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Functional Replacement of the *Tobacco rattle virus* Cysteine-rich Protein by Pathogenicity Proteins from Unrelated Plant Viruses

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Mutation of the 16K gene encoded by RNA1 of *Tobacco rattle virus* (TRV) greatly reduced the levels of viral RNA that accumulated in both infected protoplasts and plants, showing that the 16K cysteine-rich protein (CRP) is required for efficient multiplication of TRV. Overexpression of the 16K protein, either from an additional copy of the gene carried on TRV RNA2 or from a PVX vector, led to an increase in the severity of disease symptoms, suggesting that the protein has a role in the pathogenicity of the virus. Mutation of the 16K gene could be overcome by expression from RNA2 of the *Cucumber mosaic virus* 2b gene, the *Soil-borne wheat mosaic virus* 19K gene, or the *Barley stripe mosaic virus* γb gene, indicating that the proteins encoded by these diverse genes may have similar functions. One known function of the CMV 2b gene is as a suppressor of posttranscriptional gene silencing, suggesting that the TRV 16K protein may also possess this activity.

Key Words: tobacco rattle virus; 16K cysteine-rich protein; posttranscriptional gene silencing; viral silencing suppressor; virus pathogenicity.

INTRODUCTION

Tobacco rattle virus (TRV), similar to the two other tobraviruses *Pea early-browning virus* (PEBV) and *Pepper ringspot virus* (PepRSV), has a bipartite, positive-strand RNA genome, each segment of which is encapsidated separately in a rod-shaped particle (MacFarlane, 1999). The smaller RNA (RNA2) varies considerably in size and gene content between different isolates but always encodes the coat protein (CP) and may encode nonstructural 2b and 2c proteins. The 2b protein is a helper factor for transmission of the virus by root-feeding vector nematodes. The larger genomic RNA (RNA1) is closely conserved in size and gene content not only between different isolates of each virus but also between the three different viruses themselves. A characteristic of tobraviruses is that RNA1 can infect plants systemically in the absence of RNA2, i.e., without CP expression and virion formation. This type of infection, referred to as NM infection, occurs frequently in vegetatively propagated crop plants such as potato and bulbous ornamentals and is often associated with increased symptom severity. For TRV, RNA1 encodes the 134K and 194K proteins that, as judged by amino acid sequence conservation, comprise the viral replicase. RNA1 also encodes a 29K protein involved in cell-to-cell movement of the virus (Ziegler-Graff *et al.,* 1991) and a 16K cysteine-rich protein (CRP) that in earlier studies was reported not to function in virus replication or movement (Guilford *et al.,* 1991).

Other studies detected the TRV 16K protein by Western blotting in extracts of infected tobacco protoplasts (Angenent *et al.,* 1989). The protein accumulated to high levels, equivalent to that of the CP, but continued to be expressed even after CP synthesis had declined. Cell fractionation experiments, combined with sedimentation analysis, showed that the 16K protein accumulated in a high-molecular-weight complex, either as a multimer or in association with host proteins (Angenent *et al.,* 1989). In whole plants the 16K protein was only detected when infected leaves were extracted using highly denaturing reagents, although, even in these conditions, some of the protein still accumulated in higher molecular weight aggregations (Liu *et al.,* 1991). Immunogold labeling of ultrathin sections showed that the 16K protein was located mostly in the nucleus but was also present in the cytoplasm (Liu *et al.,* 1991).

The other tobraviruses, PEBV and PepRSV, also carry a small, 3′-terminal gene encoding a cysteine-rich protein. The CRP from these viruses is smaller (12K) than the TRV 16K protein; however, all of the proteins contain cysteine/histidine motifs reminiscent of zinc-binding domains present in some regulatory proteins and both have C-terminal regions rich in basic amino acid residues. Amino acid sequence identity between the 16K and 12K proteins is low (31%); however, there is a striking conservation in the arrangement of the cysteines and their flanking residues in these proteins. Computer-aided alignment suggested that there was significant amino

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acid sequence homology between the C-terminal basic domain of the TRV 16K protein and mammalian high mobility group chromatin (HMG) proteins (Koonin *et al.,* 1991). HMG proteins are nuclear proteins that bind DNA in a non-sequence-specific fashion to promote chromatin function and gene regulation (Grasser, 1998).

Mutagenesis studies of the PEBV 12K CRP showed that in pea plants this protein is required for seed transmission of the virus and that deletion of the 12K gene prevented movement of the virus into pollen grains and ovules (Wang *et al.,* 1997). Interestingly, this mutant also caused more severe symptoms on pods and leaves and accumulated to higher levels than did the wild-type virus. In contrast, the PEBV 12K deletion mutant was severely restricted in *Nicotiana* species, accumulating to only 1/60th of the level of wild-type (S. MacFarlane, unpublished data). These observations suggested that the tobravirus CRPs have a number of different functions, some of which may be tissue- or species-specific, and that reexamination of the TRV 16K CRP would be worthwhile.

