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Plants transformed with a region of the 201-kilodalton replicase gene from pea early browning virus RNA1 are resistant to virus infection

(54-kilodalton protein open reading frame-mediated resistance/virus resistance/tobravirus)

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ABSTRACT **The 3′ proximal portion of the gene encoding the 201-kDa putative replicase protein from the *Tobravirus* pea early browning virus (PEBV) can potentially be expressed separately as a 54-kDa protein. *Nicotiana benthamiana* plants transformed with the open reading frame (ORF) encoding the 54-kDa protein, designated *54K* ORF, were resistant to infection by purified PEBV at inoculum doses of up to 1 mg/ml, the highest concentration tested. However, resistance was abolished by the introduction into the *54K* ORF of mutations that would cause premature termination of translation. This suggests that the resistance mechanism requires the involvement of an intact 54-kDa protein. The *54K* ORF-transformed plants were also resistant to infection by broad bean yellow band virus and an uncharacterized isolate of British PEBV (PGRO R) but were not resistant to infection by two other tobraviruses, pepper ringspot virus and the I6 isolate of tobacco rattle virus. Additionally, two variants of PEBV which overcame *54K* ORF-mediated resistance have been isolated, the analysis of which might provide important information about both the resistance mechanism itself and the process of normal virus replication.**

Pea early browning virus (PEBV) is a member of the *Tobravirus* group of plant viruses, which have a genome consisting of two positive-sense RNAs separately encapsidated in rod-shaped particles. Analysis of the sequence of the viral RNAs revealed that the genetic organization of the larger RNA (RNA1) of PEBV resembles that of the monopartite virus tobacco mosaic virus (TMV) (1, 2). PEBV RNA1 encodes two proteins with molecular masses of 141 and 201 kDa, which share amino acid sequence homology with the TMV 126-kDa and 183-kDa replicase proteins. These proteins are NH_2-coterminal, the larger protein being expressed by the read-through of an opal (UGA) termination codon at the COOH terminus of the 141-kDa protein (1, 3). Additionally, PEBV RNA1 encodes a 30-kDa protein with homology to the TMV 30-kDa cell-to-cell spread protein. However, whereas the 3′ proximal gene of TMV encodes the virus coat protein, this region in PEBV RNA1 encodes a cysteine-rich 12-kDa protein. PEBV coat protein is encoded by the 5′ proximal gene of RNA2, which also encodes putative 29.6-kDa and 23-kDa proteins of unknown function (4).

Closer inspection of the PEBV RNA1 sequence revealed that the COOH-terminal region of the 201-kDa protein could potentially be expressed separately as a 54-kDa protein by translation of an internal open reading frame (ORF) initiating at an AUG codon situated 147 nucleotides (encoding 49 amino acids) downstream of the termination codon (nucleotide 3866) for the 141-kDa protein. The PEBV ORF encoding the 54-kDa protein, designated *54K* ORF,† encodes amino acid sequences that are highly conserved among the RNA-dependent RNA polymerases from a wide range of plant, animal, and bacterial viruses. Several studies have presented detailed alignments of these proteins and identified a number of highly conserved sequence motifs that presumably are regions of common functional importance (5, 6). However, at present there are no data to confirm the expression of the PEBV *54K* ORF at either the protein or RNA level.

The concept of parasite-derived resistance has been described in which it was proposed that the transformation of a host organism with a nucleic acid sequence, such as a portion of a replicase gene, derived from a pathogen of that host might selectively interfere with the multiplication of the pathogen without having a deleterious effect on the host (7). The success of this approach has been shown with the demonstration that plants transformed with the homologous *54K* ORF from TMV were resistant to subsequent infection by very high concentrations of virus or viral RNA (8). In this respect *54K* ORF-mediated resistance appears to be superior to coat protein-mediated resistance (9). In an attempt to discover whether *54K* ORF-mediated resistance might be applicable to viruses other than TMV and to provide an insight into the mechanism of resistance, we transformed *Nicotiana benthamiana* plants with the *54K* ORF from PEBV. The generation and subsequent analysis of these transgenic plants is discussed.

