Modification of *Tobacco rattle virus* RNA1 to Serve as a VIGS Vector Reveals That the 29K Movement Protein Is an RNA Silencing Suppressor of the Virus

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Tobacco rattle virus (TRV) has a bipartite, positive-sense single-stranded RNA genome and is widely used for virus-induced gene silencing (VIGS) in plants. RNA1 of TRV that lacks the gene for the cysteine-rich 16K silencing-suppression protein infects plants systemically in the absence of RNA2. Here, we attempted to engineer RNA1 for use as a VIGS vector by inserting heterologous gene fragments to replace 16K. The RNA1 vector systemically silenced the phytoene desaturase (PDS) gene, although less efficiently than when the original VIGS vector system was used, which consists of wild-type RNA1 and engineered RNA2 carrying the heterologous gene. Infectious RNA1 mutants with a dysfunctional 16K suppressed silencing and enhanced transgene expression in green fluorescent protein-transgenic *Nicotiana benthamiana* following inoculation by agroinfiltration, unlike mutants that also lacked 29K, a movement protein (MP) gene. The 30K MP gene of *Tobacco mosaic virus* complemented in *cis* the movement defect but not the silencing suppression functions of TRV 29K. Silencing suppression by 29K occurred in the context of RNA1 replication but not in an agroinfiltration assay which tested 29K alone for suppression of sense-mediated silencing. Both 29K and 16K were needed to avoid necrotic symptoms in RNA1-infected *N. benthamiana*. The results shed new light on virulence factors of TRV.

Tobacco rattle virus (TRV) has a bipartite, positive-sense single-stranded RNA (ssRNA) genome and is a type member of the genus *Tobravirus* (family *Virgaviridae*) (Adams et al. 2012). RNA1 contains four open reading frames (ORF), one of which encodes a 194K protein, including the methyltransferase, helicase, and RNA-dependent RNA polymerase (RdRp) domains, and is translated from the genomic RNA (Hamilton et al. 1987; MacFarlane 1999). The 29K and 16K proteins are translated from the respective subgenomic RNAs (sgRNA) and function as

a movement protein (MP) (Hamilton and Baulcombe 1989) and a suppressor of RNA silencing (Ghazala et al. 2008; Martín-Hernández and Baulcombe 2008; Martínez-Priego et al. 2008; Reavy et al. 2004), respectively. RNA silencing is a basal cellular antiviral defense mechanism that targets double-stranded RNA, including the replicative forms of viral RNA (Wu et al. 2010). TRV RNA2 can vary in size and gene content but always encodes a coat protein (CP), which encapsidates RNA1 and RNA2 into different rod-shaped particles (Cornelissen et al. 1986; Hamilton et al. 1987; MacFarlane 1999). RNA2 also encodes the proteins 2b and 2c, which are implicated in transmission of TRV by root nematodes (Hernández et al. 1997; MacFarlane et al. 1999), and 2b also in invasion of meristems in roots (Valentine et al. 2004). All proteins encoded by RNA2 are translated from sgRNAs (MacFarlane 1999). TRV does not require CP and viral particles for viral long-distance movement in plants and, hence, systemic infection with RNA1 alone can occur (Swanson et al. 2002).

