MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 5, No. 2, pp. 144-153, 1992

Pathogen-Derived Resistance to a Potyvirus: Immune and Resistant Phenotypes in Transgenic Tobacco Expressing Altered Forms of a Potyvirus Coat Protein Nucleotide Sequence

John A. Lindbo[1] and William G. Dougherty[1,2]

[1]Department of Microbiology and [2]Center for Gene Research and Biotechnology, Oregon State University, Corvallis 97331-3804 U.S.A.
Received 11 September 1991. Accepted 5 November 1991.

Transgenic *Nicotiana tabacum* 'Burley 49' plants containing one of six different forms of the tobacco etch virus (TEV) coat protein (CP) nucleotide sequence have been generated. In whole plant studies, R1 and R2 progeny were inoculated mechanically with TEV, and the appearance and severity of symptoms were recorded. Symptom phenotype was altered, ranging from near wild type susceptibility to apparent immunity. Protoplasts derived from wild type and transgenic Burley 49 plant lines were transfected with TEV RNA. Protoplasts from transgenic plants expressing full-length or truncated forms of TEV CP supported virus replication. Protoplasts from certain transgenic plants, producing plus- or minus-sense CP transcripts but no CP, did not support virus replication at wild type levels. A model is proposed to account for these observations.

Additional keywords: coat protein-mediated resistance.

The theory of pathogen-derived resistance (Sanford and Johnston 1985) predicts that a "normal" host-pathogen relationship can be disrupted if the host organism expresses essential pathogen-derived genes. It has been proposed that host organisms expressing pathogen gene products in excess amounts, at the inappropriate developmental stage or in a dysfunctional form, may disrupt the normal replicative cycle of the pathogen and result in an attenuated or aborted infection of the host.

It has been demonstrated that transgenic plants expressing a plant virus coat protein (CP) can be resistant to infection by the homologous virus. This type of pathogen-derived resistance has come to be known as CP-mediated resistance and has been demonstrated for the tobamo-, potex-, cucumo-, tobra-, carla-, poty-, and alfalfa mosaic virus groups (for review see Beachy *et al.* 1990) and more recently for the luteovirus group (Kawchuk *et al.* 1990, 1991). We have undertaken experiments to investigate the efficacy and mechanism of this form of pathogen-derived resistance for the potyvirus group.

The potato virus Y, or potyvirus, family represents a large number of plant viral pathogens that collectively can infect most crop species and compromise crop yield and/or quality (Hollings and Brunt 1981; Matthews 1982; Francki *et al.* 1985). Potyviruses have a single-stranded, "plus sense" RNA (of about 10,000 nucleotides) that has a virus-encoded protein linked to the 5′ end and a 3′ polyadenylate region. A single open reading frame (ORF) codes for a 351,000-Da (351-kDa) polyprotein that is proteolytically processed into mature viral gene products (Dougherty and Carrington 1988). The RNA is encapsidated by approximately 2,000 copies of a CP monomer to form a virion (Hollings and Brunt 1981). The capsid protein is encoded by the sequence present at the 3′ end of the large ORF (Allison *et al.* 1985b)

We have generated a series of transgenic plants expressing either full-length or truncated forms of the CP of the potyvirus tobacco etch virus (TEV). Additionally, transgenic plants were generated that expressed either an antisense (AS) form of the CP RNA sequence or a "sense" strand CP RNA molecule (RC) with a frameshift mutation, rendering it untranslatable. R1 and R2 progeny plants were screened by western and/or northern blot analysis for expression of the transgene. Expressing plant lines were inoculated mechanically with TEV, and symptoms were recorded. Truncated forms of the TEV CP tended to confer greater protection than the full-length CP. Plant lines expressing either AS or RC transcripts were highly resistant. In protoplast transfection studies, all lines that produced CP supported viral replication, whereas some lines that expressed high levels of AS or RC RNA did not support replication at wild type levels. These results suggest that the resistant phenotypes observed function via at least two different mechanisms in these transgenic plant lines.

MATERIALS AND METHODS

All restriction enzymes were purchased from New England Biolabs, unless specified, and used according to manufacturer's instructions. Plasmids were maintained in *Escherichia coli* strains HB101 or TG1. Radioactive isotopes were purchased from New England Nuclear, Westwood, MA. T7 and SP6 RNA polymerases were purchased from Bethesda Research Laboratories, Gaithersburg, MD. Rabbit reticulocyte lysate was purchased from Green

Hectares, Oregon, WI. Plasmid pCGN2113 was a kind gift from Calgene (Davis, CA). *Agrobacterium tumefaciens* (Smith and Townsend) Conn strains A136/pCIB542, pRK2013, pCIB710, and pCIB200 were kind gifts of Ciba Geigy Corporation (Research Triangle Park, NC).

Mutagenesis of pTL37/8595. Plasmid pTL37/8595 (Carrington and Dougherty 1987; Carrington *et al.* 1987) contains a cDNA copy of the genomic sequence of the highly aphid transmissible (HAT) isolate of TEV corresponding to nucleotides (nt) 1–200 and nt 8,462–9,495. The first and last codons of the CP coding region in the TEV genome are nt 8,518–8,520 (Ser) and 9,307–9,309 (opal), respectively. (For numbering of TEV nucleotides, see Allison *et al.* 1986). pTL37/8595 was subjected to *in vitro* site-directed mutagenesis using the method developed by Taylor *et al.* (1985a,b). In all cases, nucleotide changes were confirmed by dideoxy-nucleotide sequencing (Sanger *et al.* 1977).

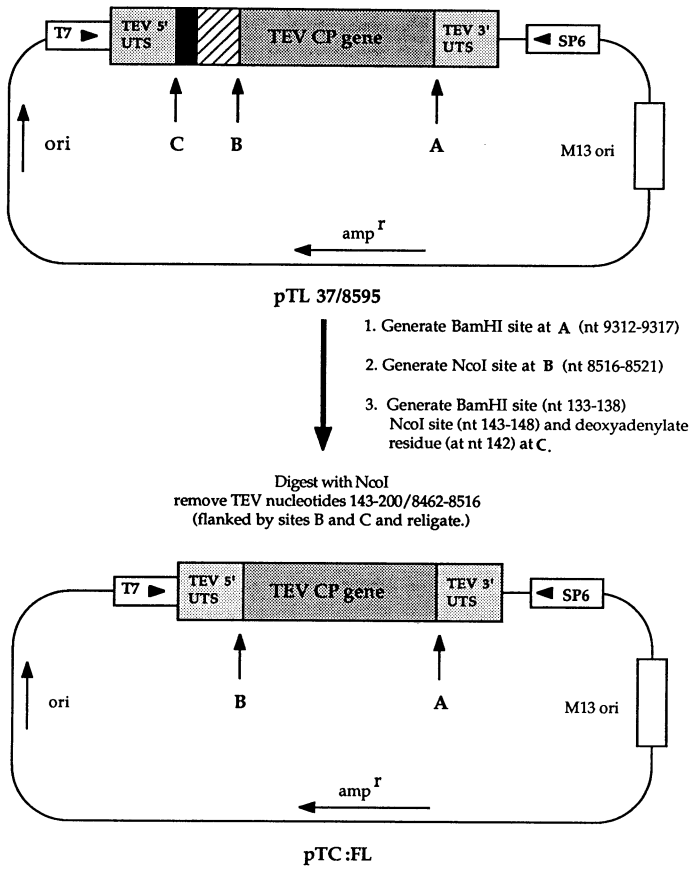

Fig. 1. Schematic representation of pTC:FL and its construction. Restriction endonuclease sites were introduced into pTL37/8595 at positions A, B, and C in above diagram. Following these nucleotide changes the mutated pTL37/8595 was digested with the restriction enzyme *Nco*I, the DNA fragment delineated by the restriction enzyme sites at B and C was removed, and the plasmid was religated to generate pTC:FL. pTC:FL contains the tobacco etch virus (TEV) coat protein (CP) nucleotide sequence flanked by *Bam*HI restriction sites and the TEV 5′ and 3′ untranslated sequences (UTS). T7 and SP6 promoters are also shown. Abbreviations used in this diagram are as follows: T7, T7 RNA polymerase promoter sequence; SP6, SP6 RNA polymerase promoter sequence; ori, origin of replication; M13 ori, bacteriophage M13 single-stranded origin of replication; amp^r, β-lactamase gene. Lightly stippled areas are TEV 5′ and 3′ untranslated sequences; solid black area, TEV genome cDNA nucleotides 144–200; striped area, a portion of the TEV NIb gene (TEV nt 8,462–8,517); heavily stippled areas, cDNA of TEV CP nucleotide sequence (TEV nt 8,518–9,309).