METHODS

Clone Construction. Restriction enzymes were used according to the manufacturers' instructions. Recombinant DNA procedures were carried out by standard methods. The PEBV *54K* ORF (nucleotides 4013–5425) carried on the plasmid pT7FL3 (10) was subjected to PCR amplification with *Taq* polymerase (AmpliTaq; Perkin–Elmer/Cetus). The upstream oligonucleotide used for the amplification was 5′-GGGTACCACGAGCCTTGAC-3′, which inserts a *Kpn* I restriction site adjacent to nucleotide 3994 of the virus sequence. The downstream oligonucleotide was 5′-CGAGCTCCCAACTGGGATT-3′, which inserts an *Sst* I site adjacent to nucleotide 5473. Amplification was carried out in a 50-μl reaction mixture containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 9.3), 200 μM each dNTP, 20 pmol of each primer, and 2.5 units of *Taq* polymerase. The DNA was denatured by incubation at 93°C for 7 min; amplification was at 93°C for 1 min, 55°C for 1 min, and 72°C for 2 min, repeated for 25 cycles. The PCR products were precipitated with

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Abbreviations: PEBV, pea early browning virus; TMV, tobacco mosaic virus; PRV, pepper ringspot virus; BBYBV, broad bean yellow band virus; TRV, tobacco rattle virus; p.i., postinoculation; ORF, open reading frame.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M90705).

ethanol, digested with *Kpn* I and *Sst* I, extracted with phenol/chloroform mixtures, reprecipitated, and ligated into similarly digested transformation vector pRok2 (11). Recombinants were selected in *Escherichia coli* strain JM83, and DNA minipreps were checked by restriction mapping. Three clones (54.1, 54.2, and 54.3) were mobilized into *Agrobacterium tumefaciens* strain LBA4404 by standard methods (12). The *54K* ORF was subcloned from each of the three clones into the vector M13mp18 prior to DNA sequencing (13).

DNA Sequence Analysis. DNA sequence assembly and analysis was carried out by using University of Wisconsin Genetics Computer Group programs (14).

Preparation of Virus Inoculum. *N. benthamiana* plants infected with the SP5 isolate of British PEBV-B were harvested 10 days postinoculation (p.i.) and homogenized in 30 mM sodium phosphate buffer (pH 7.0) (1 g wet weight per ml of buffer). Aliquots were stored frozen at −20°C until required. Subsequently, the frozen homogenate was thawed to room temperature and diluted in an equal volume of sodium phosphate buffer before inoculation onto plants. Purified virus was isolated from PEBV-infected *N. benthamiana* plants by the method of MacFarlane *et al.* (1). Pepper ringspot virus (PRV) was a gift from A. Siegel (Wayne State University). Broad bean yellow band virus (BBYBV), the I6 isolate of tobacco rattle virus (TRV), and the PGRO R isolate of PEBV were gifts from D. Robinson (Scottish Crop Research Institute, Invergowrie). Inocula containing these viruses were prepared exactly as described above for PEBV-B.

Plant Transformation. *N. benthamiana* leaf discs were transformed by using the procedure of Horsch *et al.* (15). Transformed calli were selected by growth on medium containing kanamycin at 100 μg/ml. Primary transformed plants (t^1) were potted into a peat-based medium and transferred to the greenhouse. The plants were self-fertilized, and seeds (t^2) from each were collected and germinated in Petri dishes containing 2% agarose, MS salts (16), and kanamycin at 500 μg/ml. After 6 days, unbleached seedlings were potted as above. Plants at the six-to-eight leaf stage were inoculated with a homogenate of *N. benthamiana* infected with PEBV strain SP5.

Nucleic Acid Extractions. *Small-scale preparation.* RNA was extracted from leaf discs taken from "systemic" leaves (younger than the inoculated leaf) by using the method of Verwoerd *et al.* (17) and was collected by LiCl precipitation. Samples were analyzed by electrophoresis through formaldehyde/agarose gels (18), transferred to nitrocellulose, and probed with PEBV-specific cDNA labeled with [^{32}P]dCTP as described (19).

