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Resistance to Tobacco Mosaic Virus Induced by the 54-kDa Gene Sequence Requires Expression of the 54-kDa Protein

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Tobacco plants transformed with the sequence encoding the 54-kDa putative replicase protein of tobacco mosaic virus were resistant to systemic virus disease (D. B. Golemboski, G. P. Lomonossoff, and M. Zaitlin, Proc. Natl. Acad. Sci. USA 87:6311-6315, 1990). Resistance was due to a marked suppression of virus replication at the site of inoculation (J. P. Carr and M. Zaitlin, Mol. Plant-Microbe Interact. 4:579-585, 1991). Although RNA transcripts encoding the 54-kDa protein were present in resistant plants, the 54-kDa protein itself was not observed *in vivo*. We wished to assess the relative importance of the 54-kDa protein versus its RNA in mediating resistance. Further attempts to detect the 54-kDa protein in plant tissues were unsuccessful; therefore, an indirect approach was taken using a protoplast-based transient gene expression system. Electroporation of protoplasts with plasmids capable of expressing the wild-type 54-kDa protein gene sequence or a mutant lacking the first AUG initiation codon of the 54-kDa open reading frame and encoding a slightly truncated protein reduced virus replication in protoplasts. In contrast, a frameshift mutant that was capable of directing synthesis of a protein only 20% the size of the 54-kDa protein, did not produce resistance in protoplasts. These results show that expression of the 54-kDa protein gene sequence at the RNA level alone is insufficient for resistance, and they implicate the 54-kDa protein itself in mediating this resistance phenomenon.

Additional keywords: nonstructural gene, replicase, plant virus, protection, replicase-mediated resistance.

Tobacco plants transformed with the DNA sequence encoding a putative nonstructural protein of tobacco mosaic virus (TMV), the 54-kDa protein, have been shown to be highly resistant to systemic infection with TMV (Golemboski *et al.* 1990). This novel form of genetically engineered resistance to a plant virus differed in several respects from the resistance engendered in transgenic plants expressing the coat protein (CP) of TMV (Powell-Abel *et al.* 1986; Beachy *et al.* 1990): It was exhibited against both TMV RNA and TMV particles; 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 sequence was obtained (strain U1), but not against other TMV strains such as U2, or against other viruses (Golemboski *et al.* 1990). More recently, it was shown that the resistance caused by expression of the 54-kDa protein gene sequence acted at the level of the single cell and that it caused a very marked, general (but not a total) suppression of virus replication (Carr and Zaitlin 1991). It was concluded that resistance was not a direct result of restriction of virus movement, but that the inhibition of replication of the virus at the infection site was effective enough to prevent subsequent spread of TMV and the production of systemic disease (Carr and Zaitlin 1991).

The strategy of using nonstructural replicase genes for protection seems applicable to other virus-host systems. MacFarlane and Davies (in press) have reported that transformation of *Nicotiana benthamiana* plants with an analogous 54-kDa protein sequence from pea early browning virus (PEBV) renders these plants highly resistant to that virus, and Anderson *et al.* (in press) have reported that tobacco plants transformed with a truncated form of the open reading frame (ORF) for the replicase encoded by RNA 2 of cucumber mosaic virus (CMV) exhibit resistance to CMV.

The genome organization of TMV is well known (reviewed by Palukaitis and Zaitlin 1986). It is generally agreed that the single-stranded, plus-sense RNA encodes four proteins, two of which, the 126- and 183-kDa proteins, are enriched in partially purified preparations of the viral replicase complex and are therefore considered to be viral-coded subunits of the replicase enzyme (Young *et al.* 1987). The 183-kDa protein is synthesized by occasional read through of the termination codon of the sequence coding for the 126-kDa protein (Pelham 1978). More controversially, it has been proposed that a fifth protein (54-kDa) is also encoded by TMV (Palukaitis and Zaitlin 1986). Synthesis of the 54-kDa protein would be directed by an ORF present within the read-through region of the sequence coding for the 183-kDa protein. Importantly, the 54-kDa protein sequence is in frame with the 183-kDa protein sequence and shares several sequence motifs characteristic of viral RNA-dependent RNA polymerases (Argos 1988; Habili and Symons 1989; Bruenn 1991), implying that the 54-kDa protein might be a third viral-coded replicase subunit or a replicase-associated regulatory protein. Although single-stranded and double-stranded forms of a subgenomic RNA (I_1 RNA) corresponding to the ORF for the 54-kDa protein have been found in TMV-infected cells (Zelcer *et al.* 1981; Sulzinski *et al.* 1985) with the single-stranded I_1 RNA

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occurring on polysomes (Sulzinski *et al.* 1985), no 54-kDa protein has been detected *in vivo* (our own unpublished data; Saito *et al.* 1986). Even in the transgenic plants harboring the 54-kDa protein gene sequence, no 54-kDa protein could be detected, although the RNA transcript corresponding to the 54-kDa protein gene was present (Golemboski *et al.* 1990). Although up to this time the 54-kDa protein has been observed only in cell-free *in vitro* translation systems and has remained elusive *in vivo,* there still remains the possibility that the 54-kDa protein may be synthesized in trace amounts or may occur only ephemerally in the plant cell.