TEV nt 9,312–9,317 were first mutated (Fig. 1) to generate a *Bam*HI restriction site. TEV nt 8,516–8,521 were then altered to generate an *Nco*I site, changing the first codon of the TEV CP coding region from AGT (Ser) to ATG (Met). A single oligonucleotide was then used to mutate TEV nt 133–138 to a *Bam*HI restriction site, nt 143–148 to an *Nco*I restriction site, and nt 142 to a deoxyadenylate residue. These mutations generated an *Nco*I site centered on the first codon of the TEV ORF and in a good translational start context as described by Kozak (1984). Digestion of the resulting plasmid with the restriction enzyme *Nco*I, removing TEV nt 143–200/8,462–8,516, and religation generated plasmid pTC:FL. pTC:FL contained only the TEV CP gene flanked by *Bam*HI restriction sites and TEV 5′ and 3′ untranslated sequences (see Fig. 1).

Plasmid pTC:ΔN29 was constructed as follows: TEV nt 8,600–8,605 in pTC:FL were mutated to an *Nco*I restriction site. Digestion of the resulting plasmid with the restriction enzyme *Nco*I, removal of TEV nt 8,517–8,601, and religation generated pTC:ΔN29, a plasmid lacking the nucleotides that coded for the N-terminal 29 amino acids of the TEV CP gene.

pTC:FL and pTC:ΔN29 were modified further to generate plasmids pTC:ΔC18 and pTC:ΔN/C, respectively. Originally these two plasmids were mutated to create a premature stop codon at TEV nt 9,253–9,255, such that the 18 carboxy-terminal codons would not be translated. However, subsequent nucleotide sequence analysis of the mutagenized region revealed that a single nucleotide deletion (TEV nt 9,249) and three point mutations (at TEV nt 9,250–9,252) had also occurred. The resulting frameshift, 20 codons upstream of the wild type TEV CP stop codon, negated the introduced TAG stop condon at TEV nt 9,253–9,255 and created an in-frame TGA stop codon eight codons downstream of the frameshift. Translation of these genes resulted in a truncated TEV CP with the addition of seven non-TEV amino acids (EPRQRTM) after TEV CP amino acid number 243.

Plasmid pTC:RC (RNA control) was generated by insertion of a single deoxythymidylate residue after TEV nt 8,529, and point mutations of TEV nt 8,522 (G→C), 8,534 (C→A), 8,542 (G→A), and 8,543 (A→G) to create a frameshift mutation immediately followed by three stop codons. An *Nhe*I restriction site was simultaneously generated, for screening purposes, at nt 8,539–8,544.

All plasmids described above were linearized with *Hin*dIII, transcribed with T7 RNA polymerase (Melton *et al.* 1984), and translated in a rabbit reticulocyte lysate containing ^{35}S methionine (Dougherty and Hiebert 1980a). Radiolabeled translation products were analyzed by electrophoretic separation on a 12.5% acrylamide gel containing sodium dodecyl sulfate (SDS) (Laemmli 1970) and detected by autoradiography. Transcripts of plasmid pTC:RC produced no detectable protein products, whereas transcripts from all other plasmids produced proteins of the expected sizes (data not shown).

The various forms of the CP nucleotide sequence were then inserted as *Bam*HI cassettes into the plant expression vector pPEV (see below and Fig. 2). The full-length TEV CP ORF was inserted in the reverse orientation to make the antisense (AS) construct.

Construction of pPEV. The vector pPEV is part of a binary vector system for *A. tumefaciens*-mediated plant cell transformation. Plasmid pPEV was constructed from