Large-scale preparations. Leaves were frozen in liquid nitrogen, ground to a powder in a precooled mortar, and repeatedly extracted with phenol/SDS buffer (17). Total nucleic acids were collected by precipitation with sodium acetate/ethanol and resuspended in sterile distilled water. RNA was recovered by two precipitations with LiCl followed by an additional precipitation with ethanol and was analyzed as described above. Total DNA (the LiCl-soluble fraction) was reprecipitated from ethanol, resuspended in 100 mM NaCl/1 mM EDTA/10 mM Tris chloride, pH 7.6, and digested with DNase-free RNaseA at 0.1 mg/ml. After RNase digestion, the DNA was extracted with 1:1 (vol/vol) phenol/chloroform, collected by precipitation with ethanol, and analyzed by Southern blotting (20).

Analysis of *54K* ORF mRNA. *54K* ORF mRNAs were identified by RACE PCR by the method of Frohman *et al.* (21). Total RNA from both untransformed and *54K* ORF transgenic plants was hybridized to the 3′ oligonucleotide primer 5′-GACTCGAGTCGACATCGA(T_{17})-3′. cDNA was synthesized by using Superscript reverse transcriptase (GIBCO/BRL) according to the manufacturer's instructions. *54K* ORF-specific cDNA was amplified by using the oligonucleotides 5′-GGATCCTGTGAAAACTG-3′ (nucleotides 5110–5126) and 5′-GACTCGAGTCGACATCG-3′ with a 94°C 4-min initial denaturation followed by 40 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. PCR products were analyzed by probing DNA blots with [^{32}P]dCTP-labeled *54K* ORF-specific cDNA.

RESULTS

Regeneration of Transformed Plants. In an initial experiment, 11 primary transformed plants (t^1) were successfully rooted. Four were derived from clone 54.1 (lines 3, 5, 7, and 9), six were derived from clone 54.2 (lines 1, 2, 6, 8, 10, and 11), and one plant was derived from clone 54.3 (line 4). All 11 plants were self-fertilized, and the t^2 progeny were tested for resistance to PEBV infection. Subsequently, more detailed molecular studies were carried out with progeny from lines 7 (54.1), 6 (54.2), and 4 (54.3). In a second transformation experiment, an additional two lines (12 and 13) containing clone 54.3 were generated.

Plants Transformed with Clone 54.3 Are Resistant to Infection by PEBV. Seeds (t^2) from line 4 (54.3) were germinated on medium containing kanamycin. All seedlings exhibited kanamycin resistance, indicating that the primary transformant contained multiple inserts. At the outset 19 plants were inoculated with purified virus at a range of concentrations extending from 50 μg/ml to 1 mg/ml. Fourteen plants (75%) were resistant to infection, including four of five plants inoculated with the highest inoculum concentration used (1 mg/ml). In later experiments plants were challenged with a