Uncertainty regarding the existence *in vivo* of the 54-kDa protein represents a significant obstacle to our understanding of the mechanism(s) underlying the phenomenon of resistance in transgenic plants expressing the 54-kDa protein gene sequence. In the present study we renewed efforts aimed at the direct detection of the 54-kDa protein *in vivo*. However, because these experiments proved inconclusive we decided to take an indirect approach to determine the importance of the 54-kDa protein in resistance. A transient gene expression system in protoplasts was developed to test derivatives of the 54-kDa protein sequence for their ability to confer resistance to TMV. Using this procedure, we determined that expression in protoplasts of the 54-kDa protein gene sequence at the RNA level alone is insufficient to generate resistance to the virus, implying that it is the 54-kDa protein that mediates resistance.

MATERIALS AND METHODS

Plants and virus strains. Tobacco plants (*Nicotiana tabacum* L.) cvs. Xanthi NN and Samsun nn were maintained under greenhouse conditions. One week before use in protoplast experiments, Xanthi NN plants were moved to a growth chamber and grown under conditions described previously (Carr and Zaitlin 1991). TMV U1 and U2 (also known as tobacco mild green mosaic virus) were purified by the method of Asselin and Zaitlin (1978), and viral RNA was prepared from virion preparations by phenol extraction and ethanol precipitation.

Detection methods for the 54-kDa protein. Samsun nn tobacco plants were inoculated on their upper and lower leaf surfaces with TMV U1 at a concentration of 500 $\mu g \cdot ml^{-1}$. Protein was extracted from the leaves at 4, 5, and 6 days postinoculation by the method of Young and Zaitlin (1986). Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970) and electroblotted to nitrocellulose for western analysis (Harlow and Lane 1988). Three classes of antisera were used to attempt to detect 54-kDa protein by western analysis: A rabbit polyclonal serum raised against a fusion protein including 432 amino acids of the read-through region of the 183-kDa protein (Saito *et al.* 1986; a generous gift of Y. Okada, University of Tokyo, Japan), rabbit polyclonal antisera raised against a 15-residue synthetic oligopeptide (sequence: LDISKYDKSQ-NEFHC) corresponding to nucleotides 4221 to 4265 in the TMV genome, and a guinea pig polyclonal antiserum raised against an extract of a baculovirus system expressing a fusion protein containing the 54-kDa protein (a gift of J. I. Cooper, Institute of Virology, Oxford, U.K.). Antibody binding was detected by three methods: ^{125}I-protein A and autoradiography (Berry *et al.* 1985); goat anti-rabbit IgG or protein A conjugated to horseradish peroxidase, detection of enzyme activity was with an enhanced chemiluminescence kit (ECL; Amersham Corp., Arlington Heights, IL); or goat anti-rabbit IgG conjugated to alkaline phosphatase, detecting enzyme activity with an alkaline phosphatase detection kit (Promega Inc., Madison, WI).

DNA manipulations. All DNA manipulations were performed essentially as described by Sambrook *et al.* (1989). Oligonucleotide-directed *in vitro* mutagenesis of M13 subclones derived from the plasmid pRTT1, which contains the 54-kDa protein gene sequence (Golemboski *et al.* 1990), was carried out as described by Kunkel (1985). Mutated 54-kDa protein gene DNA sequences were initially subcloned into pBS(−) (Stratagene, La Jolla, CA). These plasmids were used as templates for *in vitro* transcription with T7 polymerase (Sambrook *et al.* 1989). Subsequently, the inserts from these pBS(−) derived plasmids were subcloned into the polylinker region of pFF19 (a gift of M. C. P. Timmermans, Rutgers University). This plasmid contains an expression cassette constructed from the cauliflower mosaic virus 35S 5′ regulatory region with the enhancer duplicated, and the 35S polyadenylation signal (Timmermans *et al.* 1990). A polylinker inserted between the transcriptional start and polyadenylation sites permitted convenient cloning of the wild-type and mutant versions of the 54-kDa protein gene sequence. Another pFF19 derivative, pFF19G (Timmermans *et al.* 1990), which contains the ORF for β-glucuronidase (GUS; Jefferson 1987) was also a gift of M. C. P. Timmermans.

