysis of RNA. At various times after electroporation, protoplasts were harvested, washed in sterile 0.7 M mannitol, disrupted in 50 mM Tris HCl, pH 8.0, 10 mM EDTA, 2% SDS, and extracted with phenol/chloroform/isoamyl alcohol (50:50:1) (Berry *et al.* 1985). In some cases, following ethanol precipitation, lithium chloride-soluble (enriched in dsRNA) and -insoluble (enriched in ssRNA) fractions were prepared (Berry *et al.* 1985). Leaf RNA was prepared in the same manner starting with leaf tissue pulverized in liquid N_2.

RNAs were separated on formaldehyde-containing, 1.2% agarose gels and were blotted to nitrocellulose (Sambrook *et al.* 1989), which was probed with *in vitro*-synthesized, [^{32}P]-labeled, ssRNA transcripts. Northern blots were prehybridized and hybridized for 24 hr at 45° C in 5× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 5× Denhardt's solution, 50 mM sodium phosphate, pH 7.0, 0.1% SDS, 250 μg/ml of denatured salmon sperm DNA, 100 μg/ml of yeast RNA, and 50% formamide, and were washed five times in 0.1× SSC, 0.2% SDS at 65° C. Relative amounts of specifically hybridizing RNA bands were quantified by excising the appropriate areas of the nitrocellulose filter using an autoradiograph as a template and determining the amount of radioactive probe bound using a liquid scintillation spectrometer (LS-230, Beckman Instruments, Fullerton, CA).

In vitro-synthesized RNA probes were prepared from two DNA templates: 1) T3 polymerase transcription (Sambrook *et al.* 1989) of pBS126 (obtained from G. P. Lomonossoff, John Innes Institute), a derivative of pBSM13− (Stratagene, La Jolla, CA) containing an insert corresponding to nucleotides 350–3,785 of strain U1 TMV, part of the 126-kDa protein reading frame, yields a (+) sense transcript corresponding to this region of TMV genomic RNA and complementary to the 3′ region of full-length (−) sense TMV RNA; 2) SP6 polymerase transcription (Sambrook *et al.* 1989) of pSP64CP (obtained from D. Sleat, Cleveland Clinic Foundation), a pSP64 (Promega Corp., Madison, WI) derivative containing an insert corresponding to the CP gene of TMV (from nucleotide 5,663 to the 3′ end). This yields a (−) sense transcript complementary to the (+) sense, full-length, as well as the subgenomic TMV RNAs, all of which possess the same 3′ terminus (Palukaitis and Zaitlin 1986). Additionally, T7 transcripts of pRTT-1, containing the sequence encoding the strain U1 TMV 54-kDa protein (Golemboski *et al.* 1990) were prepared and used to program wheat-germ (Roberts and Paterson 1973) and reticulocyte lysate-derived (Pelham and Jackson 1976), *in vitro* translation systems (both obtained from Promega).

RESULTS

Protoplasts from 54-kDa transgenic plants resist infection with strain U1 TMV. Protoplasts from 54-kDa transgenic plants that were electroporated 24 or 48 hr earlier with strain U1 TMV RNA did not contain any infectious virus detectable by bioassay on local lesion indicator plants (data not shown), but under the same experimental conditions these protoplasts replicated infectious strain U2 TMV. In contrast, control protoplasts from nontransformed plants replicated both strains of TMV. According to bioassay data, protoplasts derived from 54-kDa transgenic plants remained resistant to strain U1 TMV RNA even when the inoculum concentration was increased from 10 to 100 μg/ml of RNA. Consistent with the bioassay data, western blot analysis (Fig. 1) of protoplast proteins showed that 54-kDa transgenic protoplasts accumulated no detectable strain U1 TMV CP, although under the same conditions these cells accumulated strain U2 TMV CP in amounts similar to those in the control, nontransgenic tobacco protoplasts. It should be noted that the apparently low intensity signal obtained for strain U2 TMV CP is due to use of a polyclonal antiserum raised against strain U1 TMV CP, which reacts less strongly with strain U2 TMV CP (data not shown).