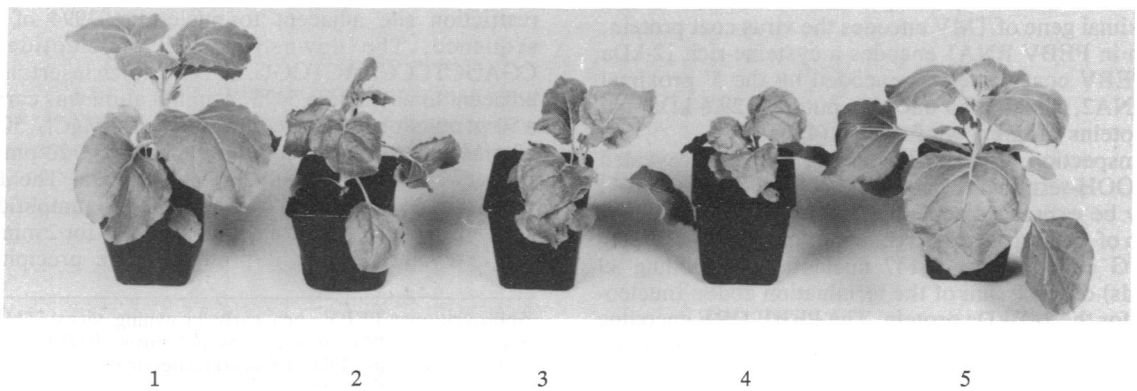

FIG. 1. The effect of inoculation with PEBV on *N. benthamiana*. Plants: 1, uninoculated, 54.3-transformed; 2, untransformed, inoculated; 3, inoculated, 54.1-transformed; 4, inoculated, 54.2-transformed; 5, inoculated, 54.3-transformed.

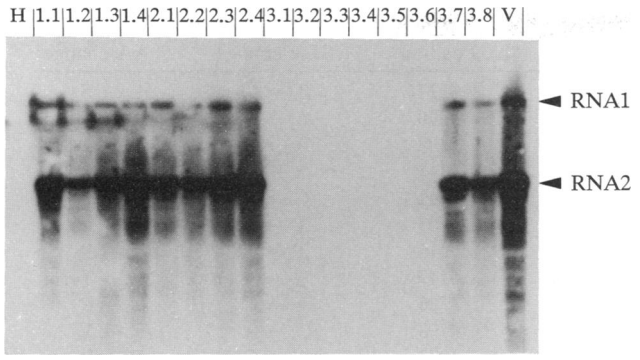

FIG. 2. Northern blot of plants inoculated with PEBV. Lanes: H, RNA from an uninoculated, untransformed plant; 1.1–1.4, 54.1-transformed plants; 2.1–2.4, 54.2-transformed plants; 3.1–3.8, 54.3-transformed plants; V, an untransformed, inoculated plant. Arrows mark the positions of PEBV RNAs 1 and 2. The blot was probed with full-length cDNA of both viral RNAs.

homogenate of infected plants that reproducibly gave 100% infection when used to inoculate control plants.

In another experiment, eight kanamycin-resistant seedlings transformed with clone 54.3 were inoculated with virus and transferred to the greenhouse. Mild disease symptoms (curling of upper leaves, slight mosaic) appeared 4 days p.i. on two of the plants, which showed clear disease symptoms 9 days p.i., while the remaining six inoculated plants were completely free of symptoms (Fig. 1). Leaf discs (17-mm diameter) were taken from expanding leaves of all eight plants, and a sample of RNA from each plant was analyzed by Northern blotting. PEBV RNA was present in the two plants (54.3.7 and 54.3.8) that showed disease symptoms, whereas the six symptom-free plants (3.1–3.6) were not infected with PEBV (Fig. 2). Five of the PEBV-resistant t^2 plants (54.3.2, 54.3.3, 54.3.4, 54.3.5, and 54.3.6) were self-fertilized to produce t^3 progeny. When challenged with virus, 7 of 34 (20%) 54.3 t^3 plants became infected.

By analogy to the previously reported studies of plants transformed with the TMV *54K* gene, it might be expected that the resistance of the progeny of line 4 to infection by PEBV was attributable directly to the PEBV *54K* sequence rather than to the insertion of exogenous DNA into a region of the plant genome that is required for virus replication. The former hypothesis was confirmed by the generation, in a second series of transformations, of an additional two plants containing clone 54.3 (lines 12 and 13) that were resistant to infection when inoculated with a homogenate of PEBV-infected plants.