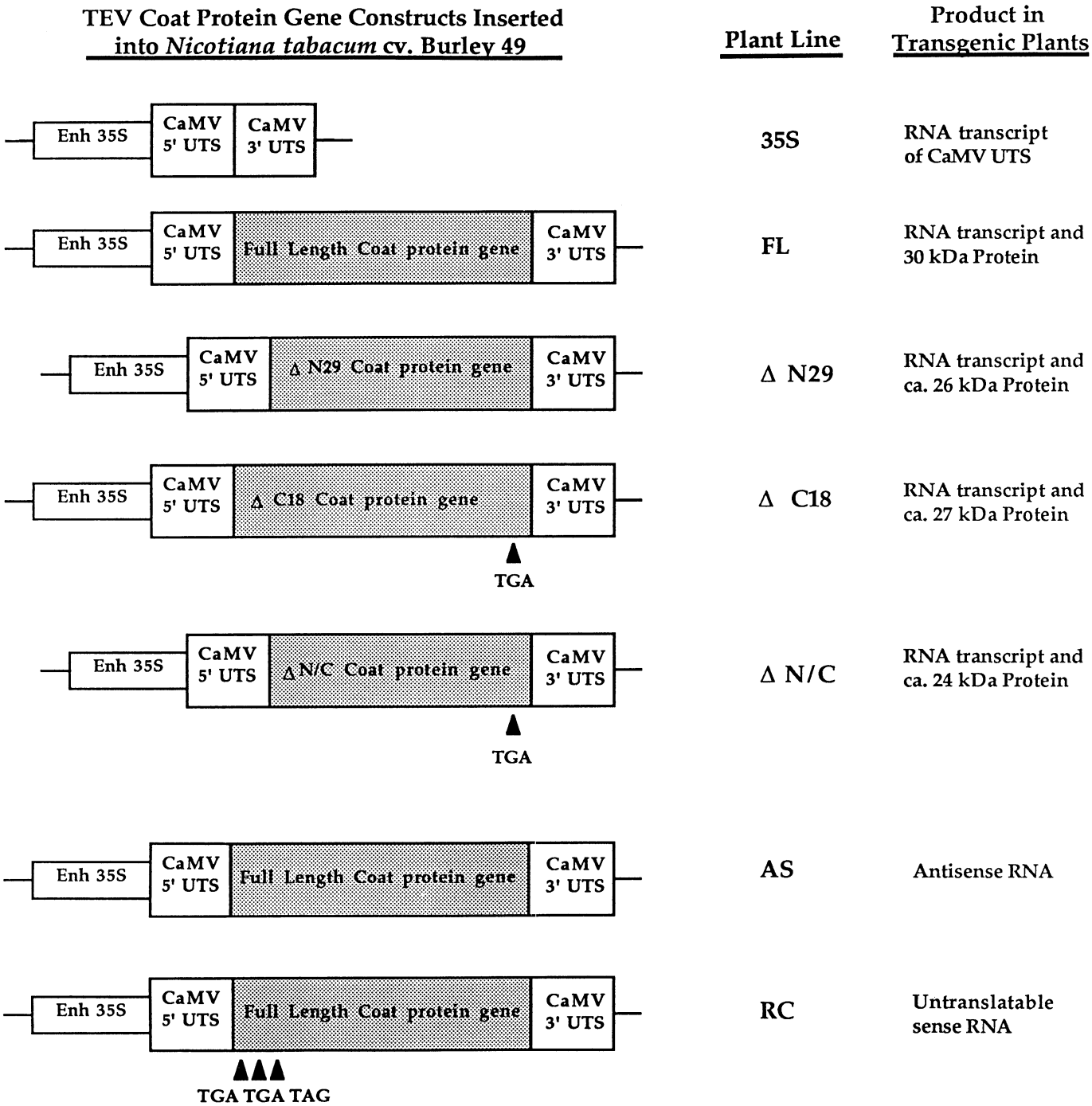

Fig 2. Forms of the tobacco etch virus (TEV) coat protein (CP) gene inserted into *Nicotiana tabacum* 'Burley 49.' All constructs contained the enhanced cauliflower mosaic virus (CaMV) 35S (Enh 35S) promoter, CaMV 35S 3′ untranslated sequence (UTS) of 50 bp and the CaMV 35S 3′ UTS/polyadenylation site of 110 bp. The nomenclature used to describe the transgenic plant lines is presented along with the gene products produced in those plant lines (far right column). Abbreviations are as follows: 35S, transgenic plants containing the CaMV 35S promoter and 5′ and 3′ UTS only; FL, ΔN29, ΔC18, ΔN/C, transgenic plants containing the transgene coding for full-length TEV CP. TEV CP lacking the amino-terminal 29 amino acids (aa), TEV CP lacking the carboxy-terminal 20 aa, or TEV CP lacking both the amino-terminal 29 aa and carboxy-terminal 20 aa, respectively. AS and RC transgenic plants contain the transgene expressed as an antisense form of the TEV CP gene or an untranslated sense form of the TEV CP gene, respectively. Stippled areas represent various forms of the TEV CP nucleotide sequence.

the plasmids pCGN2113 (Calgene), pCIB710, and pCIB200 (Ciba Geigy Corp.). pCGN2113 contains the "enhanced" cauliflower mosaic virus (CaMV) 35S promoter (CaMV sequences −941 to −90/−363 to +2, relative to the transcription start site) in a pUC-derived plasmid backbone. pCIB710 has been described (Rothstein *et al.* 1987) and pCIB200 is a derivative of the wide host range plasmid pTJS75 (Schmidhauser and Helinski 1985), which contains left and right *A. tumefaciens* T37 DNA borders, the plant-selectable NOS/NPTII chimeric gene from the plasmid pBin6 (Bevan 1984) and part of a pUC polylinker. The small *Eco*RI-*Eco*RV DNA fragment of pCIB710 (Rothstein *et al.* 1987) was ligated into *Eco*RI-*Eco*RV digested pCGN2113. This regenerated the enhanced CaMV 35S promoter (Kay *et al.* 1987) of pCGN2113 and introduced the CaMV 35S 5′ and 3′ untranslated sequences into pCGN2113. The CaMV 35S promoter-terminator cassette of the resulting plasmid was isolated as an *Eco*RI-*Xba*I DNA fragment and ligated into *Eco*RI-*Xba*I digested pCIB200 to generate pPEV. TEV CP ORFs were cloned as *Bam*HI cassettes into *Bam*HI-digested pPEV, and orientation of inserts was confirmed by digestion with appropriate restriction endonucleases.

Plant transformation. pPEV plasmids containing TEV CP ORFs were mobilized from *E. coli* HB101 into *A. tumefaciens* A136 containing plasmid pCIB542 (Ciba Geigy), using the helper plasmid pRK2013 in *E. coli* HB101 and the tri-parental mating system of Ditta *et al.* (1980). Plasmid pCIB542 supplied *vir* functions necessary for T-DNA transfer.

Leaf disks of *Nicotiana tabacum* L. 'Burley 49' were transformed, and whole plants were regenerated according to Horsch *et al.* (1985). Transformed tissue was selected by culturing callus on MS plates (Murashige and Skoog 1962) containing 1 μg/ml of 6-benzylaminopurine (Sigma Corp., St. Louis, MO), 0.1 μg/ml of α-naphthaleneacetic acid (Sigma Corp.), 500 μg/ml of carbenicillin, and 100 μg/ml of kanamycin sulfate (Sigma Corp.). Shoots were rooted on MS plates containing 500 μg/ml of carbenicillin and 100 μg/ml of kanamycin sulfate, and plantlets were transplanted into soil and transferred directly into the greenhouse approximately 2–3 wk after rooting.

R0, R1, and R2 plants were screened by western and/or northern blot analyses. R2 seed (approximately 100 seeds per R2 plant) was screened for the kanamycin resistant (Kan^r) phenotype by surface sterilizing seed in 10% bleach for 5 min, washing twice in sterile water, and germinating on MS plates containing 100 μg/ml of kanamycin sulfate. R2 seed lines, which were 100% Kan^r, were screened by western blot analysis for expression of TEV coat protein. Those transgenic plant lines generated and their nomenclature are presented in Figure 2.

Western blot analysis. Tissue samples of regenerated plants were ground in 10 vol of 2× Laemmli (Tris-glycine) running buffer (Laemmli 1970) and clarified by centrifugation in a microcentrifuge for 10 min at 10,000 × *g*. Protein concentration was estimated by the dye-binding procedure of Bradford (1976) using bovine serum albumin (BSA) as a standard. Protein samples (50 μg of total protein) were separated on a 12.5% polyacrylamide gel containing SDS