These results show that the resistance displayed by whole, intact 54-kDa transgenic plants is retained by protoplasts prepared from them, and that the resistance mechanism functions at the level of the single cell. This implies that resistance at the whole plant level is not due primarily to a block in cell-to-cell or long distance virus spread, but must act either by preventing the initiation of virus infection or by inhibiting virus replication once infection has taken place. This conclusion is consistent with data showing that plasmodesmata (the routes of cell-to-cell virus spread; Wolf *et al.* 1989) in 54-kDa transgenic plants appear to be unmodified and have normal molecular exclusion limits (W. J. Lucas, University of California, Davis; personal communication).

Synthesis of the 126-kDa protein was undetectable in 54-kDa transgenic tobacco protoplasts infected with strain U1 TMV. The 126-kDa protein is the more abundant of the two known viral-coded TMV replicase components and its synthesis, directed by the 5′ proximal ORF of TMV genomic RNA, is probably the first step in infection after (or during) virus uncoating (Wilson *et al.* 1990). The 126-kDa protein was not apparent among [^{35}S]-labeled proteins extracted from 54-kDa transgenic protoplasts infected with strain U1 TMV (Fig. 2). However, under the same conditions the 126-kDa protein was present in extracts of [^{35}S]-labeled protein from nontransgenic tobacco protoplasts infected with strain U1 TMV. The equivalent, faster moving protein encoded by strain U2 TMV (Fraile and Garcia-Arenal 1990) was synthesized in both transgenic and nontransgenic protoplasts infected with that strain of TMV (Fig. 2). Similarly, synthesis of strain U2 TMV CP was observed in both cell types. Synthesis of strain U1 TMV CP could not be observed in this way because it contains no methionine (Goelet *et al.* 1982). Attempts to improve the sensitivity of detection of the 126- and 183-kDa proteins by immunoprecipitation with appropriate antisera were unsuccessful (data not shown).

Low levels of TMV-specific RNAs occur in 54-kDa transgenic tobacco protoplasts infected with strain U1 TMV. Although direct methods were inadequate to demonstrate

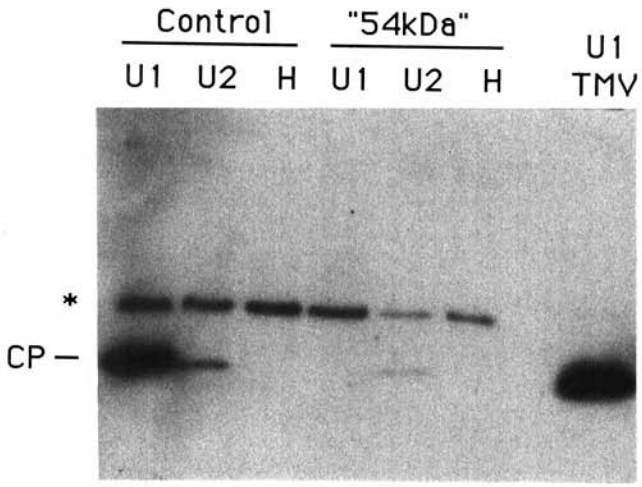

Fig. 1. Accumulation of tobacco mosaic virus (TMV) coat protein (CP) in transgenic and nontransgenic protoplasts. Protein extracted from approximately equal numbers (~ 30,000) of protoplasts prepared from nontransgenic (control) or 54-kDa transgenic tobacco ("54 kDa") plants, which were inoculated by electroporation with RNA from either strain U1 TMV or strain U2 TMV, or which had been electroporated with buffer only (H), was analyzed by SDS-PAGE and western blotting using polyclonal rabbit anti-U1 TMV CP serum. Purified strain U1 TMV CP (U1 TMV; 0.25 μg) was run as a marker. Note that the antiserum was raised against strain U1 TMV CP and cross-reacts less well with strain U2 TMV CP (data not shown). The antiserum also reacts with a polypeptide unrelated to TMV CP, which is present in all protoplast extracts (*). Position of TMV CP indicated by CP.