RESULTS

The 16K gene is required for virus multiplication

The initial step in this work was the construction of a full-length cDNA clone of RNA1 of TRV isolate PpK20. Transcripts derived from this clone, pTRV1, were infectious when inoculated to plants either alone or in combination with transcripts of TRV RNA2 and, when coinoculated with TRV RNA2, were encapsidated into virus particles and could be transmitted by the natural nematode vector of TRV (data not shown). A modified clone of TRV RNA1 was created, in which the 16K gene was flanked by novel restriction sites (Fig. 1). Transcripts from this clone, pTRV1NB, behaved in an identical way to those derived from the wild-type clone pTRV1. A second clone, pTRV1-16Δ, was created in which the entire 16K gene was deleted. Transcripts derived from clones pTRV1NB or pTRV1-16Δ were mixed with RNA2 transcripts from clone pK20-GFPc (MacFarlane and Popovich, 2000), which encodes the virus coat protein and the green fluorescent protein (GFP), and inoculated to *Nicotiana tabacum* (var. Samsun NN). More than 30 fluorescent lesions were visible by 3 days postinoculation (d.p.i.) on four of six plants inoculated with wild-type (pTRV1NB) RNA1 and RNA2-GFP; however, no fluorescent lesions were visible on any plant inoculated with RNA1 carrying the 16K gene deletion even at 5 d.p.i. or later (Fig. 2A). Northern blot analysis showed that, although both TRV RNAs were clearly evident at 7 d.p.i. in these four plants infected using transcripts of pTRV1NB, neither RNA1 nor RNA2 could be detected in plants inoculated with transcripts from the 16K deletion mutant (Fig. 2B). Thus, in tobacco plants the presence of the 16K gene is essential for accumulation of TRV RNAs.

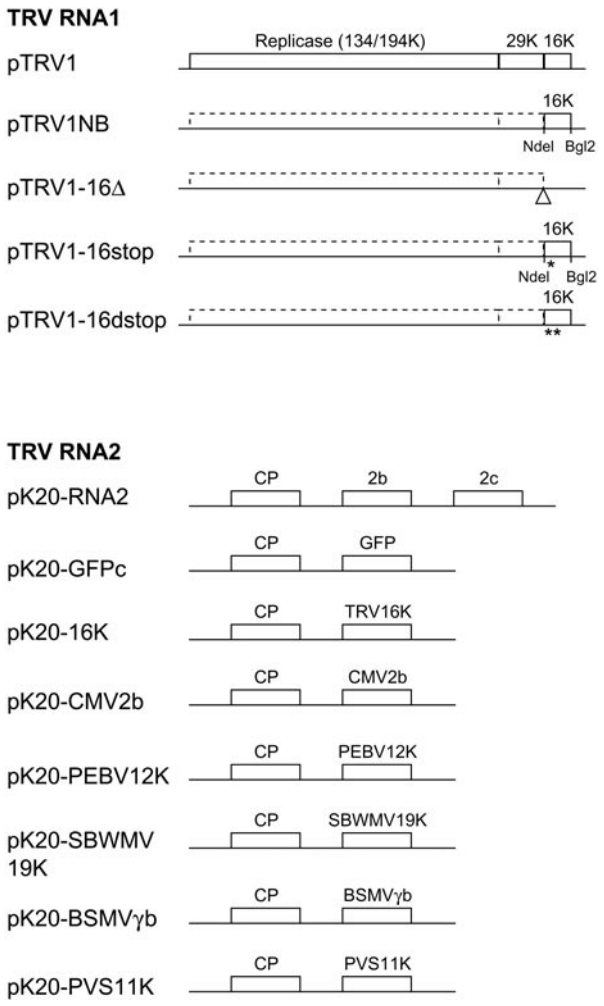
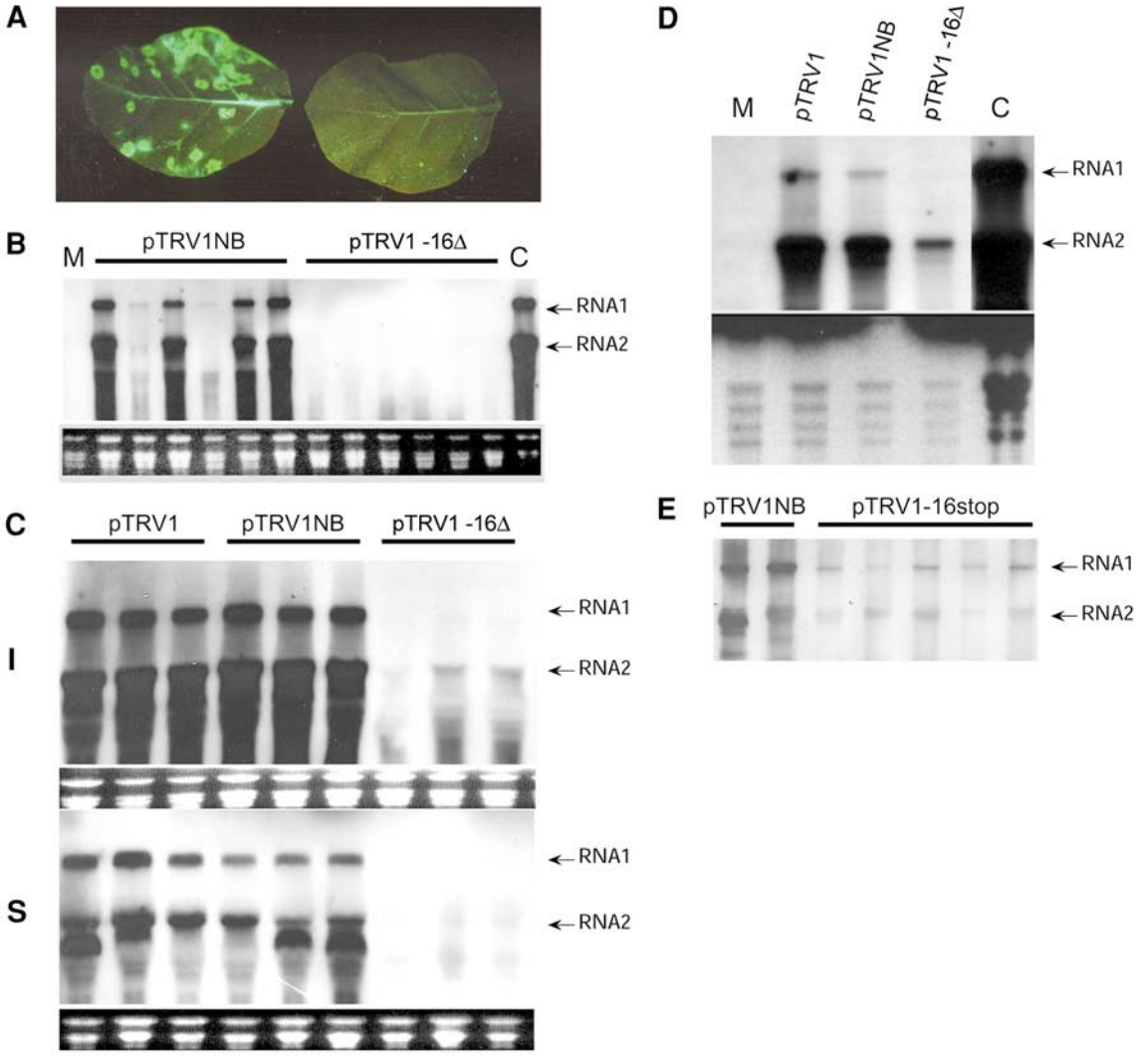