Protoplast preparation and electroporation with plasmid DNAs and viral RNAs. Protoplasts were obtained from Xanthi NN tobacco as described previously (Hills *et al.* 1987; Carr and Zaitlin 1991). The simultaneous introduction of TMV RNA and plasmid DNA into protoplasts was carried out by a modification of the procedure of Carr and Zaitlin (1991) for electroporation of tobacco protoplasts with viral RNA. Electroporation was performed in a final volume of 1.5 or 2.0 ml of sterile 0.7 M mannitol, using a single-ring electrode (2.5 mm high, 1 cm gap) connected to a ProGenetor I electroporation apparatus (Hoefer Scientific Instruments, San Francisco, CA), by discharge of four 10 msec pulses of 425 V. The viral RNA (TMV U1 or U2) concentration used was 0.5 μg/ml, and the concentration of plasmid DNA ranged from 50 to 100 μg/ml depending on the experiment. Between 0.5 and 1.0×10^6 protoplasts (counted in a hemacytometer) were present in each electroporation cell. After electroporation, the protoplasts were kept on ice for 15–20 min before transfer to incubation medium under conditions described previously (Carr and Zaitlin 1991). At the end of the incubation period (24 h) protoplasts were washed in 0.7 M mannitol and counted again before further procedures were carried out.

Detection of GUS activity and virus infection in protoplasts. GUS activity was detected by taking an aliquot of approximately 5,000 protoplasts newly electroporated with pFF19G or pFF1954 (negative control) and incubating them for 24 hr in 2 ml of protoplast incubation buffer

(Carr and Zaitlin 1991) containing (per milliliter) 10 μg of rimocidin, 100 μg of carbenicillin, 10 μg of chloramphenicol, and the GUS substrate X-GLUC (Jefferson 1987) at 1 mM. Samples of protoplasts were allowed to dry onto glass slides coated with Meyer's albumin. The dried protoplasts were decolorized by dipping the slide sequentially into 95% ethanol, 50% ethanol, and 95% ethanol. The slide was allowed to air dry and was examined with a microscope.

The proportion of cells infected with TMV present in a batch of protoplasts was determined by a modification of the tissue blotting method of Cassab and Varner (1987). Nitrocellulose was prepared by boiling it in water for 3 min, soaking it at room temperature in 0.2 M $CaCl_2$ for 30 min, and air drying it. Protoplasts (24 hr postelectroporation) were spotted onto the pretreated nitrocellulose and allowed to dry. The nitrocellulose was placed in Tris-buffered saline (TBS; Harlow and Lane 1988) containing 1% bovine serum albumin for 1 hr, then incubated for 30 min in TBS containing 0.05% Tween 20 and polyclonal rabbit anti-TMV CP serum at a dilution of 1:5,000. After washing for 5 min in TBS-Tween, the nitrocellulose was incubated for 30 min in a 1:10,000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega). After the nitrocellulose was washed in TBS-Tween and water, alkaline phosphatase activity was detected using an alkaline phosphatase substrate kit (Promega) according to the manufacturer's instructions. Cell staining was observed with a microscope.

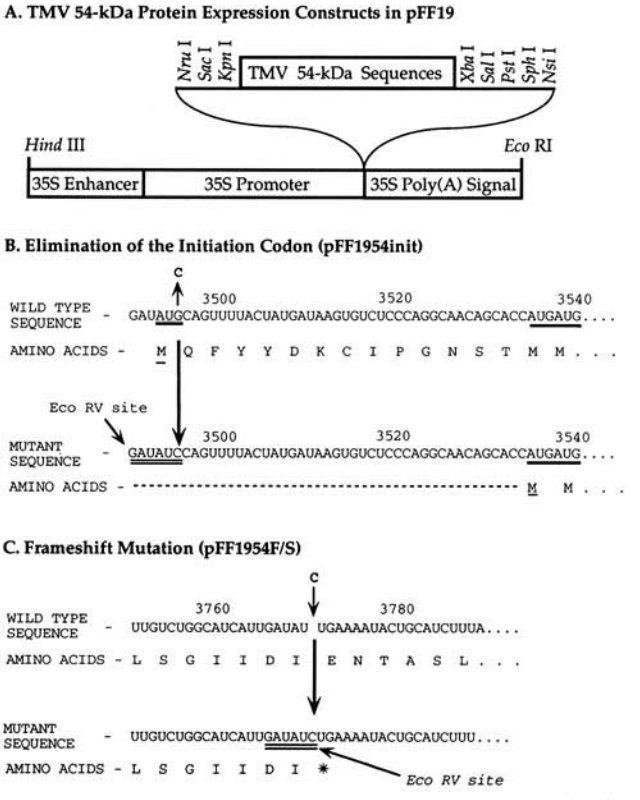

Fig. 1. Insertion of the 54-kDa protein gene sequence and derivatives into a transient expression vector. **A,** The 54-kDa protein gene sequence and its derivatives were cut out of pBS(−) by digestion with *Pst*I and *Kpn*I and ligated into the polylinker region within the expression cassette of pFF19. **B,** Nucleotide 3497 of the TMV sequence was converted from G to C, eliminating the putative initial AUG initiation codon (underlined) of the 54-kDa protein gene sequence and generating an *Eco*RV site (double underlined) but leaving two downstream AUG codons at 3535 and 3538 (underlined) unaffected. This construct was subcloned into pBS(−) to give pRTTinit and then further subcloned into pFF19 to give pFF1954init. **C,** An extra C was inserted between TMV nucleotides 3772 and 3773 shifting the reading frame and resulting in formation of a termination codon (*) and generation of an *Eco*RV site (double underlined). This construct was subcloned into pBS(−) to give pRTTF/S and then further subcloned into pFF19 to give pFF1954F/S.