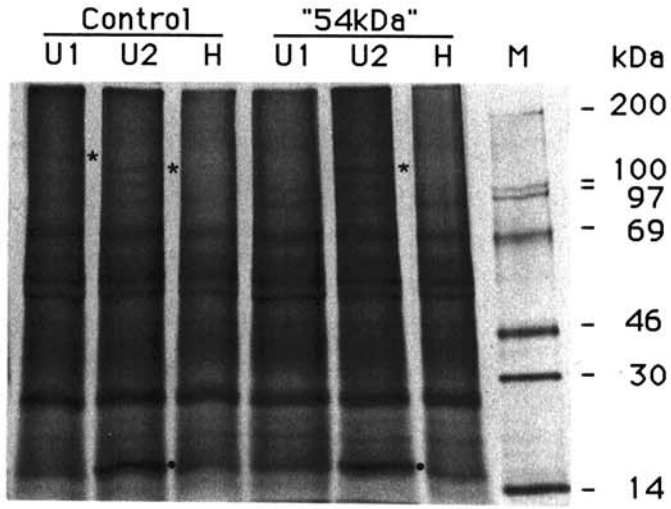

Fig. 2. Synthesis of host and viral proteins in transgenic and nontransgenic protoplasts. Protoplasts from nontransgenic ("control") or 54-kDa transgenic ("54 kDa") plants were inoculated by electroporation with RNA of either strain U1 TMV or strain U2 TMV, or electroporated with buffer only (H) and incubated with [^{35}S]-methionine for 21 hr. Labeled proteins were extracted from equivalent numbers of protoplasts and analyzed by SDS-PAGE and autoradiography. The 126-kDa protein is marked with *; strain U2 TMV CP is marked with ●. Lane M was loaded with [^{14}C]-labeled protein molecular weight standards (Amersham Corp., Arlington Heights, IL).

synthesis of viral-coded replicase proteins in 54-kDa transgenic tobacco protoplasts infected with strain U1 TMV, there remained an indirect way of detecting the presence of these proteins. Specifically, any products of replicase activity will betray the presence of small levels of all of the replicase components. Because the initial product of replication is (−) sense RNA generated from the input, (+) sense, genomic TMV RNA, RNA from strain U1 TMV-infected 54-kDa transgenic tobacco protoplasts was probed for the presence of full-length, (−) sense TMV RNA (Fig. 3). By 21 hr post-inoculation (hpi), trace amounts of ss, (−) sense, full-length TMV RNA were found to be present in the strain U1 TMV-infected 54-kDa transgenic tobacco protoplasts (Fig. 3A), although its ds form was not detectable (Fig. 3C). Therefore, some small quantity of virus-coded replicase components must have been synthesized after infection and must have been functional to some extent in these cells.

Detection of (−) sense TMV RNA in the 54-kDa transgenic tobacco protoplasts prompted the question: Does replication proceed beyond (−) strand synthesis and result in any (+) strand synthesis? Northern analysis of protoplast RNAs with a probe specific for (+) sense, 3′ sequences of TMV RNA detected the presence of low levels of (+) sense TMV RNAs by 5 hpi and the full complement of full-length and subgenomic TMV RNAs by 21 hpi in strain U1 TMV-infected 54-kDa transgenic tobacco protoplasts (Fig. 4A). The full complement of ds forms of the full-length and subgenomic TMV RNAs were also observed in the TMV-infected nontransgenic protoplasts (Fig. 4C). However, northern blot analysis was not sensitive enough to detect TMV dsRNAs in the TMV-infected 54-kDa transgenic protoplasts (Fig. 4C). Counting of the radioactive probe bound to specific ssRNA bands (data not shown) showed that the levels of full length (+) sense TMV RNAs, which accumulated in strain U1 TMV-infected 54-kDa transgenic tobacco protoplasts, were between 20- and 80-fold less than those in infected nontransgenic proto-