FIG. 1. Schematic drawing of TRV constructs used in this work. For TRV RNA1, 29K denotes the cell-to-cell-movement protein. To highlight the alterations made in and around the 16K gene, the replicase and movement protein genes in the modified RNA1 constructs are outlined with dashed lines. The single and double translation terminators inserted into the 16K genes of pTRV1-16stop and pTRV1-16dstop appear as asterisks below the 16K gene. *Nde*I and *Bgl*II are artificial restriction sites inserted into pTRV1NB and pTRV1-16stop. For TRV RNA2, a series of constructs was made in which the 2b and 2c nonstructural genes were replaced either with the GFP gene or with different genes from other plant viruses.

When transcripts from these clones were inoculated to *Nicotiana benthamiana*, similar results were obtained. In Northern blots wild-type, TRV1, or TRV1NB viral RNAs were clearly detectable in both inoculated and systemically infected leaves (Fig. 2C). However, with mutant TRV1-16Δ, viral RNAs were barely detectable in inoculated leaves at 6 d.p.i. and were not detected in upper, uninoculated leaves at 11 d.p.i. Lack of systemic infection of mutant TRV-16Δ was confirmed by RT-PCR analysis of samples from these plants (data not shown). The TRV 16K gene was also necessary for efficient virus multiplication in *N. benthamiana* protoplasts, where