Analysis of RNA. Total RNA was extracted from protoplasts and separated by lithium chloride precipitation into ss- and ds-RNA-enriched fractions as described previously (Berry *et al.* 1985; Carr and Zaitlin 1991). Plus-sense TMV-specific RNAs were detected by northern blotting: Protoplast RNAs were electrophoresed in formaldehyde-containing 1.2% agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P-labeled SP6 transcripts of pSP64CP (obtained from D. Sleat, Cleveland Clinic Foundation) which are complementary to the (+) sense, full-length, as well as all the subgenomic TMV U1 RNAs (Carr and Zaitlin 1991). TMV U2 RNAs were detected using ^{32}P-labeled cDNA generated from TMV U2 genomic RNA by reverse transcription (Palukaitis 1988) using random primers (Pharmacia). ^{32}P-labeled T3 transcripts of pRTT1 were used to probe northern blots for the presence of 54-kDa protein gene-specific RNAs. Each northern blot lane was loaded with RNA extracted from an equal number of protoplasts, typically 3,000. Relative amounts of hybridization to specific RNA bands were quantified by excising the appropriate areas of the nitrocellulose filter using an autoradiograph as a template and determining the amount of bound radioactive probe by liquid scintillation spectrometry (Carr and Zaitlin 1991).

T7 transcripts of pRTT1 (Golemboski *et al.* 1990) and other derivatives of pBS(−) containing mutated 54-kDa protein gene sequences were used to program a wheat-germ cell-free *in vitro* translation system (Roberts and Paterson 1973) containing L-[^{35}S]methionine (Amersham) according to the instructions of the manufacturer (Promega). Radiolabeled proteins were analyzed by SDS-PAGE (Laemmli 1970) and visualized by autoradiography.

RESULTS

The search for the 54-kDa protein in TMV-infected leaf tissue. Evidence of expression of the putative nonstructural 54-kDa protein of TMV was sought in several ways. The principal method was to subject proteins extracted from heavily TMV-inoculated (500 $\mu g \cdot ml^{-1}$) tobacco leaves to western blotting. Out of a number of antiserum preparations it was found that two antisera, both raised against a 15-residue synthetic oligopeptide, in combination with an enhanced chemiluminescence or alkaline phosphatase detection system were the most effective in detecting the accumulation of the TMV 183-kDa replicase protein (data not shown). However, despite the fact that the 54-kDa protein possesses the same 15-residue stretch of amino acid sequence as the 183-kDa protein, and therefore should react with the antisera, it was never detected (data not shown). Reconstruction experiments in which differing amounts of the 15-residue oligopeptide were subjected to western analysis suggested that the lower limit of detection of this

sequence was 100 ng. Approximately 60 μg of leaf protein was loaded per lane for western blotting, implying that about 0.1–0.15% of this protein would have had to consist of the reactive sequence for a signal to have been detected. Because the 15-residue sequence is only about one twenty-fifth of the whole 54-kDa protein sequence, approximately 2–3% of the protein loaded would have had to have been 54-kDa protein for a signal to be detected with these antisera. This would be a level of accumulation comparable to that attained by CP and one that would be unlikely for a nonstructural protein of TMV. None of the antiserum preparations that we have tested are sufficiently sensitive to detect the trace amounts of 54-kDa protein that may be present in TMV-infected leaf tissue or in plants transformed with the 54-kDa protein gene sequence.

Additionally, both leaf tissue and infected protoplasts were labeled with L-[^{35}S]methionine and proteins were immunoprecipitated with a 183-kDa antiserum. This serum was raised in rabbits (by David Dunigan) against 183-kDa protein contained in polyacrylamide gel slices, using methods described in Hills *et al.* (1987). Such antibodies were capable of precipitating 54-kDa protein from a reticulocyte lysate *in vitro* translation reaction (with TMV RNA as template) but did not detect 54-kDa protein in leaf tissue or in protoplasts (M. Zaitlin and T. M. A. Wilson, unpublished). This antiserum was also used to probe tissue sections of TMV-infected tissue by immunogold tagging in the electron microscope. Using techniques applied successfully in this laboratory to detect ubiquitin (Gaspar *et al.* 1990), no tagging was observed to the 183-kDa protein, indicating the insensitivity of this method, and the improbability of success when using immunogold tagging to seek the 54-kDa protein in tissue sections.

Construction of 54-kDa protein gene derivatives. Starting with the wild-type 54-kDa protein gene sequence in pRTT1 (Golemboski *et al.* 1990) two mutants were prepared by site-directed mutagenesis (Fig. 1). The first derivative, pRTTinit, contained a mutation in the AUG initiation codon intended to prevent synthesis of the 54-kDa protein (Fig. 1). The second derivative, pRTTF/S, contained a frameshift mutation caused by insertion of an extra C residue between TMV nucleotides 3772 and 3773 (Fig. 1) that would lead to premature termination of protein synthesis yielding a protein only 20% as long as the wild-type 54-kDa protein.