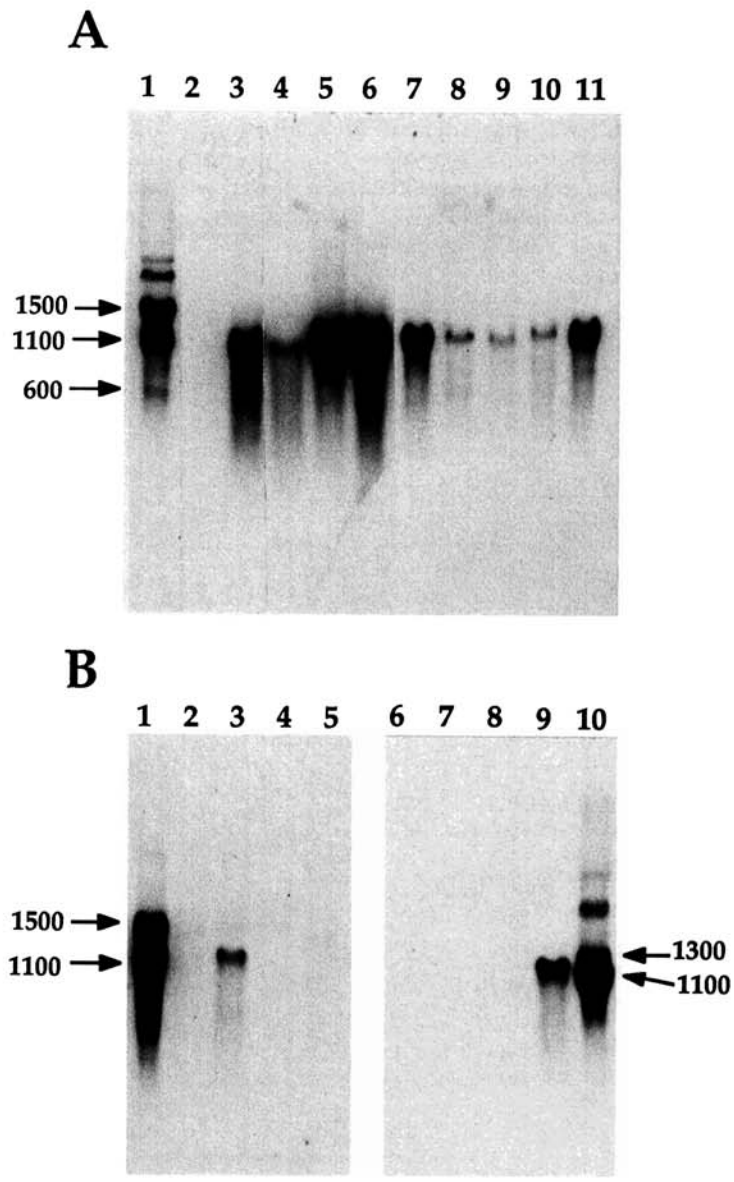

Fig. 3. Northern blot analysis of total RNA extracted from transgenic plants. Samples (10 μg) of total RNA from R2 (coat protein [CP] lines) or R1 (AS#3 and RC#5) transgenic plants were separated on a 1.2% agarose gel containing 6% formaldehyde. RNAs were transferred to nitrocellulose membranes and hybridized with either a minus-sense tobacco etch virus (TEV) CP riboprobe (A, and lanes 1–5 of B) or with a plus-sense TEV CP riboprobe (B, lanes 6–10). After washing under stringent conditions, filters were exposed to Kodak X-Omat film at −70° C. Plus- or minus-sense transcripts of pTL37/8595 were used as markers. RNA present in each lane is as follows: **A,** total RNA from 35S#4 leaf tissue with marker transcripts derived from T7 polymerase transcription of pTL37/8595 added (lane 1), total RNA samples from leaf tissue of R2 transgenic plants from lines 35S#4 (lane 2), FL#3 (lane 3), FL#24 (lane 4), ΔC18#7 (lane 5), ΔC18#15 (lane 6), ΔN29#1 (lane 7), ΔN29#2 (lane 8), ΔN29#8 (lane 9), ΔN/C#69 (lane 10), and ΔN/C#61 (lane 11). Marker transcript sizes are presented on the left side of the figures. **B,** total RNA from leaf tissue of the following transgenic plant lines: 35S#4 with sense-strand transcripts derived from pTL37/8595 added (lanes 1 and 6), 35S#4 (lanes 2 and 7), RC#5 (lanes 3 and 8), AS#3 (lanes 4 and 9), 35S#4 with antisense strand transcripts (from SP6 transcription of pTL37/8595) added (lanes 5 and 10). The sizes of the marker RNAs are presented on the left and right sides of the figure.

and subjected to the immunoblot transfer procedures described by Towbin *et al.* (1979). Anti-TEV coat protein polyclonal primary antibodies, alkaline phosphatase-conjugated secondary antibodies, and the chromogenic substrates NBT (para-nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indoyl phosphate para-toluidine salt) were used to detect bound antigen.

Northern blot analysis. Total nucleic acids were isolated from tissue and RNA precipitated with LiCl as described by Verwoerd *et al.* (1989). RNAs were electrophoretically separated on 1.2% agarose gels containing 6% (v/v) formaldehyde and transferred to nitrocellulose. Prehybridization and hybridization conditions were as described in Sambrook *et al.* (1989). Strand-specific riboprobes were generated from SP6 or T7 DNA-dependent RNA polymerase transcription reactions of pTL37/8595 linearized with the restriction enzymes *Asp*718 (Boehringer Mannheim Corp., Indianapolis, IN) or *Hin*dIII, respectively, using α-labeled [^{32}P]CTP ribonucleotide and suggested procedures (Promega Corp., Madison, WI).

Inoculation of transgenic plants. Eight-week-old (approximately 15 cm tall) R1 and R2 plants were inoculated with either purified virus preparations or infected plant sap. Inoculum was applied with sterile, premoistened cotton swabs. Infected plant sap inoculum was prepared by grinding TEV-infected *N. tabacum* 'Burley 21' leaf tissue (2 wk post-inoculation) in Carborundum and 50 mM sodium phosphate buffer (pH 7.8) at a ratio of 1 g:0.2 g:10 ml, respectively, and filtering the homogenate through cheesecloth. TEV virions were purified as described by Dougherty and Hiebert (1980b). One leaf per plant was dusted lightly with Carborundum (320 grit) and inoculated at two interveinal locations with 50 μl (total) of inoculum. Inoculated plants were examined daily, and the appearance and severity of systemic symptoms were recorded. Symptoms on any leaf above the inoculated leaf were considered to be systemic. Ten R2-expressing plants of each of the CP-expressing lines were inoculated with infected plant sap and 20 R1 plants of lines AS#3 and RC#5 were inoculated with 50 μl of a 5-μg/ml solution of purified TEV. Identical results were obtained when AS#3 and RC#5 R1 plants were inoculated with TEV-infected plant sap, as described above.

Preparation, inoculation, and analysis of protoplasts. Protoplasts were prepared from transgenic plants and electroporated according to the procedure of Luciano *et al.* (1987). Protoplasts (1×10^6) were resuspended in 450 μl of electroporation buffer (330 mM mannitol, 1 mM KPO_4, pH 7.0, 150 mM KCl) and electroporated using a BTX Transfector 300 (San Diego, CA) (950 microFarads, 130-V pulse amplitude, 3.5-mm electrode gap) in the presence or absence of 6 μg of purified TEV RNA. After electroporation, protoplasts were incubated for 96 hr in incubation medium as described in Luciano *et al.* (1987). Protoplasts were extracted in 2× Laemmli (Tris-glycine) running buffer and 5×10^4 extracted protoplasts were then subjected to western blot analysis as described above. Protoplast viability was measured by dye exclusion as described in Luciano *et al.* (1987). All electroporated protoplast samples had equivalent viability counts.

RESULTS

Analysis of transgenic plants. We first sought to determine if the resulting transgenic Burley 49 tobacco plants were producing the expected RNA and protein products.