Southern Blot and RACE PCR Analysis of 54.3 Transgenic Plants. A further 20 54.3 t^2 seedlings were selected for more detailed analysis. DNA was extracted from each plant and

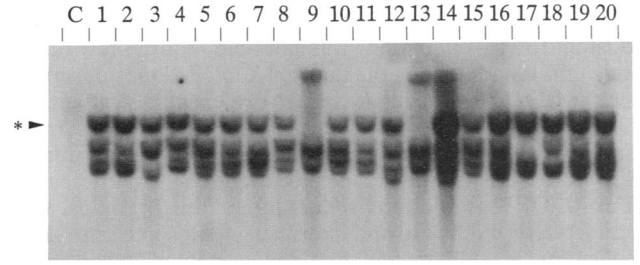

FIG. 3. Southern blot of genomic DNA from 20 54.3-transformed plants. Each lane contained 5 μg of DNA digested with *Hin*dIII. Lane C contained 5 μg of DNA from an untransformed control plant. The asterisk indicates the position of the 5.5-kilobase fragment that is absent from the virus-susceptible plants 9 and 13. The blot was probed with *54K*-specific cDNA.

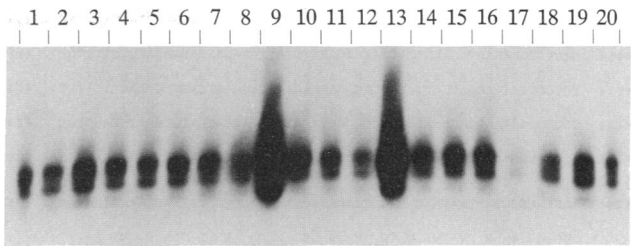

FIG. 4. Southern blot of PCR-amplified 54K ORF cDNA from 20 54.3-transformed t^2 plants. The blot was probed with *54K*-specific cDNA. The two bands in each lane are single-stranded and double-stranded DNA molecules, which can be differentiated by treatment with mung bean nuclease (data not shown; see ref. 21).

analyzed by Southern blotting. Digestion with *Hin*dIII, which cuts the integrated DNA at a single site upstream of the cauliflower mosaic virus promoter, revealed that each plant was transformed with two or more copies of the *54K* ORF (Fig. 3). Expression of the *54K* ORF in the 54.3 t^2 plants was analyzed by 3′ RACE PCR amplification of cDNA synthesized from total RNA isolated from these plants. A *54K*-specific 5′ primer was selected to permit amplification of a 365-base-pair (bp) cDNA fragment [not including the mRNA poly(A) tail] derived from the 3′ terminus of the *54K* ORF. Transcription of the *54K* ORF was detected in each plant (Fig. 4). All 20 plants appeared to express the *54K* ORF at similar levels, with the exceptions of plants 9 and 13, which had much higher levels of *54K* message, and plant 17, which had a lower level of *54K*-specific mRNA. As an additional control, total RNA from a virus-resistant plant (54.3.1) was included in a cDNA synthesis reaction in the absence of reverse transcriptase. PCR amplification of this mixture did not produce a *54K* DNA fragment, showing that the 3′ RACE technique was not amplifying residual genomic DNA or virus inoculum RNA (data not shown).

Plants Transformed with Clones 54.1 and 54.2 Are Not Resistant to Infection. Progeny from all 10 lines transformed with clones 54.1 and 54.2 were not resistant to infection by PEBV regardless of whether the inoculum was purified virus, virion RNA, or virus-infected plant homogenate. Southern blot analysis of lines 6 and 7 showed these plants to be transformed with PEBV *54K* DNA; moreover, RACE PCR showed these plants to contain *54K*-specific mRNA (data not shown).

Sequence Analysis Revealed the Presence of Premature Termination Codons in Clones 54.1 and 54.2. The PCR-amplified PEBV *54K* ORF was inserted between the *Kpn* I and *Sst* I restriction sites of the plant transformation vector pRok2. Three clones (54.1, 54.2, and 54.3) were isolated from this procedure and used directly to transform *N. benthamiana*. The *54K* ORF from each clone was subsequently transferred to M13mp18 for DNA sequencing. Sequence analysis showed that the PCR amplification procedure had introduced a number of nucleotide changes in the three clones (Fig. 5, Table 1).