Infectious cDNA clones of TRV are used as tools for functional genomic studies on plants (MacFarlane 2010). For virus-induced gene silencing (VIGS), 2b and 2c are removed from RNA2, and a convenient multiple cloning site is engineered to facilitate insertion of the target plant gene or its fragment (Liu et al. 2002b; Ratcliff et al. 2001; Ruiz et al. 1998). The *Cauliflower mosaic virus* (CaMV) 35S promoter-driven cDNAs of RNA1 and RNA2 have been cloned into binary vectors, transformed into *Agrobacterium*, and introduced into plants by agroinfection (Grimsley et al. 1986) by, for example, infiltration of leaves (Kapila et al. 1997) or tooth-pick inoculation of stems with agrobacteria (Elmer et al. 1988). As the infection spreads, the replicating VIGS vector, which includes the plant-derived sequence, is targeted by Dicers (DCL) that recognize long double-stranded RNA and cleave it into small interfering RNAs (siRNAs) (21 to 24 nucleotides [nt]) (Xie et al. 2004). siRNAs bind to and guide the cellular ssRNA-specific RNases (Argonaute [AGO]) to cleave the homologous ssRNA molecules (i.e., the VIGS vector) and the plant mRNA that is homologous to the insert. The use of VIGS vectors enables rapid inoculation of a large number of plants and high-throughput examination of gene functions (Deng et al. 2012; Senthil-Kumar and Mysore 2011).

A VIGS vector should have rapid replication and systemic movement in plants to be an efficient gene-silencing tool. The vector virus also needs to circumvent RNA silencing to cause systemic infection and uniformly invade plant tissues but it should not suppress RNA silencing too efficiently, because that would negatively affect gene silencing. TRV is a preferred VIGS vector because it has a wide host range and is able to

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invade meristems (Martín-Hernández and Baulcombe 2008; Robinson and Harrison 1989), which allows gene silencing at early stages of organ development. However, as with viruses in general, infection with TRV-based VIGS vectors is dependent on the host genotype. For example, many potato (*Solanum tuberosum*) and tobacco (*Nicotiana tabacum*) varieties express various types of resistance to TRV infection or respond to TRV infection with necrotic or other types of symptoms, hampering observation of gene-silencing phenotypes in the host (Brigneti et al. 2004; Ghazala and Varrelmann 2007; MacFarlane 2010; Peart et al. 2002). TRV-susceptible plant genotypes may be used to overcome the problem in some cases (Deng et al. 2012) but not for plant genotype-specific research (Senthil-

Kumar and Mysore 2011). TRV RNA1 can also infect plants in the absence of RNA2, and the elimination of RNA2 may be escalated when RNA2 carries a host gene and becomes the prime target of silencing. Consequently, silencing of the target gene in systemically infected leaves may be aberrant.

The aim of this study was to test whether TRV RNA1 alone could be used as a VIGS vector carrying silencing-inducing foreign gene insertions in place of 16K. Partial or complete removal of 16K does not prevent systemic movement of TRV (Guilford et al. 1991; Martín-Hernández and Baulcombe 2008). Furthermore, because 16K is a suppressor of RNA silencing, replacement of 16K with a heterologous gene was anticipated to enhance silencing. In an attempt to alleviate the necrotic symptoms induced by TRV in many tobacco and potato genotypes, we tested whether the TRV 29K MP, which elicits necrotic symptoms (Ghazala and Varrelmann 2007; Guilford et al. 1991), could be replaced with the 30K MP of *Tobacco mosaic virus* (TMV), which complements in trans viral movement in 29K-defective TRV mutants (Ziegler-Graff et al. 1991) but whose functionality when integrated into TRV RNA1 has not been reported.

RESULTS

Disruption of TRV 16K enhances necrotic symptoms.