In principle, both mutants were expected to be capable of generating transcripts of the same length and nucleotide sequence with only one nucleotide difference from the wild-type 54-kDa protein gene sequence. *In vitro*-synthesized T7 transcripts of these clones were translated in cell-free wheat-germ extracts (Fig. 2). As expected, the major translation product of the transcript of pRTTF/S was a protein of less than 14 kDa. Surprisingly, the transcript of pRTTinit directed the synthesis of a protein of approximately 50–52 kDa, only slightly smaller than the wild-type 54-kDa protein encoded by pRTT1 (Fig. 2). A similar result was found using a rabbit reticulocyte *in vitro* translation system (data not shown). Examination of the sequence of pRTTinit, however, indicated that translation was most likely initiated (in-frame) on a downstream AUG codon at TMV nucleotide 3537 (Fig. 1; Goelet *et al.* 1982), yielding a protein lacking only the first 14 *N*-terminal amino acids of the wild-type 54-kDa protein, consistent with the result of the *in vitro* translation experiment.

The DNA inserts of pRTT1, pRTTinit, and pRTTF/S were subcloned into the polylinker region of the transient expression vector pFF19 (Timmermans *et al.* 1990) between the *Kpn*I and *Pst*I sites to produce, respectively, pFF1954, pFF1954init, and pFF1954F/S (Fig. 1).

Development of a transient gene expression assay system in tobacco protoplasts. The efficiency of uptake of DNA into protoplasts was assessed by electroporation with pFF19G (Timmermans *et al.* 1990) containing the ORF for GUS (Jefferson 1987), and efficiency of infection with TMV RNA was assessed immunologically with anti-TMV CP serum. Unfortunately, as is common in protoplast experiments (Lepetit *et al.* 1991), there was variation in the viability of different batches of protoplasts, in nucleic acid uptake, and in protoplast tolerance to electroporation. Initially, some experiments were attempted in which protoplasts were first electroporated with DNA followed 12–15 hr later by a second electroporation with TMV RNA. However, most batches of protoplasts were insufficiently robust to survive this regime. On the other hand, in about two-thirds of our experiments using simultaneous electroporation with DNA and RNA, batches of protoplasts were obtained in which survival was between 10 and 50% and in which substantial nucleic acid uptake occurred.

To determine whether the protoplasts were capable of expressing the 54-kDa protein gene and its derivatives, the protoplasts were electroporated with the DNA constructs pFF1954, pFF1954init, or pFF1954F/S. Northern analysis of total RNA extracted from the protoplasts 24 hr post-electroporation showed, as expected, that all three

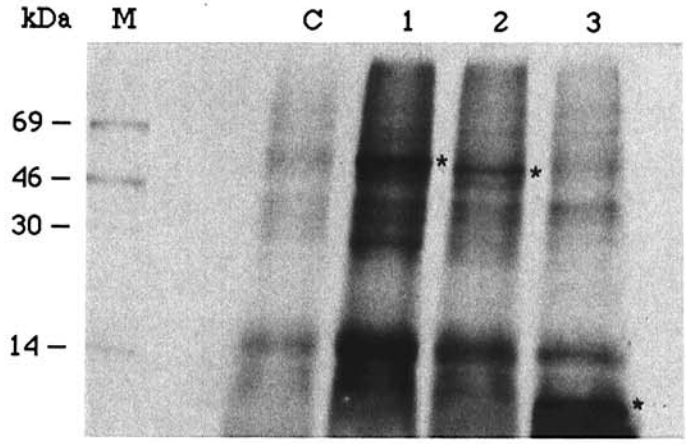

Fig. 2. Transcription/translation analysis of wild-type and mutant 54-kDa protein gene sequences. T7 transcripts of pRTT1 (lane 1; encoding the unmodified 54-kDa protein gene sequence), pRTTinit (lane 2; sequence with first initiation codon eliminated), and pRTTF/S (lane 3; frameshift mutation) were used to program wheat germ *in vitro* translation extracts. ^{35}S-labeled *in vitro*-synthesized polypeptides were analyzed by electrophoresis on an SDS/17.5% polyacrylamide gel and visualized by autoradiography. The *in vitro*-synthesized wild-type and mutated versions of the 54-kDa protein are marked with *. Lane C (control) was loaded with a wheat germ extract in which water was substituted for RNA transcripts. Lane M was loaded with ^{14}C-labeled protein molecular weight standards (Amersham).

constructs generated transcripts of approximately the same length as the 54-kDa protein gene transcript found in 54-kDa transgenic plants (Fig. 3).