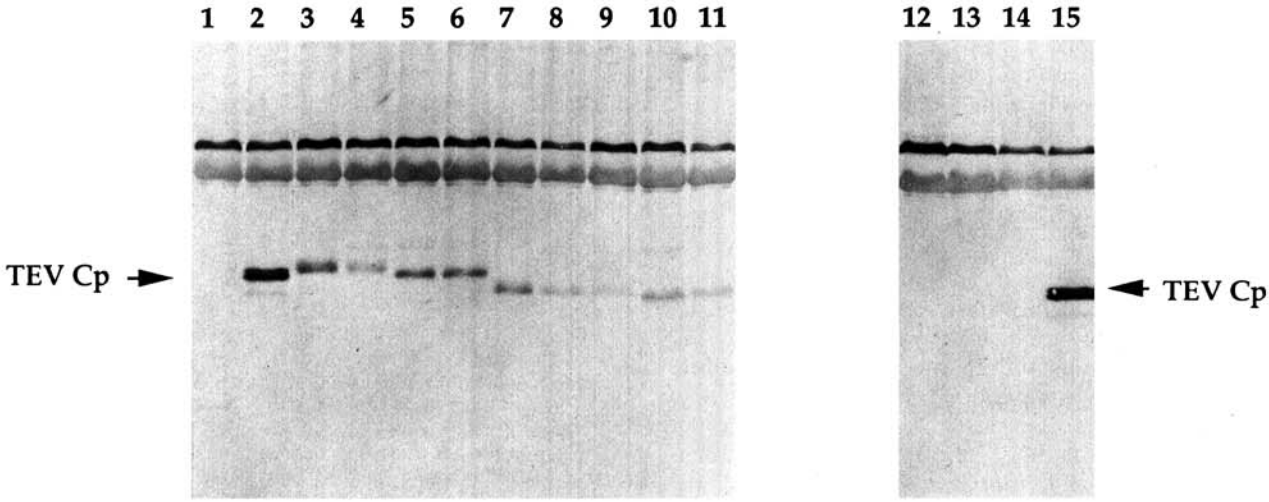

Fig. 4. Western blot analysis of transgenic plants. Protein samples (50 μg per lane) from leaf tissue of transgenic plants were separated on a 12.5% polyacrylamide gel containing sodium dodecyl sulfate (SDS), and transferred to nitrocellulose. After blocking with 3% gelatin, bound antigen was detected with rabbit anti-tobacco etch virus (TEV) polyclonal antisera, goat anti-rabbit (alkaline phosphatase conjugate) antibody, and the chromogenic substrates NBT (para-nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indoyl phosphate para-toluidine salt). Ten nanograms (ng) of purified TEV was used as a size marker (lanes 2 and 15, labeled on left and right sides of the figure). Specific degradation of TEV coat protein (CP) occurs during virus purification and repeated freezing/thawing resulting in a doublet of 30 and 27 kDa and a smaller, fainter band of about 24 kDa. Lanes 1–15 contain protein samples from the following transgenic plant lines: 35S#4 (lane 1), 35S#4 plus 10 ng TEV (lane 2), FL#3 (lane 3), FL#24 (lane 4), ΔC18#7 (lane 5), ΔC18#15 (lane 6), ΔN29#1 (lane 7), ΔN29#2 (lane 8), ΔN29#8 (lane 9), ΔN/C#69 (lane 10), ΔN/C#61 (lane 11), AS#3 (lane 12), RC#5 (lane 13), 35S#4 (lane 14), 35S#4 plus 10 ng TEV (lane 15).

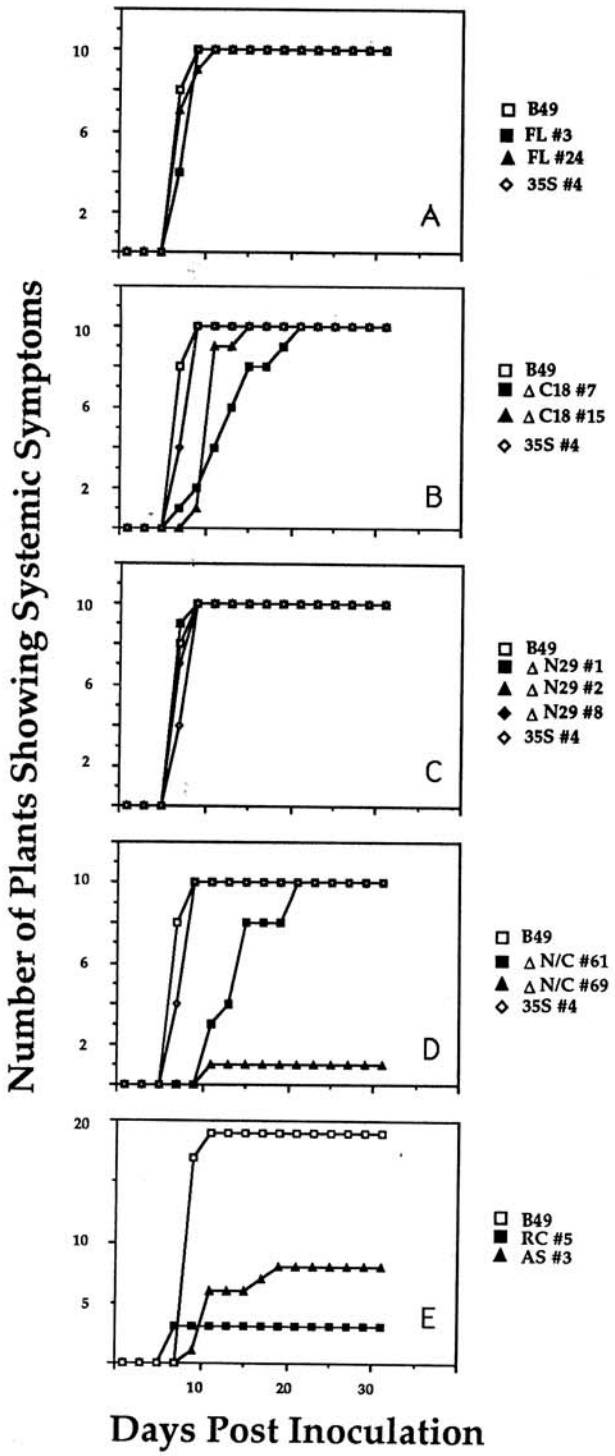

Fig. 5. Time of appearance of systemic symptoms in transgenic plants. Ten B49 (wild type) plants and 10 R2 plants of transgenic plant lines 35S#4, FL#3, FL#24 (A), ΔC18#7, ΔC18#15 (B), ΔN29#1, ΔN29#2, ΔN29#8 (C), ΔN/C#69, and ΔN/C#61 (D), homozygous for the inserted tobacco etch virus (TEV) gene, were mechanically inoculated with 50 μl of 1:10 dilution of infected plant sap. Twenty B49 plants and 20 R1 plants of lines AS#3 and RC#5 were mechanically inoculated with 50 μl of 5-μg/ml TEV (E). Plants were examined daily for the appearance of systemic symptoms. Plants were evaluated daily, and any plant displaying systemic symptoms (attenuated or wild type) were recorded as symptomatic.

Total RNA samples isolated from the various transgenic lines were analyzed in northern blot hybridization studies. An RNA transcript of approximately 1,000 nt was expected with all transgenic plant lines. Such a TEV CP transcript was detected in CP-expressing plant lines by using a "minus"-sense riboprobe containing the TEV CP sequence (Fig. 3A,B, lanes 1–5). A similar transcript was detected in AS plants by using a "plus"-sense riboprobe containing the TEV CP sequence (Fig. 3B, lane 9). However, the transcript in the RC line (Fig. 3B, lane 3), while detected with a minus-sense riboprobe, may have migrated as a slightly larger (about 1,100–1,200 nt) RNA species, possibly due to termination at an alternately selected site and/or a longer poly-A tail on the transcript. Differing levels of CP transcript accumulation were observed among different transgenic plant lines (Fig. 3).

Transgenic plant lines expressing either full-length or truncated TEV CP were identified by western blot analysis using polyclonal antisera to TEV CP. The various CP products produced in plants were stable and accumulated to different levels in individual transgenic plant lines. It was estimated by western blot analysis that between 0.01 and 0.001% of total extracted protein was TEV CP. Full-length and the three truncated forms of TEV CP could be readily distinguished in this analysis. Additionally, the antisera cross-reacted with two high molecular weight proteins present in Burley 49. No CP was detected in extracts of the AS or RC transgenic plant lines screened. The results from selected lines are presented in Figure 4.

In general, the expected RNA and protein products were detected in the various transgenic plant lines examined. Transcript expression levels generally correlated with protein expression levels as determined by western blot analysis. Different expression levels between different transgenic plant lines were also noted.

Inoculation of transgenic plants with virus. To estimate virus resistance in transgenic plants expressing different forms of the TEV CP, we challenged plants with TEV. Typically, inoculation of Burley 49 plants with TEV (either purified virus or plant sap) resulted in severe chlorosis and mosaic and mottle on systemically infected leaves approximately 6–7 days after inoculation. Severe etching of the leaf followed within a few days. We observed that transgenic plants containing only the CaMV promoter and untranslated sequences (i.e., 35S plant line) responded to challenge inoculation in a manner similar to wild type Burley 49, developing extensive chlorosis and etching at the same rate (Fig. 5 and Fig. 6B,C). Plant lines that expressed either FL or ΔN29 forms of TEV CP showed little or no delay in the appearance of symptoms when inoculated with infected plant sap (Fig. 5A,C). However, FL and ΔN29 transgenic plants did show a slight attenuation of symptoms and eventually (2–4 wk after initial appearance of symptoms) younger leaf tissue emerged devoid of symptoms and virus as demonstrated by back inoculation experiments (data not shown). Typically chlorosis and etching on older systemic leaves was limited (Fig. 6E,F). In contrast, transgenic plant lines that expressed ΔC18 or ΔN/C forms of TEV CP showed significant delays in the appearance of systemic symptoms

(Fig. 5B,D). ΔC18 and ΔN/C plant lines also showed a pronounced modification of symptoms in systemically infected leaves as only a mild mosaic was observed (note lack of etching in Fig. 6G,H). However, ΔC18 and ΔN/C plants did not completely outgrow symptoms, and a mild mosaic was detected on most leaf tissue.