Three RNA1 constructs were generated to prevent 16K expression. A premature translation stop codon was introduced into 16K by site-directed mutation of the fourth codon (GTA → TGA; mutant 16Kstop) (Fig. 1). Additionally, two deletions were made within 16K of the 16Kstop mutant that removed either the 5'-proximal region (72% of 16K) or 3'-proximal part (59%) downstream from the introduced premature stop codon (constructs M1 and M2, respectively) (Fig. 1). Construct M1 is identical to the previously described mutant pTR16D4 of TRV RNA1 (Guilford et al. 1991) and was compared with the new construct M2, in which the 5'-proximal part of the 16K ORF was retained. Agroinfiltration of leaves of *N. benthamiana* with M1, M2, and 16Kstop constructs induced necrotic symptoms that were most severe with 16Kstop, whereas infiltration with wild-type RNA1 (wtRNA1) caused no necrosis (Fig. 2A, panel i). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis indicated that the titers of wtRNA1 and mutants initially increased in the upper noninoculated leaves and subsequently declined (Supplementary Fig. S1). Concentrations of wtRNA1 reached the highest level at 7 days postinoculation (dpi), whereas the highest concentration of 16Kstop RNA1 was observed at 10 dpi. Systemic accumulation of M1 and M2 reached the highest level at 13 dpi. Although wtRNA1 caused no symptoms, 16Kstop induced severe malformation in the systemically infected leaves and stunting of the plants. M1 and M2 caused milder, albeit similar, symptoms (Fig. 2A, panels ii and iii). These and other experiments of this study generally showed similar results with M1 and M2 and, hence, M1 results are often shown to represent both constructs also in the following experiments.

Detection of wtRNA1, 16Kstop, and M1 in the systemically infected leaves by Northern blot hybridization revealed the expected RNA1, sgRNA1a, and sgRNA1b sizes which, in M1 (Fig. 3A) and M2, were shorter because of a deletion in 16K. At 7 dpi, these RNAs were detected only in plants inoculated with wtRNA1 (Fig. 3A) whereas, at 13 dpi, the corresponding RNAs of M1 (Fig. 3A) and M2 were present at higher titers than were those for wtRNA1, consistent with results obtained by qRT-PCR. These results indicated that there are 16K-dependent differences in the accumulation dynamics of the viruses in the systemically infected leaves.

Co-inoculation of leaves with the wtRNA1, M1, M2, or 16Kstop together with RNA2 ("empty vector") (Fig. 2B, panel

Fig. 1. Schematic presentation of the T-DNA organization of plant binary transformation plasmids containing cDNA clones of *Tobacco rattle virus* (TRV) RNA1 and RNA2 and the TRV constructs made for this study. The viral cDNA clones, which include the 3'-untranslated region (3' UTR), are positioned between the left and right border (LB and RB) of the T-DNA, and between the *Cauliflower mosaic virus* (CaMV) 35S promoter (35s) and a transcriptional terminator (T). The TRV RNA2-based vector (pTV00) (Ratcliff et al. 2001) encodes a coat protein (CP), whereas the two genes involved in nematode transmission of TRV (2b and 2c) have been removed and replaced with a multiple cloning site (Mcs). In RNA2:GFP, the gene for *Aequoria victoriae* GFP was inserted into the Mcs, and the CP subgenomic RNA promoter (sgP) derived from *Pea early-browning virus* has been added upstream of GFP (MacFarlane and Popovich 2000). The TRV RNA1-based vector pBINTRA6 (Ratcliff et al. 2001) was modified to contain the complete TRV RNA1 cDNA encoding the RNA-dependent RNA polymerase (RdRp), the 29K movement protein (MP), and the 16K protein (16k). In TRV, RdRp is translated from the genomic RNA, whereas 29K and 16K are translated from subgenomic RNAs (sgRNA1a and sgRNA1b, respectively). Five TRV RNA1 mutants were made based on pBINTRA6. 16Kstop, M1, and M2 carried a stop codon in the fourth codon of 16K. Furthermore, most of 16K (426 nucleotides) was deleted in M1 (nucleotides 13 to 317) and M2 (nucleotides 151 to 401; deletions are indicated by black boxes). 16Kstop and M1 were similar to the mutants described by Guilford and associates (1991). In trv30K and trv30K16Kstop, all of 29K was replaced by *Tobacco mosaic virus* (TMV) 30K. Similar to 16Kstop, trv30K16Kstop carried a stop codon in the beginning of 16K.