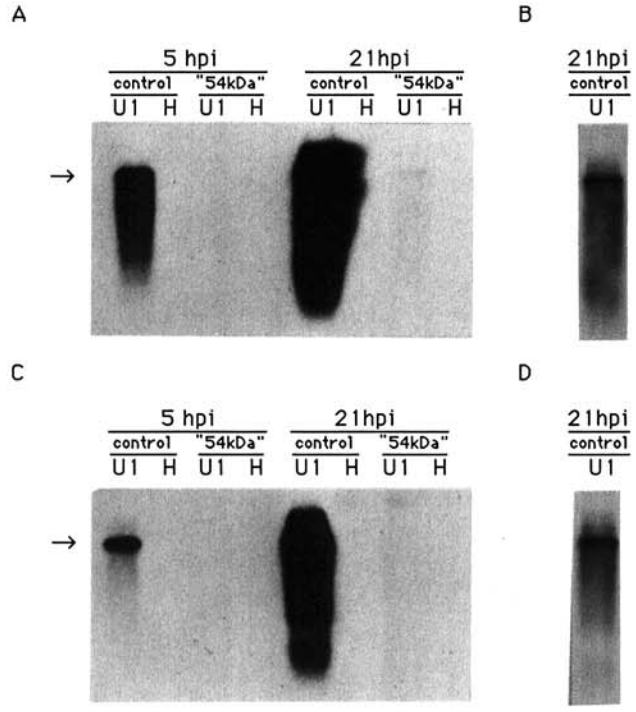

Fig. 3. Detection of full-length, (−) sense TMV transcripts in transgenic protoplasts. Equal numbers of protoplasts from nontransgenic (control) and 54-kDa transgenic ("54 kDa") tobacco plants were inoculated by electroporation with RNA from strain U1 TMV or were electroporated in the presence of buffer alone (H). At 5 or 21 hr post-inoculation (hpi), ss-enriched (A and B) and ds-enriched (C and D) RNAs were extracted from the protoplasts and subjected to northern blot analysis using as probe a [^{32}P]-labeled T3 transcript of pBS126 specific for full-length, (−) sense strain U1 TMV RNA (indicated by arrows). **A, C,** autoradiographic exposures of 72 hr with intensification screens. **B, D,** shorter exposures (4 hr without intensification screens) of the lanes loaded with RNA from strain U1 TMV-infected control protoplasts (21 hr post inoculation) from the same northern blots shown in A and B, respectively.