Expression of resistance in protoplasts electroporated with DNA constructs containing wild-type or mutated 54-kDa protein gene sequences. Because of the problems of variability in protoplast experiments, electroporation with all constructs was carried out in duplicate or more usually in triplicate. Additionally, all experiments included control transfections in which pFF19G was electroporated into protoplasts simultaneously with TMV RNA. Because it was necessary for the success of the transient expression experiments to obtain a protoplast population in which a majority of cells expressed the DNA sequences, GUS staining was important in providing a measure of DNA uptake. Thus, if fewer than 50% of surviving cells exhibited GUS staining the experiment was abandoned. Initial experiments were aimed at determining whether the construct containing the wild-type 54-kDa protein gene sequence, pFF1954, was able to confer resistance on protoplasts. It was found that protoplasts electroporated with TMV U1 RNA and pFF1954 supported less TMV replication (as assessed by northern blot analysis of TMV-specific RNAs) than protoplasts electroporated with TMV U1 RNA and pFF19G (Fig. 4). Counting of the radioactive probe bound to the ss- and dsRNA bands corresponding to the subgenomic LMC (CP) mRNA (data not shown) showed two- to fivefold less TMV replication in protoplasts electroporated with pFF1954 versus those electroporated with pFF19G. In contrast, electroporation with pFF1954 appeared to cause little or no reduction of replication of TMV U2 (Fig. 4). These results indicated that the 54-kDa protein gene sequence could produce resistance in protoplasts in a transient expression system and that the resistance was specific for TMV U1, as it is in stably transformed plants and protoplasts (Golemboski *et al.* 1990; Carr and Zaitlin 1991). The difference in TMV replication between resistant (pFF1954-transfected) and nonresistant (pFF19G-transfected) is small compared to that observed between protoplasts derived from transgenic plants harboring the 54-kDa protein gene sequence and nontransgenic plants (20- to 80-fold; Carr and Zaitlin 1991). However, this is not unexpected since the protoplasts consist of a mixed population of transfected and untransfected cells, some of which will have received only viral RNA and which thus will yield wild-type levels of virus.

When protoplasts were electroporated with pFF1954init there was a similar suppression of virus replication to that observed in protoplasts electroporated with pFF1954 (Fig. 5). However, in protoplasts electroporated with pFF1954F/S there appeared to be little or no suppression of TMV replication (Fig. 5). Thus, although all the constructs were capable of generating full-length transcripts corresponding to the 54-kDa gene sequence (Fig. 3), only those with the potential to direct synthesis of full-length or close-to-full-length 54-kDa proteins (Fig. 2) conferred resistance to TMV. This implies that expression of the 54-kDa protein gene sequence at the RNA level alone is insufficient for resistance and implies a role for the 54-kDa protein in resistance.

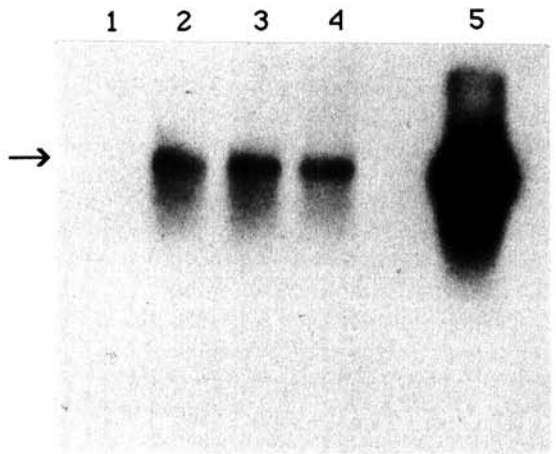

Fig. 3. Expression of 54-kDa protein gene sequence-specific RNAs in protoplasts. Protoplasts were electroporated with pFF19G (lane 1; GUS control), pFF1954 (lane 2; wild type 54-kDa protein gene sequence), pFF1954F/S (lane 3; frameshift mutant), and pFF1954init (lane 4; first initiation codon eliminated). RNA was extracted from protoplasts and analyzed by northern blot analysis using as probe a ^{32}P-labeled, T3 transcript of pRTT1 specific for transcripts of the 54-kDa protein gene sequence. The position of the 54-kDa protein gene sequence-specific RNAs is indicated with an arrow. For comparison, lane 5 was loaded with RNA extracted from leaves of a transformed tobacco plant harboring the 54-kDa protein gene sequence (line 5413; Golemboski *et al.* 1990).