Transgenic Burley 49 plant lines AS#3 and RC#5, expressing only TEV CP related RNA sequences, showed a delay in the appearance of symptoms (Fig. 5E) and a modification of symptoms when inoculated with TEV. Because the 20 R1 plants were not screened for expression of CP RNA before inoculation, some of the symptomatic plants represented nonexpressing plants in which the gene of interest had been lost during Mendelian segregation. Modified symptoms on AS#3 plants appeared as small chlorotic lesions often associated with a vein (see arrows in Fig. 6D). Most of the leaves were devoid of symptoms and virus (determined by back inoculation experiments). Approximately 15% of RC#5 plants showed symptoms that were identical to those of infected Burley 49. However, the remaining RC#5 plants were entirely asymptomatic, and virus was not detected in back inoculation studies (data not shown).

Analysis of TEV replication in protoplasts derived from transgenic plant lines. We were surprised by the results obtained when AS and RC transgenic plants were challenged with TEV and by the alteration of symptoms observed with the different CP-expressing plants. In our initial attempt to explain these results, we sought to determine if all of the transgenic plant lines we investigated would support virus replication at a level comparable to Burley 49. Accumulation of virus-encoded proteins was

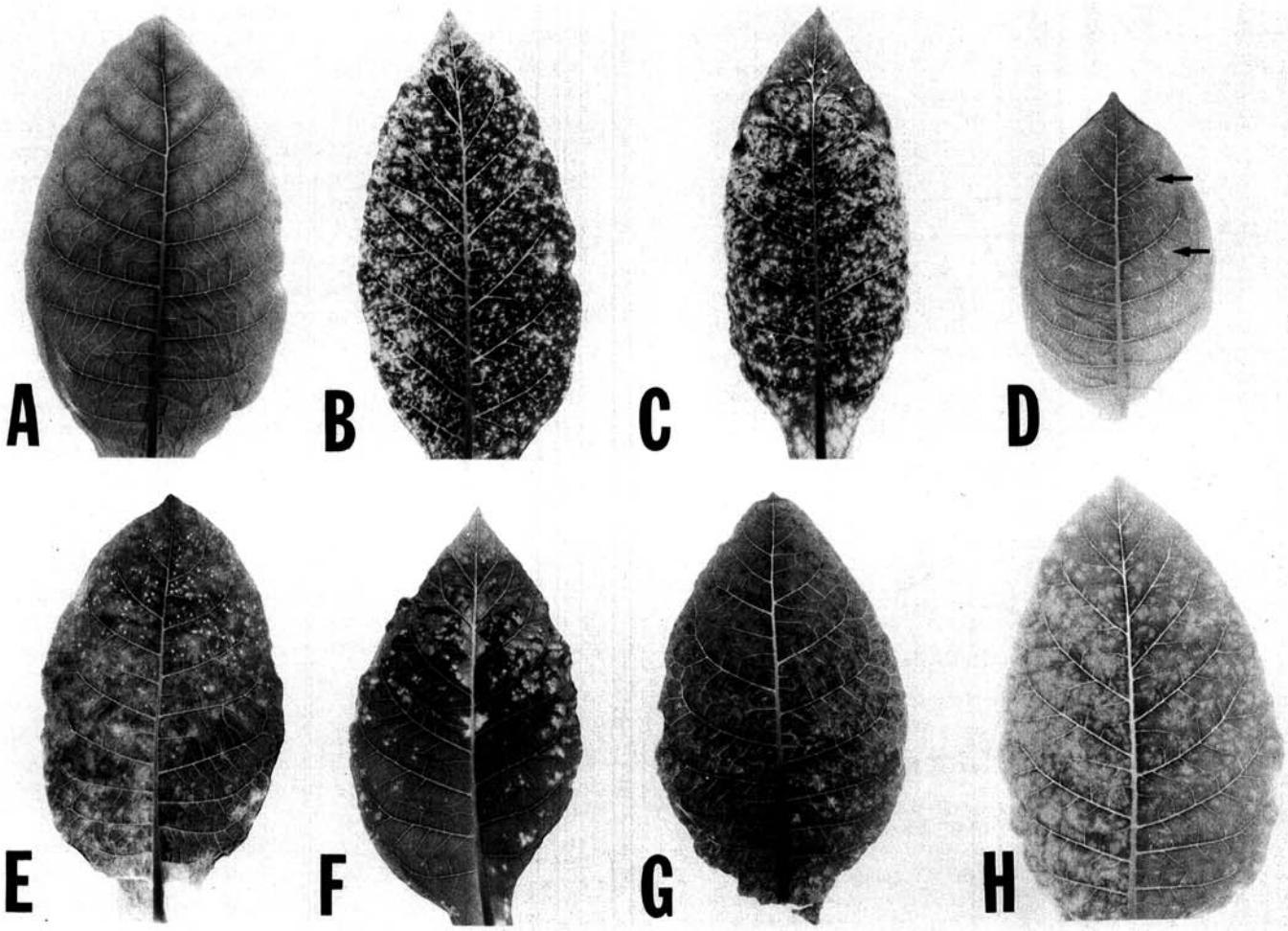

Fig. 6. Photographs of individual leaves of transgenic and wild type *Nicotiana tabacum* 'Burley 49' uninoculated or inoculated with tobacco etch virus (TEV). Individual leaves were removed from plants that had been inoculated with TEV 14 days earlier (or uninoculated Burley 49 plant) and photographed on a light box with transmitted light to illustrate symptom attenuation in transgenic plants. Symptoms in D, G, and H were not readily apparent without the use of transmitted light. Leaf samples are from **A,** uninoculated Burley 49; **B,** TEV-infected Burley 49; **C–H,** TEV-infected leaf from transgenic plant lines: 35S#4 (C), AS#3 (D), FL#3 (E), ΔN29#2 (F), ΔC18#7 (G), and ΔN/C#69 (H).

used as an indirect indicator of viral replication. Protoplasts were derived from leaf tissue of homozygous CP-expressing plants and electroporated with TEV RNA. Four days after electroporation, protoplast proteins were extracted and assayed for the presence of TEV-encoded protein, indicative of TEV gene expression and replication, by western blot analysis. These results, presented in Figure 7, indicated that protoplasts from all CP-expressing plant lines (full-length and truncated versions) supported virus replication at levels comparable to wild type Burley 49 protoplasts. R1 transgenic plants from lines AS#3 and RC#5 were initially screened by northern analysis, and leaves from positive expressors were used in the production of protoplasts. Transfected protoplasts derived from AS#3 plants supported TEV replication, albeit at a reduced level. Protoplasts derived from RC#5 transgenic plant leaf tissue did not support TEV replication at a detectable level. These results (Fig. 7) and those presented in the whole plant inoculation series (Fig. 5E) suggested AS and RC plants interfere with TEV replication.

DISCUSSION

We have generated transgenic plants expressing either full-length CP, amino-terminal truncated CP, carboxy-terminal truncated CP, or amino- and carboxy-terminal truncated CP of TEV. The amino- and carboxy-termini of potyvirus CPs reside on the exterior of intact virions. Comparisons between different potyvirus CP nucleotide sequences have revealed that the amino- and carboxy-termini are highly variable in size and sequence, whereas the internal "core" CP amino acids tend to be very conserved. Our ΔN29, ΔC18, and ΔN/C truncations maintain the highly conserved TEV CP core (Allison *et al.* 1985a; Dougherty *et al.* 1985; Shukla *et al.* 1988). We sought to determine if these truncated versions of the CP gene would be functional in CP-mediated resistance.