A
B
M
pTRV1NB
pTRV1 -16Δ
C
RNA1
RNA2
C
pTRV1
pTRV1NB
pTRV1 -16Δ
I
S
RNA1
RNA2
D
M
pTRV1
pTRV1NB
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C
RNA1
RNA2
E
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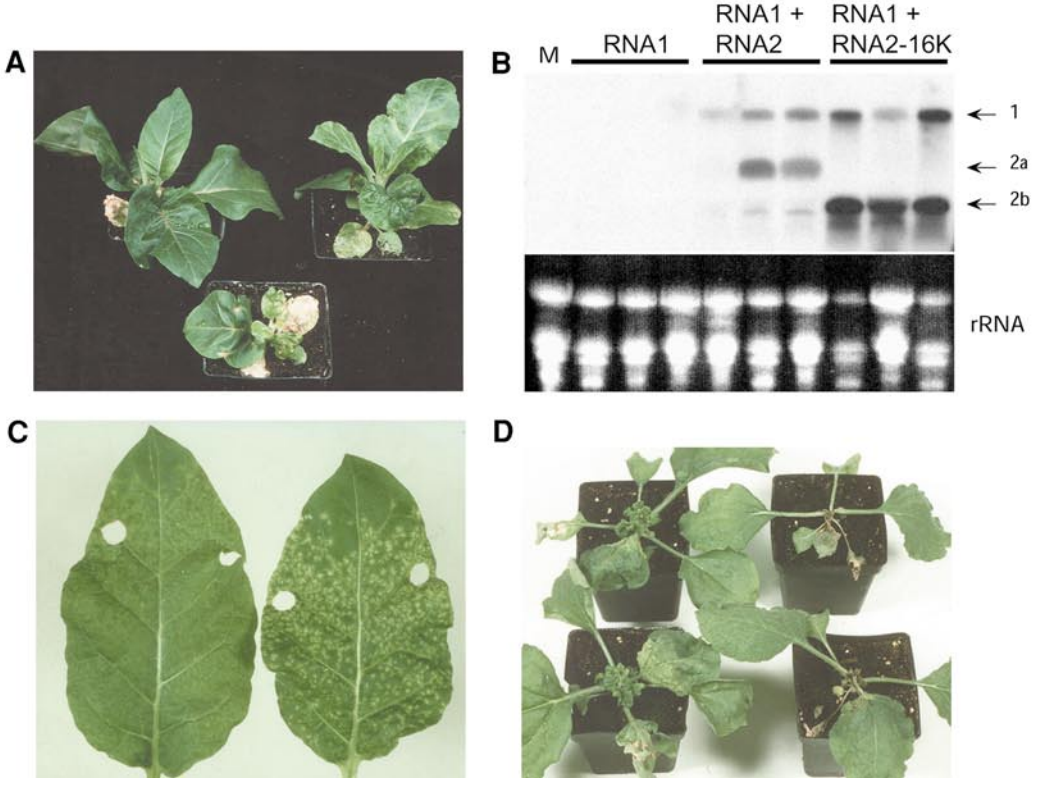
pTRV1-16stop
RNA1
RNA2

2


A
B
M
RNA1
RNA1 + RNA2
RNA1 + RNA2-16K
1
2a
2b
rRNA
C
D

3

RNA1 and RNA2 of mutant TRV1-16Δ accumulated to levels less than 10% of those of the wild-type viruses, TRV1 and TRV1NB (Fig. 2D).

The requirement of the 16K protein for efficient TRV replication was further examined by the creation of two mutants carrying premature translation termination codons in the 16K gene. In mutant TRV-16stop, the sixth codon of the 16K gene is replaced by a UAG terminator and the 16K gene is flanked by *Nde*I and *Bgl*II sites. This mutant multiplied very poorly compared to wild-type virus both in whole plants (Fig. 2E) and in protoplasts (data not shown), confirming that the 16K protein rather than the 16K RNA sequence is required for efficient virus replication. Mutant TRV-16dstop carries two early stop codons, codon four is UAA and codon six is UAG. However, in this mutant the 16K gene is not flanked by artificial *Nde*I and *Bgl*II restriction sites. This mutant also replicated poorly (see below), confirming that the restriction sites introduced into all of the previous mutants were not the cause of reduced replication efficiency.

The 16K protein is a pathogenicity determinant

TRV RNA2 can be used as a vector from which heterologous sequences are expressed at high levels using a duplicated coat protein promoter (MacFarlane and Popovich, 2000). Clone pK20-16K was constructed to examine the effects of overexpression of the 16K protein on symptom production by TRV. Infection of tobacco plants with TRV RNA1 only (NM infection) resulted in stem/midvein necrosis and slight stunting but on most systemic leaf blades no symptoms were apparent. Infection with wild-type (RNA1 and RNA2) TRV (isolate PpK20) resulted in infrequent, small necrotic patches on systemic leaves together with some leaf distortion and chlorotic mottle. Infection with TRV RNA1 and RNA2-16K caused severe stunting and distortion of systemic leaves together with widespread necrosis (Fig. 3A). Northern blot analysis of these plants showed that NM-infected plants had little or no virus RNA in systemic leaf blades. Both viral RNAs were easily detectable in wild-type virus-infected tissue; however, infection with TRV RNA1 and RNA2-16K led to an increase in the level of virus RNAs, particularly RNA2 (Fig. 3B). Thus, overexpression of the 16K protein leads to increased pathogenicity of TRV.

Expression of the 16K gene from a PVX vector

To examine whether enhancement of symptom expression by the 16K protein was specific for TRV, the 16K gene was cloned into the PVX vector (Chapman *et al.,* 1992). Inoculation of tobacco plants with PVX lacking any insert resulted in a systemic, chlorotic mottle (Fig. 3C, left). Systemic infection with PVX-16K was slower by 1 to 2 days than with PVX and produced severe, chlorotic lesions rather than mottling (Fig. 3C, right). There was an even greater contrast in symptomatology of the two viruses following inoculation to *N. benthamiana*. Both PVX and PVX-16K initially induced severe systemic leaf curling and vein chlorosis. However, by 20 d.p.i., whereas PVX-infected plants were highly stunted (Fig. 3D, left), plants infected with PVX-16K developed severe necrosis of the topmost leaves (Fig. 3D, right), which later led to the death of the plants. Thus, the TRV 16K protein is a pathogenicity determinant that can function when expressed from a different virus.