Clone 54.1 contains three base changes when compared with the sequence obtained from the cDNA clone used for PCR amplification. Two of the base changes result in amino acid substitutions in the 54-kDa protein, while the third results in premature termination of translation, giving a truncated protein lacking 12 amino acids (2.55%) at the COOH terminus. Clone 54.2 has five base changes, four resulting in amino acid substitutions and one resulting in premature termination of translation. The truncated protein lacks 93 amino acids (19.7%) at the COOH terminus. Clone 54.3 has six base changes, three introduce amino acid substitutions, while three are silent third-base changes.

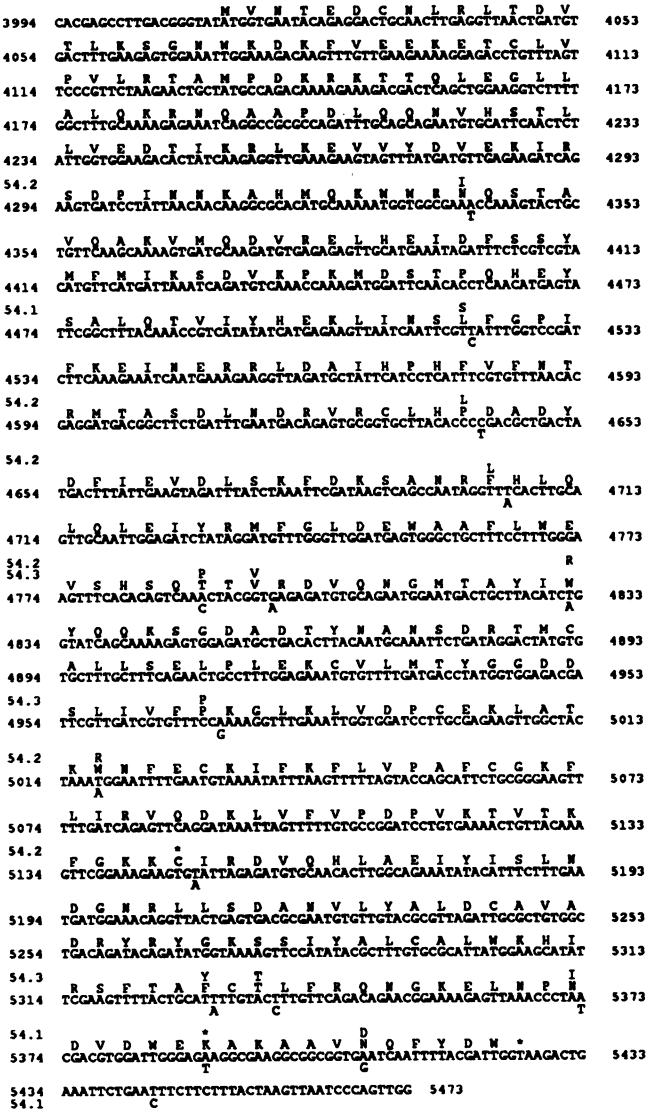

FIG. 5. Nucleotide sequence of the *54K* gene. The contiguous sequence is wild-type *54K*, and the numbers are from the sequence of a full-length DNA copy of PEBV RNA1. Amino acid residues that differ from wild-type appear above the amino acid sequence line (in single-letter code). Nucleotide changes that cause the amino acid differences appear below the nucleotide line. Asterisks (*) denote termination codons. The *54K* clones containing each particular sequence change are indicated to the left of each line.

Variants of PEBV that Overcame *54K* ORF-Mediated Resistance. The 20 54.3 t^2 plants described above were inoculated with PEBV and regularly examined for symptoms of infection. Plants 9 and 13 showed clear signs of virus infection (leaf curling and mild mosaic formation) 6 days p.i. Leaf discs were taken from the uppermost leaves of each plant 9 days p.i., and RNA extracted from these leaf samples was examined by Northern blotting. In agreement with the observable symptoms, plants 9 and 13 contained high levels of viral RNA. In addition, extended exposure of the autoradiograph revealed a small amount of viral RNA in plant 19 (Fig. 6). Symptoms of infection were detectable on plant 19 by day 16 p.i. The plants were maintained until day 34 p.i., when plants 19 and 17 showed extensive symptoms of infection. However, these symptoms differed from the normal symptoms of leaf/stem curling and mosaic formation (as exhibited by plants 9 and 13) in that both plants 17 and 19 had black necrotic regions on the leaves (especially around the leaf midrib) and discoloration of the stem. Northern blotting