Our study suggested that transgenic plants expressing mutated TEV CPs (either ΔC18 or ΔN/C forms, in particular) were more effective in CP-mediated resistance to TEV than transgenic plants that expressed FL TEV CP. We speculate that the truncated CPs are in some way dysfunctional and are more effective at disrupting the normal virus-host relationship than FL CP. This disruption may be at the level of virus movement throughout the plant. There have been reports of CP deletions in tobamovirus systems that affect virus movement (Dawson *et al.* 1988). In these studies, viral genomes encoding truncated CPs are dysfunctional in the process of systemic (long distance) movement. Perhaps in our TEV system, truncated CP molecules are dysfunctional in the process of long distance movement and, as a consequence of being incorporated into virions, generated defective virus or ribonucleoprotein complexes inhibited in systemic movement. We have not yet investigated the effect of truncated CPs on short distance movement. The reported "broad spectrum resistance" (Stark and Beachy 1989; Ling *et al.* 1991) for potyviruses may also involve this phenomenon. CPs from heterologous potyviruses may protect, because they are dysfunctional in the heterologous system, resulting in limited systemic movement of the virus.

This study represents our initial analysis of transgenic plant lines. Our goal is to select 12–15 independent lines of each construct to complete our germ plasm bank. Although a limited number of transgenic plants have been tested to date, in general it appears as though ΔC18 CPs (either ΔC18 or ΔN/C forms) confer more effective resistance to virus than transgenic plants that express either full-length or ΔN29-truncated CPs. To our knowledge, this is the first report of CP-mediated resistance in which truncated CP forms demonstrate significant virus resistance. Because protoplasts from all CP-expressing transgenic plants examined support virus replication, subsequent efforts to unravel the mechanism involved in the observed

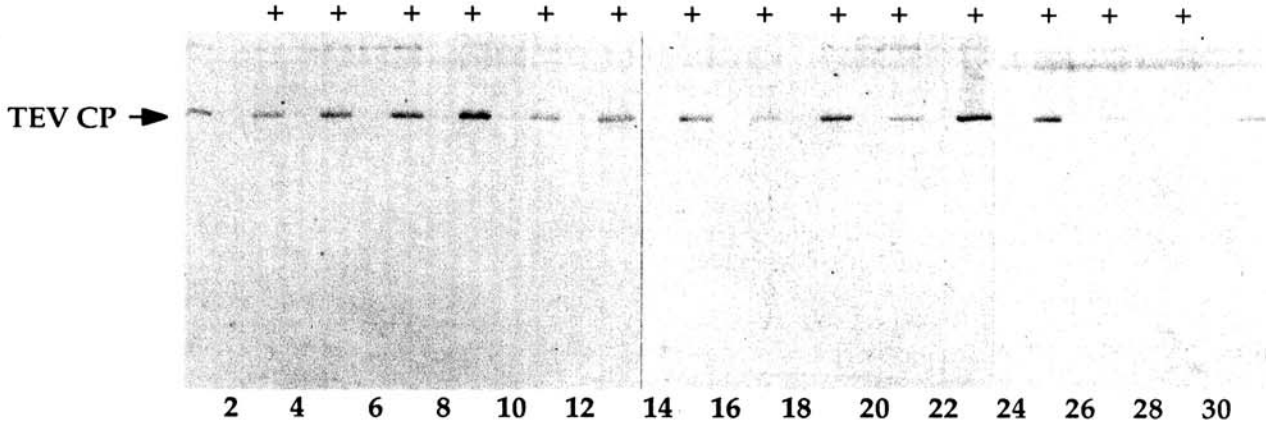

Fig. 7. Western blot analysis of tobacco etch virus (TEV) RNA transfected protoplasts. Protoplasts (1×10^6) were electroporated in the presence (lanes marked +) or absence (lanes unmarked) of 6 μg of purified TEV RNA. After 94 hr, protoplasts were collected and extracted by grinding in 2× Laemmli (1970) Tris-glycine running buffer. The equivalent of 5×10^4 viable protoplasts were loaded per lane. Proteins were separated by electrophoresis, and TEV proteins were detected by western blotting conditions identical to those described in Figure 2. Migration of TEV coat protein (CP) in this analysis, indicated on the left and right, was determined by adding 10 ng of purified TEV to a sample of 5×10^4 B49 protoplasts (lanes 1 and 30). Electroporated protoplasts were derived from the following tobacco lines: B49 (lanes 2,3,24, and 25), FL#3 (lanes 4 and 5), FL#24 (lanes 6 and 7), ΔC18#7 (lanes 8 and 9), ΔC18#15 (lanes 10 and 11), ΔN29#1 (lanes 12 and 13), ΔN29#2 (lanes 14 and 15), ΔN29#8 (lanes 16 and 17), ΔN/C#69 (lanes 18 and 19), ΔN/C#61 (lanes 20 and 21), 35S#4 (lanes 22 and 23), AS#3 (lanes 26 and 27), and RC#5 (lanes 28 and 29).

resistance will focus on how CP-expressing transgenic lines might primarily interfere with systemic movement of the replicating virus.

Transgenic plants expressing either AS or RC TEV RNAs showed a delay in symptoms and a reduction in symptom severity (AS plants), or were asymptomatic, as in plant line RC#5, which was apparently immune. Protoplasts derived from AS#3 transgenic leaf tissue did not support TEV replication at wild type levels, whereas protoplasts from RC #5 did not support detectable TEV replication (Fig. 7). We suggest that TEV resistance in these plants was due primarily to interference with a step in viral replication. Our results with these two transgenic plant lines appear to be similar to those reported by Golemboski *et al.* (1990). They have reported that plants that express an RNA sequence from the 3′ end of the 183-kDa gene of tobacco mosaic virus (TMV), but no detectable protein, were highly resistant to TMV infection. There are other examples reported in which transgenic plant lines are highly resistant to challenge virus inoculation, yet the expected protein product is not observed (Kawchuk *et al.* 1990, 1991). It may be that in these instances, as with the AS#3 and RC#5 plant lines, the resistant phenotype is mediated through a defective RNA species and not the expected translation product, as demonstrated in the extensively studied TMV-transgenic tobacco system (Powell *et al.* 1990). In our AS and RC plant lines, AS and RC RNA molecules may be interfering with viral replication by hybridizing to plus- or minus-sense genome length TEV RNA (respectively), thereby interfering with transcription and/or translation; binding to an essential host or viral factor (e.g., replicase); or interfering with virion assembly. Further studies will focus on these questions. From our preliminary studies, it appears that transgenic plants expressing defective RNAs or proteins will be among the most effective potyvirus control strategies.

At present, literature regarding CP-mediated resistance to potyviruses is limited. Two previous studies have investigated the ability of a potyvirus CP to confer resistance to a heterologous potyvirus (referred to as "broad spectrum resistance") (Stark and Beachy 1989; Ling *et al.* 1991). The only other report of potyvirus CP-mediated resistance involved transgenic potato plants expressing PVY CP (Lawson *et al.* 1990). In this study, a transgenic line (303) that expressed little, if any, PVY CP was highly resistant to PVY infection, whereas other transgenic lines expressing higher levels of PVY CP were more sensitive, showing only mild protection. In our experiments, we have observed that transgenic lines expressing FL TEV CP show little or no protection to TEV challenge, whereas plant lines that accumulate RNA, but no CP, are highly resistant (AS#3 and RC#5 lines). It may have been that in the study of Lawson *et al.* (1990) a spontaneous mutant that resulted in an untranslatable PVY CP RNA was responsible for the high degree of PVY resistance observed in the absence of PVY CP.