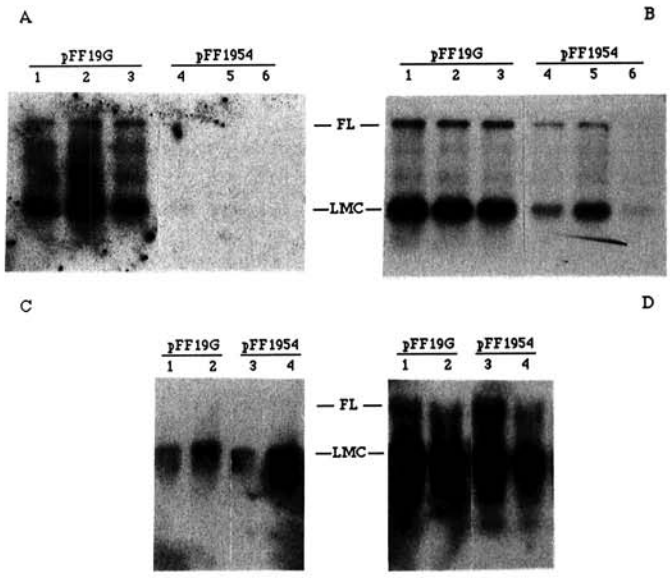

Fig. 4. Replication of TMV in protoplasts transiently expressing the 54-kDa protein gene sequence. Duplicate or triplicate samples of protoplasts were electroporated simultaneously with TMV U1 (panels A and B) or TMV U2 RNA (panels C and D) together with pFF19G (GUS control; lanes 1–3 of panels A and B, lanes 1–2 of panels C and D) or pFF1954 (containing the 54-kDa protein gene sequence; lanes 4–6 of panels A and B, lanes 3–4 of panels C and D). After incubation for 24 hr, single strand-enriched (A and C) and double strand-enriched (B and D) RNAs were extracted from the protoplasts and subjected to northern blot analysis. (+) sense TMV U1 RNAs were detected by hybridization with a ^{32}P-labeled, SP6 transcript of pSP64CP (A and B). (+) sense TMV U2 RNAs were detected by hybridization with (^{32}P)-labeled cDNA generated by reverse transcription of TMV U2 RNA (C and D). Positions of full-length genomic TMV RNA (FL) and coat protein mRNA (LMC) are indicated.

DISCUSSION

The aim of this study was to determine whether or not the 54-kDa protein itself has a direct role in mediating resistance to TMV in transgenic plants harboring the 54-kDa protein gene sequence. We tried to develop an antibody-based method for detection of the 54-kDa protein. However, none of the antisera raised against either 54- or 183-kDa protein sequences proved sufficiently sensitive for our purposes, and we decided to employ an indirect strategy for assessing the role of the 54-kDa protein. The approach chosen was to introduce site-specific mutations into the 54-kDa protein gene sequence and test the mutated genes *in vivo* for their ability to confer resistance to TMV.

To expedite the study, the mutated 54-kDa protein gene sequences were tested using a protoplast-based transient expression system rather than by using them to transform whole plants. By using electroporation conditions and relative nucleic acid concentrations described in Materials and Methods, it was possible to obtain 50–90% of surviving protoplasts taking up DNA and 5–20% taking up viral RNA. An excess of cells expressing DNA sequences over those infected with virus was required for the success of the transient expression experiments. In this way it could be assumed that the majority of virus-infected cells also contained a 54-kDa protein gene or derivative so that any effect of the construct on virus replication could be observed more easily. In contrast, if virus-infected cells outnumbered cells harboring a DNA construct, any reduction in replication occurring in a few protoplasts would be masked by the overwhelming production of virus in the protoplasts transfected with the viral RNA alone. We found that in protoplasts electroporated with either of two constructs, pFF1954 or pFF1954init, which encode, respectively, a full-length and a close-to-full-length 54-kDa protein, TMV replication was suppressed more than in protoplasts coelectroporated with a control plasmid (pFF19G). However, a frameshift mutant, pFF1954F/S, which encodes a polypeptide representing only the N-terminal 20% of the native 54-kDa protein did not suppress TMV replication in protoplasts. These results suggest that the 54-kDa protein itself is indeed involved in mediating resistance to TMV.

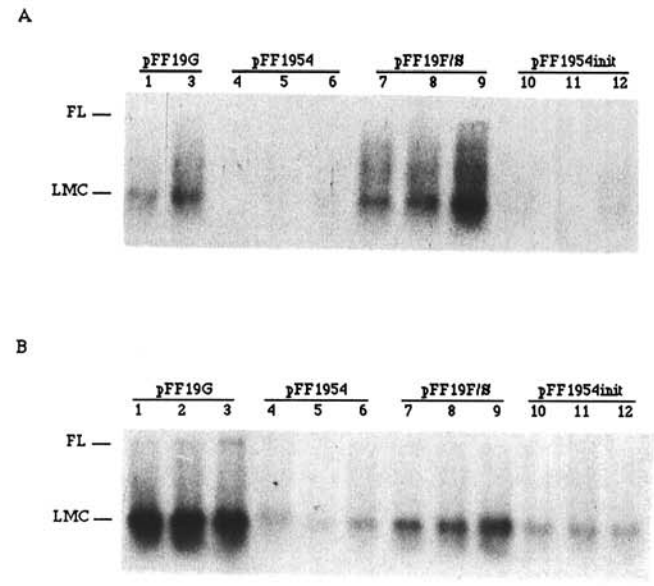

Fig. 5. Replication of TMV U1 in protoplasts transiently expressing mutants of the 54-kDa protein gene sequence. Protoplasts were electroporated simultaneously with TMV U1 RNA together with pFF19G (GUS; lanes 1–3), pFF1954 (wild-type 54-kDa; lanes 4–6), pFF1954F/S (frameshift mutant; lanes 7–9) or pFF1954init (initiation codon mutant; lanes 10–12). After incubation for 24 hr corresponding single strand-enriched (A) and double strand-enriched (B) RNAs were extracted from the protoplasts, subjected to northern blot analysis and probed for the presence of (+) sense TMV U1 RNAs as described for Figure 4. Positions of full-length genomic TMV RNA (FL) and coat protein mRNA (LMC) are indicated. The ss enriched RNA loaded onto lane 2, panel A was degraded and has been omitted from the figure, the ds enriched RNA from the same set of protoplasts (lane 2, panel B) is shown.