Table 1. Base changes in clones 54.1, 54.2, and 54.3

Clone	Position	Base change	AA change
54.1	4521	T → C	L → S
	5390	A → T	K → Term
	5408	A → G	N → D
54.2	4341	A → T	N → I
	4641	C → T	P → L
	4705	T → A	F → L
	5018	T → A	W → R
	5149	T → A	C → Term
54.3	4790	A → C	T → P
	4798	G → A	V → V
	4972	A → G	P → P
	5331	T → A	F → Y
	5338	T → C	T → T
	5373	A → T	N → I

Term, termination; AA, amino acid.

analysis showed that plants 9, 13, and 19 contained high levels of viral RNA, plant 3 (which exhibited no symptoms of infection) had lower levels of viral RNA, and plant 17 contained very low levels of viral RNA.

The uppermost leaves of plants 9, 13, 17, and 19 were collected, homogenized in buffer, and inoculated onto more 54.3 t^3 seedlings. After 7 days, symptoms of infection were apparent on several plants inoculated with isolates 9 and 13. At the same time all plants inoculated with isolates 17 and 19 exhibited symptoms of infection. RNA samples were extracted from all the plants on day 9 p.i. Northern analysis showed that 2 of 14 and 2 of 13 plants were infected by isolates 9 and 13, respectively. In contrast, 13 of 13 and 14 of 14 plants were infected by isolates 17 and 19, respectively.

Resistance Extends to Other Isolates of PEBV. Plants transformed with clone 54.3 were examined for resistance to other, related viruses. The plants were resistant to infection by an uncharacterized isolate (PGRO R), of British PEBV (D. Robinson, personal communication). These plants were also resistant to BBYBV, which has substantial nucleotide sequence homology with PEBV but is serologically unrelated (22, 23). However, 54.3 plants were not resistant to infection by either PRV (24) or the I6 isolate of TRV (25).

DISCUSSION

The results presented in this study confirm that the normally very susceptible host, *N. benthamiana*, is resistant to infection by PEBV when transformed with the *54K* ORF from the virus. Our results mirror those obtained with TMV (8) in that transformed plants are resistant to very high inoculation doses of virus and suggest that the agricultural potential of *54K* ORF-mediated resistance might be widespread.

In addition, we have shown that plants transformed with a mutant *54K* sequence containing a premature termination codon express *54K*-specific RNA transcripts but are not resistant to virus infection. It is possible that the few nucleotide differences among the three *54K* ORF clones described here (54.1, 54.2, and 54.3) might preferentially affect the secondary structure of 54.1 and 54.2 mRNA and interfere directly with the resistance mechanism. We consider it more likely that *54K*-mediated resistance requires the participation of a *54K* ORF translation product interacting, perhaps, with viral RNA or binding to and interfering with some part of the replication machinery and that the truncated proteins encoded by clones 54.1 and 54.2 are functionally inactive.

While it might be expected that the prematurely terminated protein encoded by clone 54.2 (lacking 93 amino acids) might no longer be functional, it is perhaps more surprising that the product of clone 54.1, which is missing only 12 amino acids at the COOH terminus, also appears to be nonfunctional.