i) induced symptoms in inoculated leaves similar to those observed with inoculation with RNA1 alone. However, symptoms in systemically infected leaves (Fig. 2B, panels ii and iii) were somewhat more severe. The mildest leaf symptoms (mosaic, mild chlorosis, and some epinasty) were caused by wtRNA1 + RNA2, with M1 and M2 causing more severe epinasty. 16Kstop induced the most severe symptoms, including

vein necrosis, necrotic lesions, epinasty, and chlorosis, in the systemically infected leaves, and plants were stunted (Fig. 2B, panel iii). Symptoms in systemically infected leaves appeared 2 to 3 days earlier than those following inoculation with RNA1 alone, and accumulation of viral RNAs reached the highest concentrations 2 to 3 days earlier than following infection solely with RNA1 (Fig. 3B). Northern blot hybridization re-

Fig. 2. Symptoms induced by *Tobacco rattle virus* (TRV) wild-type (wt)RNA1 and the RNA1 mutants M1, M2, and 16Kstop in *Nicotiana benthamiana*. **A**, Infections with RNA1 alone i) in the leaves inoculated by agroinfiltration and ii) in the systemically infected leaves 10 days postinoculation (dpi); and iii) an overall view of the morphology and symptoms at 15 dpi. **B**, Infections with RNA1 + RNA2 as shown in A. Bars in A and B indicate 2 cm.

vealed RNA1, sgRNA1a, sgRNA1b, and RNA2 in the systemically infected leaves of all inoculated plants at 7 and 13 dpi. The concentrations of M1 (and M2) RNA at 7 dpi were lower than those of wtRNA1 and 16Kstop, which were similar (Fig. 3B). These differences were in accordance with differences in the rate of systemic accumulation of wtRNA1 and the RNA1 mutants and, additionally, indicated that RNA2 enhanced systemic infection.

All of the aforementioned experiments were carried out three times independently with similar results. Mutations introduced into RNA1 were verified in the progeny viruses by RT-PCR and sequencing of the products.

Mutation of 16K does not enhance VIGS.

VIGS vectors M1:PDS and M2:PDS were generated by cloning a 200-bp fragment of the *N. benthamiana* phytoene desaturase (PDS) gene into the M1 and M2 constructs to replace the deleted part of 16K (Fig. 1). Agroinfiltration of these constructs without RNA2 into leaves of *N. benthamiana* induced symptoms similar to those obtained with M1 and M2 in the infiltrated leaves. No visually detectable PDS silencing occurred until 30 dpi in three experiments.

Following agroinfiltration for expression of RNA2 together with M1:PDS (Fig. 4A) or M2:PDS, the systemically infected leaves became chlorotic and occasionally developed white “photobleached” tissues (Kumagai et al. 1995) along the veins (Supplementary Fig. S2). Expression levels of PDS mRNA were 65% in the chlorotic tissues as compared with the control. However, when wtRNA1 + RNA2:PDS was used, extensive photobleaching was observed in the systemically infected leaves, and PDS mRNA levels were only 4% of those detected in the control (Fig. 4A).

Subsequently, the M1, M2, 16Kstop, and wtRNA1 were coinoculated with RNA2:PDS into *N. benthamiana* (Fig. 4B). Infection with M1 + RNA2:PDS (Fig. 4B, panel i, leaf 1) and M2 + RNA2:PDS induced similar chlorosis and photobleached patches in the systemically infected leaves of most plants by 20 dpi, whereas 16Kstop + RNA2:PDS induced somewhat

more extensive photobleaching in all infected plants (Fig. 4B, panel i, leaf 2). Infection with 16Kstop + RNA2:PDS also induced severe disease symptoms, including extensive necrosis in systemically infected leaves, at 20 dpi (Fig. 4B, panel i, leaf 2'). However, systemic infection with wtRNA1 + RNA2:PDS induced no necrosis (Fig. 4B, panel i, leaf 3'), although this combination caused the most extensive photobleaching among all constructs. qRT-PCR analysis showed that infection with M1 + RNA2:PDS, 16Kstop + RNA2:PDS, and wtRNA1 + RNA2:PDS decreased PDS mRNA levels to 43, 37, and 4%, respectively, of the amounts detected in the leaves of noninoculated control plants (Fig. 4B, ii). Results were similar in three independent experiments.