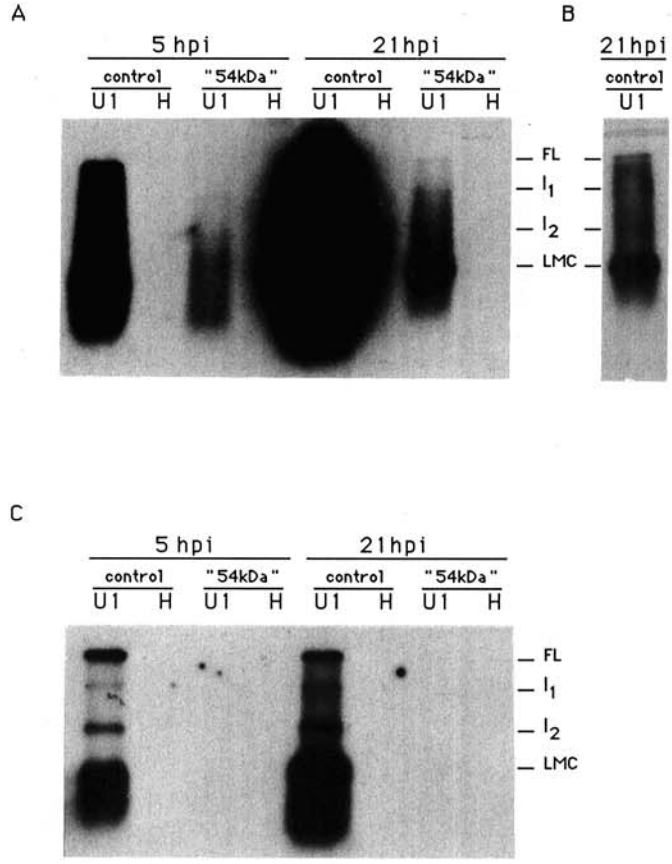

Fig. 4. Detection of (+) sense TMV transcripts in transgenic and nontransgenic protoplasts. Equal numbers of protoplasts from nontransgenic (control) and 54 kDa transgenic ("54 kDa") tobacco plants were inoculated by electroporation with RNA from strain U1 TMV or were electroporated in the presence of buffer alone (H). At 5 or 21 hr post-inoculation (hpi), ss-enriched (A and B) and ds-enriched (C) RNAs were extracted from the protoplasts and subjected to northern blot analysis using as probe a [^{32}P]-labeled, SP6 transcript of pSP64CP specific for (+) sense TMV RNAs. TMV RNAs are indicated by: FL (full-length genomic TMV RNA), I_1 RNA (RNA encoding the 54-kDa protein sequence), I_2 RNA (RNA encoding the 30-kDa movement protein) and LMC (RNA encoding the TMV CP) (Palukaitis and Zaitlin 1986). **A, C,** autoradiographic exposures of 24 hr with intensification screens. **B,** a shorter exposure (15 min without intensification screens) of the lane loaded with ss-enriched RNA from strain U1 TMV-infected control protoplasts (21 hr post-inoculation) from the same northern blot shown in A.

plasts depending on the specific experiment (data not shown). Similar results were obtained when either our laboratory U1 strain TMV RNA or clone-derived U1 TMV RNA were used. The results indicate that a low level of strain U1 TMV replication can occur in 54-kDa transgenic tobacco protoplasts.

Low levels of TMV-specific RNAs accumulate in inoculated leaves of 54-kDa transgenic plants heavily infected with strain U1 TMV. The above studies of 54-kDa transgenic tobacco protoplasts with strain U1 TMV indicated that these cells permit the synthesis of trace amounts of TMV-specific RNAs. However, did the results obtained with protoplasts truly reflect the characteristics of the resistance phenomenon in leaf cells of whole 54-kDa transgenic plants? To address this question, leaves of 54-kDa transgenic tobacco plants were inoculated on their upper and lower surfaces with strain U1 TMV particles at concentrations of 0.05 and 0.5 mg/ml. These highly concentrated inocula (approximately two and three orders of magnitude greater, respectively, than those typically employed to infect nontransgenic tobacco plants) were used to maximize the number of leaf cells infected and thus increase the chances of detecting virus-specific RNAs.