In summary, we have developed a series of transgenic plant lines that express various forms of the TEV CP gene sequence. Delay in symptom formation and altered symptom phenotype correlated with the TEV sequence being expressed. AS and RC transgenic plant lines displayed a high level of resistance and in one case, apparent immunity. The level of protection achieved in these plants may represent a new and effective way of generating potyvirus resistant germ plasm. Collectively, our work would appear to validate the concept of pathogen-derived resistance (Sanford and Johnston 1985) as a viable approach to the development of potyvirus resistant plants.

ACKNOWLEDGMENTS

Special thanks to Jim Hay and his greenhouse staff and to T. Dawn Parks for assistance with protoplast studies and for critical reading of the manuscript. This work was supported by a Tartar Research Fellowship to J. A. Lindbo and grants from the Department of Energy and the National Science Foundation to W. G. Dougherty. This is Oregon Agricultural Experiment Station technical paper 9695.

LITERATURE CITED

Allison, R. F., Johnston, R. E., and Dougherty, W. G. 1986. The nucleotide sequence of the coding region of tobacco etch virus genomic RNA: Evidence for the synthesis of a single polyprotein. Virology 154:9-20.

Allison, R. F., Dougherty, W. G., Parks, T. D., Willis, L., Johnston, R. E., Kelly, M. E., and Armstrong, F. B. 1985a. Biochemical analysis of the capsid protein gene and capsid protein of tobacco etch virus: N-terminal amino acids are located on the virion's surface. Virology 147:309-316.

Allison, R. F., Sorenson, J. G., Kelly, M. E., Armstrong, F. B., and Dougherty, W. G. 1985b. Sequence determination of the capsid protein gene and flanking regions of tobacco etch virus: Evidence for the synthesis and processing of a polyprotein in potyvirus genome expression. Proc. Natl. Acad. Sci. USA 82:3969-3972.

Beachy, R. N., Loesch-Fries, S., and Tumer, N. E. 1990. Coat protein-mediated resistance against virus infection. Annu. Rev. Phytopathol. 28:451-474.

Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. 12:8711-8721.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Carrington, J. C., and Dougherty, W. G. 1987. Small nuclear inclusion protein encoded by a plant potyvirus gene is a protease. J. Virol. 61:2540-2548.

Carrington, J. C., Cary, S. M., Parks, T. D., and Dougherty, W. G. 1987. Vectors for cell-free expression and mutagenesis of protein coding sequences. Nucleic Acids. Res. 15:1066.

Dawson, W. O., Bubrick, P., and Grantham G. L. 1988. Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement, and symptomatology. Phytopathology 78:783-789.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad-host-range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.

Dougherty, W. G., and Carrington, J. C. 1988. Expression and function of potyviral gene products. Annu. Rev. Phytopathol. 26:123-143.

Dougherty, W. G., and Hiebert, E. 1980a. Translation of potyvirus RNA in a rabbit reticulocyte lysate: Reaction conditions and identification of the capsid protein as one of the products of *in vitro* translation of tobacco etch and pepper mottle viral RNAs. Virology 101:466-474.

Dougherty, W. G., and Hiebert, E. 1980b. Translation of potyvirus RNA in a rabbit reticulocyte lysate: Cell-free translation strategy and a genetic map of the potyviral genome. Virology 104:183-194.

Dougherty, W. G., Willis, L., and Johnston, R. E. 1985. Topographic analysis of tobacco etch virus capsid protein epitopes. Virology 144:66-72.

Francki, R. I. B., Milne, R. G., and Hatta, T. 1985. Atlas of Plant Viruses, Vol II. CRC Press, Boca Raton, FL.

Golemboski, D. B., Lomonossoff, G. P., and Zaitlin, M. 1990. Plants transformed with a tobacco mosaic virus nonstructural gene sequence are resistant to the virus. Proc. Natl. Acad. Sci. USA 87:6311-6315.

Hollings, M., and Brunt, A. A. 1981. Potyviruses. Pages 731-807 in: Handbook of Plant Virus Infection and Comparative Diagnosis. E. Kurstak, ed. Elsevier/North. Amsterdam, Holland.

Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. 1985. A simple and general method for transferring genes into plants. Science 227:1229-1231.

Kawchuk, L. M., Martin, R. R, and McPherson, J. 1990. Resistance in transgenic potato expressing the potato leafroll virus coat protein gene. Mol. Plant-Microbe Interact. 3:301-307.

Kawchuk, L. M., Martin, R. R, and McPherson, J. 1991. Sense and antisense RNA-mediated resistance to potato leafroll virus in Russet Burbank potato plants. Mol. Plant-Microbe Interact. 4:247-253.

Kay, R, Chan, A., Daly, M., and McPherson, J. 1987. Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236:1299-1302.

Kozak, M. 1984. Point mutations close to the AUG initiation codon affect the efficiency of translation of rat preproinsulin *in vivo*. Nature (London) 308:241-246.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

Lawson, C., Kaniewski, W., Haley, L., Rozman, R., Newell, C., Sanders, P., and Tumer, N. E. 1990. Engineering resistance to mixed virus infection in a commercial potato cultivar: Resistance to potato virus X and potato virus Y in transgenic Russet Burbank. Bio/Technol. 8:127-134.

Ling, K., Namba, S., Gonsalves, C., Slighton, J. L., and Gonsalves, D. 1991. Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. Bio/Technol. 9:752-758.

Luciano, C. S., Rhoads, R. E., and Shaw, J. G. 1987. Synthesis of potyviral RNA and proteins in tobacco mesophyll protoplasts inoculated by electroporation. Plant Sci. 51:295-303.

Matthews, R. E. F. 1982. Classification and nomenclature of viruses. Intervirology 17:1-199.

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SF6 promoter. Nucleic Acids Res. 12:7145-7156.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473-497.

Powell, P. A., Sanders, P. R., Tumer, N., Fraley, R. T., and Beachy, R. N. 1990. Protection against tobacco mosaic virus infection in transgenic plants requires accumulation of coat protein rather than coat protein RNA sequences. Virology 175:124-130.

Rothstein, S. J., Lahners, K. N., Lotstein, R. J., Carozzi, N. B., Jayne, S. M., and Rice, D. A. 1987. Promoter cassettes, antibiotic-resistance genes and vectors for plant transformation. Gene 53:153-161.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanford, J. C., and Johnston, S. A. 1985. The concept of pathogen derived resistance: Deriving resistance genes from the parasites own genome. J. Theor. Biol. 113:395-405.

Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

Schmidhauser, T. F., and Helinski, D. R. 1985. Regions of broad host-range plasmid RK2 involved in replication and stable maintenance in nine species of Gram-negative bacteria. J. Bacteriology 164:446-455.

Shukla, D. D., Strike, P. M., Tracy S. L., Gough, K. H., and Ward, C. W. 1988. The N and C termini of the coat proteins of potyviruses are surface located and the N terminus contains the major virus-specific epitopes. J. Gen. Virol. 69:1497-1508.

Stark, D. M., and Beachy, R. N. 1989. Protection against potyvirus infection in transgenic plants: Evidence for broad spectrum resistance. Bio/Technol. 7:1257-1262.

Taylor, J. W., Schmidt, W., Cosstick, R., Odruszed, A., and Eckstein, F. 1985a. The use of phosphorothioate-modified DNA in restriction enzyme reactions to prepare nicked DNA. Nucleic Acids Res. 13:8749-8764.

Taylor, J. W., Ott, J., and Eckstein, F. 1985b. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucleic Acids Res. 13:8765-8785.

Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.

Verwoerd, T. C., Deker, B. M. M., and Hoekema, A. 1989. A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res. 17:2362.