Complementation of 16K mutation by the gene encoding the CMV 2b silencing suppressor

As expression of the 16K gene from TRV RNA2 enhanced the replication (and symptom production) of wild-type TRV RNA1, experiments were carried out to test the effect of this RNA2 on the replication of the 16dstop

FIG. 2. Analysis of the effects of mutation of the TRV 16K gene. (A) GFP expression, viewed under UV light at 5 d.p.i., in tobacco leaves inoculated with transcripts of pK20-GFPc and pTRV1NB (left) or pTRV1-16Δ (right). (B) Northern blot of RNA extracted from *N. tabacum* leaves 7 d.p.i. inoculated with wild-type RNA1 (transcript from pTRV1NB) and RNA2-GFP (transcript from pK20-GFPc) or transcripts of pTRV1-16Δ and pK20-GFPc. M is RNA from a healthy plant. C is RNA of *N. benthamiana* infected with wild-type, uncloned TRV PpK20. Blot hybridised with probes specific for TRV RNA1 and RNA2. Location of RNAs 1 and 2 is indicated. Relative amounts of RNA in each lane are indicated by ethidium bromide staining of rRNAs in the lower panel. (C) Northern blot of RNA extracted from *N. benthamiana* plants at 6 d.p.i. (I, inoculated leaf) and 11 d.p.i. (S, systemic leaf). Plants inoculated with RNA2-GFP transcripts (pK20-GFPc) and transcripts either of wild-type RNA1 (pTRV1 or pTRV1NB) or of a 16K deletion mutant (pTRV1-16Δ). (D) Northern blot of RNA extracted from *N. benthamiana* protoplasts 48 h after inoculation with transcripts of wild-type RNA2 (pK20-RNA2) and pTRV1, pTRV1NB, or pTRV1-16Δ. M is from protoplasts electroporated without transcript. C is RNA from plants infected with TRV. RNA1 can be seen in protoplasts electroporated with the 16K deletion mutant after very long exposure of the blot and in other protoplast experiments. (E) Northern blot of RNA extracted from leaves of *N. benthamiana* inoculated with transcript RNA2 from pK20-GFPc and transcript RNA1 from pTRV1NB or pTRV1-16stop.

FIG. 3. The 16K gene is a pathogenicity determinant. (A) Symptoms on *N. tabacum* var. *Samsun* NN following infection by TRV RNA1 (top, left), TRV RNA1 and RNA2 (top, right), or TRV RNA1 and RNA2-16K (bottom). Expression of an additional copy of the 16K gene from RNA2 (RNA2-16K) results in severe stunting and necrosis of the plants. (B) Northern blot of RNA extracted from systemically infected leaves of plants photographed in (A). Plants infected with RNA1 only, with RNA1 and RNA2, or with RNA1 and RNA2-16K. Position of viral RNAs is indicated by arrows. 1 is RNA1, 2a is wild-type RNA2, 2b is RNA2-16K. rRNA denotes ribosomal RNAs in these samples, labeled by ethidium bromide staining. Blot hybridised with probes specific for TRV RNA1 and RNA2. Heterologous expression of the 16K gene. (C) Upper, uninoculated leaves of tobacco inoculated with transcripts of PVX (left) and PVX-16K (right). (D) *N. benthamiana* plants 20 days after inoculation with transcripts of PVX (left) and PVX-16K (right). The plants infected with PVX continued to grow after this time, whereas the severe tip necrosis of the plants infected with PVX-16K was fatal.

RNA1 mutant. In these experiments, virus could not be detected by Northern blotting of *N. benthamiana* plants inoculated with 16dstop RNA1 and RNA2-GFP, either in inoculated or in systemic leaves (Fig. 4A, lanes 5–8). However, inoculation with 16dstop RNA1 and RNA2-16K produced a readily detectable infection (Fig. 4A, lanes 9–12). Thus, expression of the 16K protein *in trans* completely rescued the very poorly replicating 16dstop RNA1 mutant.

The results from experiments described above showed that the TRV 16K protein is a pathogenicity determinant that is required for efficient viral replication and, thereafter, systemic infection of plants. Similar properties have been described for several plant virus-encoded suppressors of PTGS (Brigneti *et al.,* 1998; Bonneau *et al.,* 1998). We hypothesised that the absence of a putative suppressor function resulting from the 16dstop RNA1 mutation could be overcome by coexpression of a known suppressor protein derived from another virus. Thus, 16dstop RNA1 was inoculated to plants together with transcripts of pK20-CMV2b, in which the CMV2b gene is expressed from TRV RNA2. Northern blotting showed that, in two of four inoculated plants, the CMV 2b gene was able to rescue TRV carrying a mutation in the 16K gene, resulting in the accumulation of high levels of viral RNAs both in inoculated and in systemic infected leaves (Fig. 4B, lanes 5 and 8). RT-PCR and sequencing confirmed that the 16dstop mutation was retained in RNA1 and that the CMV 2b gene was retained in RNA2 (data not shown). In the same series of experiments, expression of the 16K gene from TRV RNA2 rescued the 16dstop RNA1 in three of four inoculated plants (Fig. 4B, lanes 9, 11, and 12).