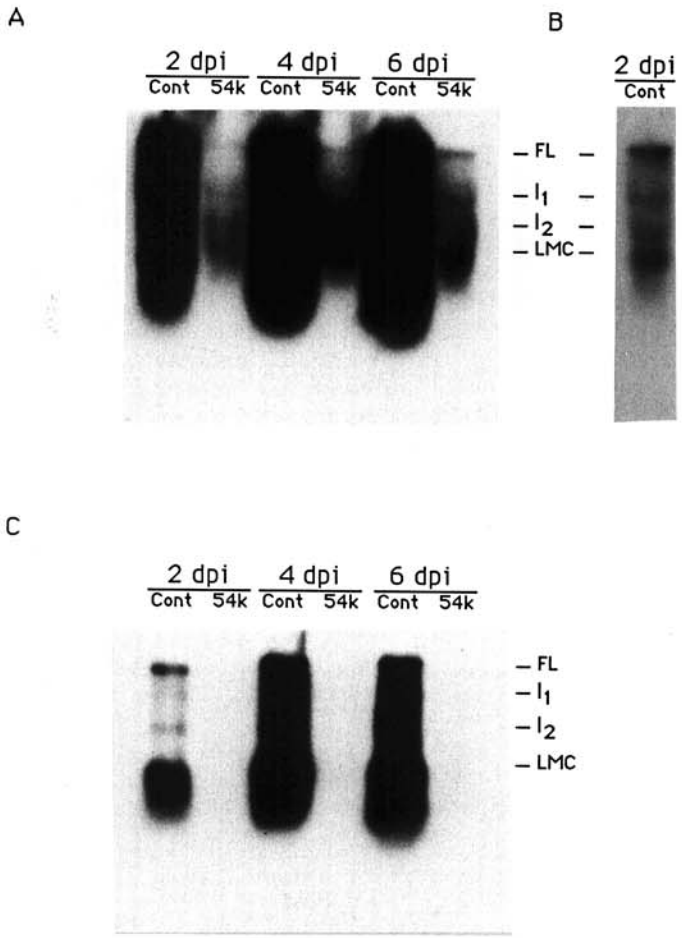

Fig. 5. Detection of (+) sense TMV RNAs in heavily inoculated transgenic and nontransgenic leaves. Leaves of nontransgenic (cont) and 54-kDa transgenic (54 k) tobacco plants were inoculated on upper and lower surfaces with strain U1 TMV at a concentration of 0.5 mg/ml. At 2, 4, and 6 days post-inoculation (dpi) leaf material was harvested and ss-enriched (A and B) and ds-enriched (C) RNAs prepared. Equal quantities of RNA were subjected to northern analysis and probed for the presence of (+) sense TMV RNAs as described for Figure 4. **A, C** autoradiographic exposures of 2 hr with intensification screens. **B,** a shorter exposure (15 min without intensification screens) of the lane loaded with ss-enriched RNA from strain U1 TMV-infected control leaves (2 dpi) from the same northern blot shown in A.

Northern blot analysis of RNA from 54-kDa transgenic tobacco leaves inoculated with strain U1 TMV at 0.05 mg/ml did not reveal any viral RNAs (data not shown). However, increasing the inoculum to 0.5 mg/ml resulted in the production of detectable levels of full-length and sub-genomic (+) sense TMV RNAs, which increased over time (Fig. 5A). Once again, any TMV dsRNAs that may have been present were at levels too low to detect using this methodology (Fig. 5C). Comparison of the amounts of radioactive probe bound to specific RNA bands in northern blots indicated that the levels of full-length (+) sense TMV RNAs that accumulated in heavily inoculated 54-kDa transgenic tobacco leaves were 17- to 20-fold less than those found in nontransgenic tobacco tissue (data not shown). Similar results were obtained both with our laboratory virus isolate and with virus propagated from an infectious TMV cDNA clone (applied at a concentration of 1 mg/ml). When the heavily inoculated areas of 54-kDa transgenic tobacco leaves were used as sources of inoculum for assay on local lesion indicator plants, small amounts of biologically active virus were sometimes detected (data not shown). No virus was detectable in other leaves of the same plant or on uninoculated parts of the same leaf when clone-derived virus was used as the inoculum.

In the case of one 54-kDa transgenic plant inoculated with noncloned strain U1 TMV at a concentration of 1 mg/ml, systemic symptoms were observed. Subsequent inoculation of the "escaped" virus onto nontransgenic tobacco resulted in symptoms characteristic of strain U2 TMV, which is unaffected by the resistance phenomenon and which occurs at low levels in preparations of naturally occurring strain U1 TMV (Siegel and Wildman 1954; Garcia-Arenal *et al.* 1984). This suggested that neither strain U1 TMV nor a U1-derived mutant had been responsible for the systemic symptoms in the 54-kDa transgenic plant.

Overall, the results obtained with heavily inoculated 54-kDa transgenic tobacco leaves appear consistent with those obtained using protoplasts in that there appears to be a major inhibition, although not a complete shutdown, of virus replication.

Can the 54-kDa protein or its RNA affect synthesis of TMV-coded proteins *in vitro*? How is the inhibition of replication of strain U1 TMV achieved in 54-kDa transgenic tobacco plants? There are two possibilities: first, the 54-kDa protein or its RNA might directly inhibit replicase activity; or second, the 54-kDa protein or its RNA may act indirectly, for instance by inhibiting synthesis of the virus-coded replicase components, the 126- and 183-kDa proteins. The second possibility was addressed by translating TMV RNA in rabbit reticulocyte or wheat-germ *in vitro* translation systems that had also been programmed or preprogrammed with an *in vitro*-synthesized RNA transcript encoding the 54-kDa protein. In both of these cell-free translation systems, synthesis of the 126-, 183-,

as well as the 54-kDa proteins occurred with no suggestion of specific inhibition of 126- or 183-kDa protein synthesis (data not shown). Thus, there is no evidence that the 54-kDa protein or its RNA affect the synthesis of virus-coded replicase components. The results are therefore consistent with the first possibility outlined above, namely that the 54-kDa protein or its corresponding RNA affect replicase activity directly.