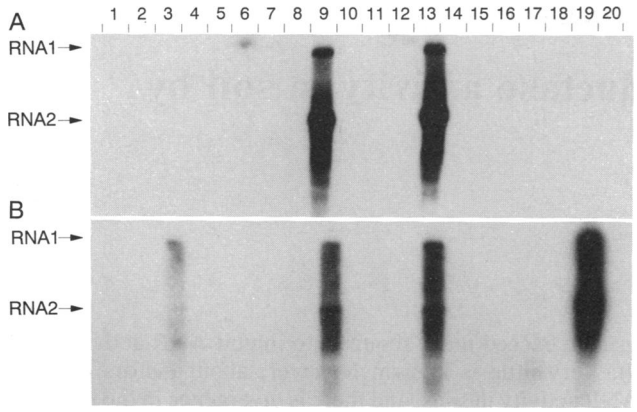

FIG. 6. Northern blot of 20 54.3-transformed plants, inoculated with PEBV and sampled on day 9 p.i. (*A*) and day 34 p.i. (*B*). The blot was probed with full-length cDNA of both viral RNAs. Arrows mark the positions of PEBV RNAs 1 and 2.

Indeed, computer alignments reveal that this region is entirely absent from the putative TMV 54-kDa protein. Both Bruenn (5) and Koonin (6) have identified eight conserved sequence motifs encompassing 21 highly conserved residues in viral RNA-dependent RNA polymerases. While none of the PCR-generated mutations identified in clones 54.1, 54.2, or 54.3 affects these residues directly, the mutation in clone 54.1 at position 4521 does lie within motif 2, as defined by Koonin, which includes 7 conserved hydrophobic residues and an absolutely conserved proline within a span of 23 amino acids. Possibly this mutation might disrupt the structural or functional integrity of that region and that this alteration might be responsible for the disability of the 54.1 protein.

Of the 20 54.3 plants analyzed in detail here, 2 were not resistant, even though they were transformed with *54K* DNA and were expressing high levels of the 54K mRNA. These plants were shown to lack a particular copy of the *54K* ORF (Fig. 3), which suggests that the products expressed from the other integrated *54K* ORFs were nonfunctional. Although we cannot at present explain this phenomenon, it is possible that during the integration process, these additional copies of the *54K* DNA underwent alteration. Alternatively, the two virus-sensitive plants, having fewer integrated copies of the *54K* gene, might not be expressing adequate amounts of the protein for the resistance mechanism to operate.

The TMV studies mentioned above reported that plants transformed with the *54K* ORF from TMV strain U1 were not resistant to infection by the only distantly related TMV strains L and U2. In this study we have shown that plants transformed with the *54K* ORF from the SP5 isolate of British PEBV were resistant to infection by other isolates of PEBV. The isolate PGRO R is only partially characterized, although it is a British serotype of PEBV and, as such, may be closely related to the SP5 isolate. BBYBV was isolated in Italy and is serologically unrelated to both British and Dutch PEBV. Hybridization studies suggested that BBYBV RNA1 shares substantial sequence identity with British and Dutch PEBV, whereas RNA2 has few sequences in common (23). Transformed plants were not resistant to infection by two other tobraviruses, PRV and the I6 isolate of TRV. The I6 isolate of TRV is anomalous in that it is a naturally occurring recombinant virus. Nucleic acid hybridization studies have shown that RNA1 is similar to TRV, whereas RNA2 has TRV-like sequences at its 5′ and 3′ termini and PEBV-like sequences between the termini (25). Thus, the replicase proteins encoded by this isolate are derived from TRV and can overcome PEBV 54K ORF-mediated resistance.

Prolonged maintenance of inoculated 54.3 plants resulted in the isolation of two virus variants that could overcome PEBV *54K* ORF-mediated resistance. Since the inoculum used in these experiments was derived by repeated passage of virus in greenhouse-maintained plants, there is a possibility that the variants represent contaminants of the virus preparation. However, the virus variants hybridized with PEBV-specific cDNA, and the experiments described above have shown that *54K*-mediated resistance is effective against more than one isolate of PEBV. Thus, we would predict that these viruses (isolates 17 and 19) have undergone mutation to escape the resistance mechanism. Identification of mutations present in isolates 17 and 19 would be extremely informative, providing data about the mechanism of resistance and perhaps giving an insight into the process of virus replication.

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