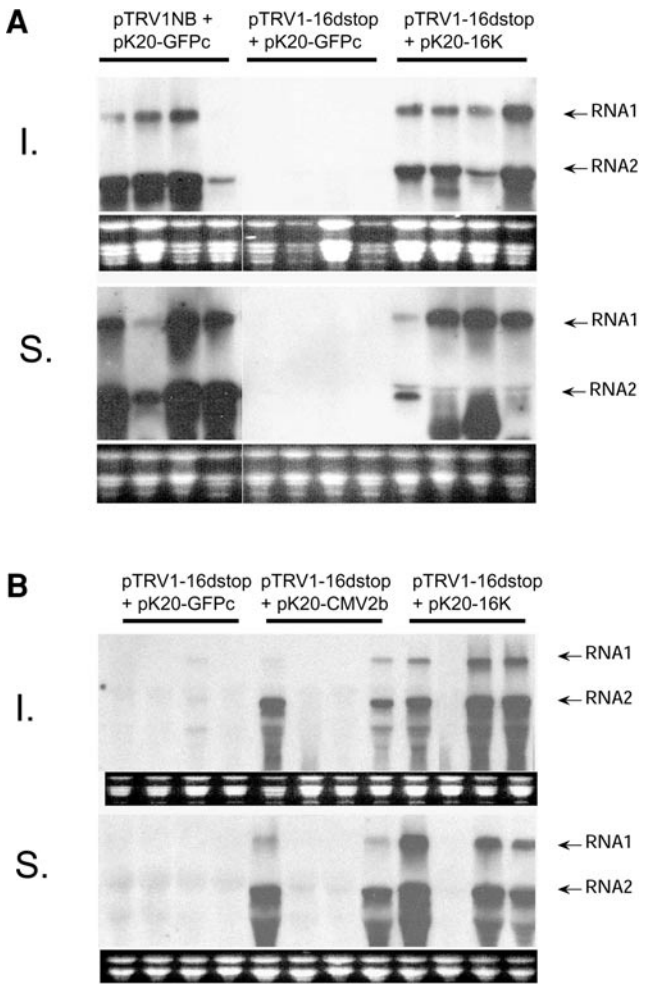


FIG. 4. Complementation of a 16K mutation *in trans*. (A) Northern blot of RNA samples of *N. benthamiana* inoculated with transcripts of pTRV1NB and pK20-GFPc, pTRV1-16dstop and pK20-GFPc, or pTRV1-16dstop and pK20-16K. Samples are from inoculated (I) and systemic (S) leaves. Expression of the 16K gene from RNA2 complements the early termination mutations in the 16K gene on RNA1. Blots hybridised with probes specific for TRV RNA1 and RNA2. The position of viral RNA 1 and 2 indicated by arrows. Relative amounts of RNA in each lane are indicated by ethidium bromide staining of rRNAs in the smaller panels. (B) Northern blot of RNA samples of *N. benthamiana* inoculated with transcripts of pTRV1-16dstop and pK20-GFPc, pTRV1-16dstop and pK20-CMV2b, or pTRV1-16dstop and pK20-16K. Expression of the CMV 2b gene from RNA2 complements the early termination mutations in the 16K gene on RNA1-16dstop.

Complementation of 16K mutation by CRP genes from diverse viruses

Similar to the tobraviruses, viruses belonging to the genera *Hordeivirus* (e.g., *Barley stripe mosaic virus*, BSMV), *Carlavirus* (e.g., Potato virus S, PVS), *Pecluvirus* (e.g., *Peanut clump virus*, PCV), *Furovirus* (e.g., *Soil-borne wheat mosaic* virus, SBWMV), and *Benyvirus* (*Beet necrotic yellow vein virus*, BNYVV) all possess a 3′-proximal gene that encodes a small (<20-kDa molecular weight) protein with an N-terminal or central cysteine-rich domain. Amino acid sequence alignment studies suggested that the tobravirus, pecluvirus, hordeivirus, and furovirus proteins, in particular, share a region of seven cysteines, with a highly conserved central motif of Cys-Gly. . .Cys-Gly-X-X-His (Diao *et al.,* 1999). TRV RNA2 constructs were made that expressed either the SBWMV 19K CRP, the BSMV γb CRP, the PVS 11K CRP, or the PEBV 12K CRP. Transcripts from these constructs were mixed with 16dstop RNA1 and inoculated to plants. Northern blotting showed that the PEBV 12K gene, the SBWMV 19K gene, and the BSMV γb gene were able to complement the TRV 16K mutation, allowing virus replication and systemic spread (Fig. 5). However, expression of the PVS 11K gene from TRV RNA2 did not overcome the effects of the 16dstop mutation.