DISCUSSION

In an earlier paper, Golemboski *et al.* (1990) demonstrated that transgenic tobacco plants expressing the 54-kDa protein gene of strain U1 TMV are able to resist the virus. We have shown in this report that resistance to strain U1 TMV is also expressed in protoplasts derived from the 54-kDa transgenic plants. This indicates that the resistance mechanism is unlikely to affect virus movement directly, either cell-to-cell or over longer distances. The detection of low levels of (+) and (−) sense TMV-specific RNAs in the transgenic protoplasts showed, moreover, that these cells do not possess a complete immunity to infection by the virus but are able to inhibit its replication greatly. The results of further experiments, in which leaves of 54-kDa transgenic tobacco plants were inoculated with high concentrations (0.5–1.0 mg/ml of TMV) of virus to saturate potential infection sites, were consistent with data obtained from protoplasts, showing that low levels of TMV replication could also take place in the initially infected cells and perhaps in a few secondarily infected cells of the intact plants. It is presumed that inoculation with high concentrations of virus succeeded in producing low levels of replication by infecting as great a number of cells as possible at once. This might appear to contradict the report of Golemboski *et al.* (1990) who were unable to detect viral RNA even in leaf tissue inoculated with high concentrations of strain U1 TMV. However, the detection method used, dot blots of crude plant material probed with TMV-specific [^{32}P]-labeled cDNA (Golemboski *et al.* 1990 and unpublished data), was much less sensitive than the hybridization techniques employed in the present study.

The results suggest that inoculation of 54-kDa transgenic tobacco plants with strain U1 TMV results in as many initially infected cells as may occur in a nontransgenic plant but that subsequent events differ considerably in the two types of plants. In the 54-kDa transgenic tobacco plant cell, viral replication proceeds but does so very poorly. If the virus does succeed in moving out of the first infected cell into adjacent cells, replication is once again inhibited thus crippling its ability to spread beyond the immediate vicinity of the infection site. Although virus replication is not halted absolutely, it is so severely impeded that little or no detectable systemic spread can occur within the plant during its lifetime.

Although the above model may provide a plausible explanation for why 54-kDa transgenic tobacco plants appear resistant to TMV, it does not suggest any specific mechanism by which expression of the TMV 54-kDa protein gene causes resistance. It still remains undetermined whether resistance is mediated by the 54-kDa protein or by its RNA. The 54-kDa protein is a truncated version of the 183-kDa replicase protein and possesses the GDD sequence common to RNA-dependent RNA polymerases of many RNA viruses (Argos 1988; Habili and Symons 1989). This sequence is, for example, essential to the functioning of phage Qβ replicase (Inokuchi and Hirashima 1990). It can be hypothesized that low levels of the 54-kDa protein are synthesized, associate with the TMV replicase complex, perhaps replacing some of the 183-kDa protein, and inhibiting replicase activity. Thus there might be a real function for 54-kDa protein in "naturally" TMV-infected nontransgenic plant cells, possibly as an inhibitory or regulatory subunit of the replicase produced at some specific stage of TMV replication; for example, the switch-over from (−) to (+) sense RNA strand synthesis, which in a normal infection occurs approximately 6–8 hr post-inoculation (Ishikawa *et al.* 1991).

Alternatively, resistance may be caused by the RNA encoding the 54-kDa protein rather than the protein itself. It is possible to envisage this RNA hybridizing to (−) TMV RNA and inhibiting further replication by a form of the (−) strand capture mechanism (Palukaitis and Zaitlin 1984). Another potential RNA-mediated mechanism is suggested by the experiments of Morch *et al.* (1987) who found that short 3′-terminal pieces of synthetic (+) sense RNAs interfered with replication of turnip yellow mosaic virus *in vitro*, perhaps by competing with authentic viral RNA for association with the replicase.

To detail precisely the mechanism by which replication is inhibited and elucidate the relative importance of the 54-kDa protein versus its RNA, technically complex experiments using a TMV *in vitro* replication system will have to be performed. Until recently, an authentic replicase system for plant RNA viruses remained elusive. However, Hayes and Buck (1990) have described such an *in vitro* system for the replication of cucumber mosaic virus and the development of a similar system for TMV may provide new insights into 54-kDa protein gene-mediated resistance as well as other aspects of TMV replication.

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