In our previous paper (Carr and Zaitlin 1991), we demonstrated that the resistance to TMV induced by expression of the 54-kDa protein gene sequence acts at the level of the single cell by suppressing virus replication. At that time it was not possible to distinguish between the contribution to resistance of the 54-kDa protein versus its RNA. However, now that the 54-kDa protein itself has been implicated in resistance, and given that it contains several sequence motifs characteristic of replicase proteins (Argos 1988; Habili and Symons 1989; Bruenn 1991) it is possible to suggest two models for the mechanism for the resistance phenomenon.

1. In the first model one might suggest that the 54-kDa protein is an authentic replicase component. It might function as a regulatory subunit perhaps controlling (+) versus (−) sense strand synthesis or by suppressing replication in general. Thus, its synthesis in inappropriate amounts or with inappropriate timing in the transgenic plant cell could disrupt replicase activity.

2. In the second potential model, the 54-kDa protein, rather than being a natural replicase subunit, is a truncated, defective version of the 183-kDa replicase protein. It would act to inhibit replicase activity by competing with the 183-kDa protein during replicase assembly. This second type of mechanism would represent a form of "dominant negative" mutation for the disruption of gene function as described by Herskowitz (1987).

Is it possible to say which of the two potential models best describes the mechanism underlying the 54-kDa protein-mediated resistance to TMV? Until the 54-kDa protein is detected in virus-infected tissue, or until experiments with an *in vitro* replicase system for TMV become practical, it will probably not be possible to be absolutely certain that model 1 describes the situation any better than model 2. However, two lines of circumstantial evidence favor model 1. First, the presence on polyribosomes of the I_1 RNA, the putative messenger RNA for the 54-kDa protein, in TMV-infected tobacco (Zelcer *et al.* 1981; Sulzinski *et al.* 1985), suggests that the 54-kDa protein is synthesized in normally infected plant cells. Second, in the case of the animal alphavirus, Sindbis virus, which has a very similar genome organization to TMV, the ORF in Sindbis virus most analogous to the ORF encoding the 54-kDa protein in TMV, has been shown to direct the synthesis of a protein (nsP4) in virus-infected cells (de Groot *et al.* 1990).

Are either of the models applicable to nonstructural gene-mediated resistance in other plant-virus systems? In the

case of resistance to PEBV, only plants transformed with a DNA construct containing an intact ORF for the PEBV 54-kDa protein were resistant to the virus, whereas plants transformed with inadvertently mutated sequences incapable of synthesizing intact 54-kDa protein were not resistant (MacFarlane and Davies 1992). This is consistent with the idea that resistance to PEBV is mediated by protein rather than RNA. Given the similarity of the genome organization of PEBV to TMV it would seem likely that the model which best describes resistance to TMV (model 1?) will also explain resistance to PEBV.

Anderson *et al.* (in press) created CMV-resistant tobacco plants by transformation with a truncated version of the replicase protein gene sequence encoded by CMV RNA2. However, the mechanism of resistance to CMV may differ from that of the 54-kDa protein-mediated resistance to TMV. CMV has an entirely different genome organization from TMV or PEBV and does not express the protein sequences analogous to the TMV 54-kDa protein via a read-through mechanism. If it can be shown that this defective nonstructural gene sequence is expressed at the protein level, model 2 would provide the most plausible explanation of replicase-mediated resistance to CMV.

Although replicase gene-mediated resistance has been demonstrated to TMV, PEBV, and CMV, simply transforming plants with replicase sequences may not always engender resistance to viruses. For example, expression of full-length RNA2 sequences from alfalfa mosaic virus in transgenic tobacco plants does not render them resistant to that virus (van Dun *et al.* 1988), and protoplasts from transgenic plants expressing full-length RNA2 sequences of brome mosaic virus do not resist brome mosaic virus replication (Mori *et al.* 1992). In fact, in both of these cases the viral genes acted *in trans* to support replication of defective viral genomes. Thus, where the approach is initially unsuccessful it may be necessary to remove or modify specific sequences from the nonstructural protein gene to give a nonfunctional replicase which can confer resistance against a virus by the mechanism described in model 2. The further analysis of viral replicase gene sequences by expression in transformed plants and protoplasts will assist in the development of replicase gene-mediated protection as a practical approach to plant protection as well as to enhance our knowledge of the viral replication process.

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