DISCUSSION

In this study, we examined the role of the 16K protein in the replication and pathogenesis of TRV. An earlier report suggested that the 16K gene was dispensable for TRV multiplication (Guilford *et al.,* 1991). Our results con-

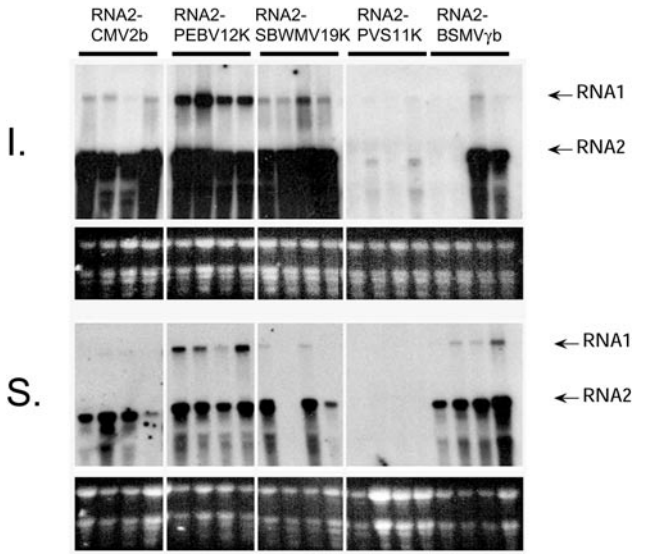


FIG. 5. Complementation of a 16K mutation by CRPs from different viruses. Northern blot of RNA samples of *N. benthamiana* inoculated with transcripts of pTRV1-16dstop and either pK20-CMV2b, pK20-PEBV12K, pK20-SBWMV, pK20-PVS11K, or pK20-BSMVγb. Samples are from inoculated (I) and systemic (S) leaves. Blots hybridised with probes specific for TRV RNA1 and RNA2. The position of viral RNA 1 and 2 indicated by arrows. Relative amounts of RNA in each lane are indicated by ethidium bromide staining of rRNAs in the smaller panels.

flict with those of this previous study, as we show that mutation of the 16K gene leads to a significant decrease in the accumulation of virus RNA in infected plants. Protoplast studies confirmed that the 16K protein is required for efficient virus replication, and overexpression of the 16K protein, whether from TRV or from PVX, led to an increase in the severity of disease symptoms. The 16K protein when expressed from TRV RNA2 functioned *in trans* to complement a mutation in the RNA1-encoded 16K gene.

Interestingly, mutation of the 16K gene was overcome by incorporation of the gene encoding the CMV 2b into TRV RNA2. Early work had identified the CMV 2b protein as a pathogenicity determinant that is required for symptom formation and systemic invasion of particular hosts (Ding *et al.,* 1995). Subsequently the CMV 2b protein was shown to be a suppressor of the posttranscriptional gene silencing (PTGS) mechanism which is a cytoplasmic, sequence-specific, RNA degradation system that in plants functions in part to combat virus infection (Beclin *et al.,* 1998; Brigneti *et al.,* 1998). These and other studies showed that many plant viruses encode proteins that can suppress PTGS (Anandalakshmi *et al.,* 1998; Kasschau and Carrington, 1998; Voinnet *et al.,* 1999, 2000). TRV was also shown to be able to suppress silencing of a GFP transgene, although the TRV silencing suppressor protein itself was not identified (Vionnet *et al.,* 1999). As the CMV 2b silencing suppressor protein can replace the TRV 16K protein for efficient virus multiplication, perhaps the TRV 16K protein is also a silencing suppressor.

Mutation of some of the other virus genes recently identified as encoding silencing suppressors results in a wide range of phenotypic effects, including reduction in accumulation of viral RNAs, reduction in symptom severity, and inhibition of long distance movement (Ding *et al.,* 1995; Cronin *et al.,* 1995; Kasschau *et al.,* 1997; Scholthof *et al.,* 1995a,b). Perhaps the results obtained following mutation of the gene encoding the *Rice yellow mottle virus* (RYMV) P1 silencing suppressor are most similar to our findings with the TRV 16K gene. The RYMV P1 protein is encoded by the 5′-terminal gene on the viral RNA. Deletion of the entire gene or insertion of a premature termination codon into the gene abolished replication of viral RNA in protoplasts. A mutant in which the P1 initiation codon was removed was able to replicate at reduced levels (ca. 50% of wild-type) in protoplast but did not accumulate either in inoculated or in upper uninoculated leaves of whole plants (Bonneau *et al.,* 1998).

Investigation of the CRPs from several other plant viruses showed that the PEBV 12K protein, SBWMV 19K protein, and the BSMV γb protein were all able to compensate for the loss of the TRV 16K protein. The PEBV 12K protein is a homolog of the 16K protein and might be expected to have the same function as the TRV protein. The complementing activity of the SBWMV and BSMV proteins is more unexpected as they have much more limited amino acid sequence homology to the TRV 16K protein. For BSMV a detailed study of the function of the CRP has been carried out. Virus in which the gene encoding the BSMV γb CRP had been deleted was able to infect barley plants systemically but viral RNAs accumulated to only 10–20% of wild-type levels and virus CP expression was reduced by three orders of magnitude (Petty *et al.,* 1990). Site-directed mutation of each of the individual cysteine and histidine residues identified above as part of the conserved CRP motif caused the same phenotype as the complete deletion mutation, emphasizing the importance of these residues in CRP function (Donald and Jackson, 1994). Other properties associated with the BSMV γb protein are seed transmission of the virus (Edwards, 1995) and RNA-binding (Donald and Jackson, 1996). Similarly, the PEBV 12K CRP also is involved in seed transmission (Wang *et al.,* 1997) and can bind RNA (D. Wang and J. Davies, personal communication).

The roles of the CRPs in SBWMV and PVS biology are not known. However, the BNYVV P14 CRP and the PCV P15 CRP are required for, respectively, accumulation of virus RNA in plants and detectable replication of virus in protoplasts (Hehn *et al.,* 1995; Herzog *et al.,* 1998). Although the TRV 16K protein is required for efficient replication of the virus, because the 16K mutation can be complemented by proteins encoded by different viruses, it seems unlikely that it functions as part of the core viral replicase. It is possible that the CRPs from TRV, SBWMV, and BSMV all act to combat a generalised plant defense

response and that, as has been shown for CMV 2b, they may suppress the plant PTGS system.

MATERIALS AND METHODS

Construction of full-length clone of TRV RNA1

Single-stranded cDNA was synthesised from total RNA extracted from *N. benthamiana* plants infected with TRV isolate PpK20 as described previously (MacFarlane *et al.,* 1991). A full-length clone of RNA1 was amplified using a proofreading polymerase with primers designed to include a T7 RNA polymerase promoter sequence and a *Sma*I restriction site at the 5′ and 3′ ends, respectively, of the virus sequence and ligated into plasmid pCR-TOPO-XL according to the manufacturer's instructions (Invitrogen). The full-length clone, pTRV1, was linearised with *Sma*I and transcribed using T7 RNA polymerase (Ambion Inc.). Transcripts were capped by addition of diguanosine triphosphate to the transcription reaction (MacFarlane *et al.,* 1991).

Mutation of the 16K gene

Inverse PCR was used to introduce a *Nde*I site immediately upstream of the 16K initiation codon and a *Bgl*II site immediately after the 16K termination codon. A fragment carrying these mutations was moved into the full-length cDNA clone to produce plasmid pTRV1NB. Subsequently, the 16K gene was deleted by digestion with *Nde*I and *Bgl*II, blunting with Klenow polymerase, and relegation to produce plasmid pTRV1-16Δ.

The *Nde*I–*Bgl*II fragment carrying the 16K gene was reamplified using a mutagenic primer (TCCATATGACGTGTGTACTC**TAG**GGTTGTGTGAATGAAGTCACTGTT) to introduce an early terminator (bold) at position 6126, 16 nucleotides downstream of the 16K initiation codon (underlined). The fragment was moved into the full-length clone pTRV1NB to produce plasmid pTRV1-16stop.

Inverse PCR was used to introduce two early terminators at positions 6120 and 6126 (bold), 10 and 16 nucleotides downstream of the 16K initiation codon (underlined) to produce the sequence ATGACGTGT**TAA**CTC**TAG**. . .. A fragment incorporating these changes but lacking the *Nde*I and *Bgl*II 16K-flanking sites was moved into the full-length clone pTRV1 to produce plasmid pTRV1-16dstop.

Expression of heterologous viral genes from TRV RNA2

The CMV 2b gene was PCR amplified to incorporate *Nco*I and *Kpn*I sites at the 5′ and 3′ ends of the gene, respectively. The *Nco*I–*Kpn*I fragment was used to replace the GFP gene carried on a similar fragment in the TRV virus vector plasmid pK20-GFPc (MacFarlane and Popovich, 2000). This new construct, pK20-CMV2b, expresses the CMV 2b protein from a duplicated tobravirus CP subgenomic promoter in TRV RNA2.

Similar strategies were used to clone the TRV 16K gene, the PEBV 12K gene, the SBWMV 19K gene, the BSMV γb gene, and the PVS 11K gene into TRV RNA2 to produce, respectively, plasmids pK20-16K, pK20-PEBV12K, pK20-SBWMV19K, pK20-BSMVγb, and pK20-PVS11K.

Inoculation and analysis of plants

Leaves of small *N. benthamiana* or *N. tabacum* cv. Samsun NN plants were dusted with Carborundum and mechanically inoculated with capped transcripts of TRV RNA1 and RNA2. RNA was isolated from inoculated and systemically infected leaves at 5–7 and 10–12 d.p.i., respectively, and aliquots from equivalent tissue samples were analysed by Northern blotting as described before (MacFarlane *et al.,* 1991) except that complementary-strand, RNA probes were prepared using a nonradioactive system (AlkPhos, Amersham Pharmacia).

Protoplasts were isolated from *N. benthamiana* plants as described before (Power and Chapman, 1985) and inoculated with transcript RNA by electroporation. RNA was extracted after 48 h and analysed by Northern blotting.

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