Northern blot analysis indicated that the amounts of PDS mRNA and PDS mRNA-derived siRNA in the systemically infected tissues were negatively correlated and were in accordance with the observed photobleaching phenotypes (Fig. 4B, iii). Significant amounts of PDS-homologous 21-nt siRNA were detected following infection with wtRNA1 + RNA2:PDS, which caused the most efficient PDS silencing (Fig. 4B, iii). These results were expected because 21-nt siRNA is produced by DCL4, which is the primary sensor of viral double-stranded RNA (dsRNA) (Xie et al. 2004). PDS-homologous 24-nt siRNA was detected following infection with all the different constructs and also in lower amounts in healthy *N. benthamiana*. Production of 24-nt siRNA by DCL3 depends on cellular RdRp 2 (RDR2) and controls transcriptional gene silencing (Kasschau et al. 2007; Matzke and Birchler 2005). The 24-nt siRNA constitutes the most abundant siRNA class in flowering plants, and its production from PDS mRNA might be associated with high expression levels of PDS.

16K stabilizes TRV RNA2:GFP.

To augment noninvasive monitoring of systemic infection, green fluorescent protein (GFP) was inserted in TRV RNA2 (construct RNA2:GFP) (Fig. 1). *N. benthamiana* plants infiltrated with wtRNA1 + RNA2:GFP showed intensive GFP fluorescence in systemically infected leaves 9 dpi (Fig. 5A). In contrast, no GFP fluorescence was observed following infection with 16Kstop + RNA2:GFP or M1 + RNA2:GFP, although apparent symptoms of viral infection such as epinasty and necrosis, respectively, were observed in systemically infected leaves (Fig. 5A).

Northern blot analysis detected a band corresponding to the expected size of RNA2:GFP in leaves that were systemically infected with wtRNA1 + RNA2:GFP (Fig. 5B). In contrast, the leaves systemically infected with M1 + RNA2:GFP and 16Kstop + RNA2:GFP contained only truncated forms of RNA2:GFP at 9 dpi (Fig. 5B). Each type of infection was analyzed in three plants in three independent experiments and results were consistent. RT-PCR using RNA2-specific primers flanking GFP and sequencing of the PCR products revealed that the GFP insert was lost from the progeny viruses of M1 + RNA2:GFP. In addition, GFP and the RNA2 sequences flanking the GFP insert were lost from the progeny viruses of 16Kstop + RNA2:GFP in many plants (Fig. 5C). However, full-length RNA2:GFP was detected in the systemically infected leaves of all plants infected with wtRNA1 + RNA2:GFP, although some plants also had shorter PCR products, indicating recombination and deletion (Fig. 5C). Each type of infection was analyzed in six plants in three independent experiments and results were consistent, indicating that an absence of 16K from RNA1 increased the instability of RNA2:GFP. On the other hand, the RNA1 mutants M1, M2, and 16Kstop were stable, as shown by analysis of the 3'-proximal part of RNA in the systemically infected leaves by RT-PCR followed by sequencing of the amplification products.

Fig. 3. Detection of *Tobacco rattle virus* (TRV) RNAs in the apical leaves of infected plants at 7 and 13 days postinoculation (dpi). The 3' untranslated region is identical in RNA1 and RNA2 and was used as a probe to detect both genomic RNAs and all subgenomic (sg)RNAs by Northern blot analysis. **A**, Infection with RNA1 alone. **B**, Infection with RNA1 + RNA2. In each panel: lane a, wild-type (wt)RNA1; lane b, 16Kstop; and lane c, M1. Note that RNA1, sgRNA1a, and sgRNA1b were shorter in M1 than wtRNA1 and 16Kstop because of deletion in the 16K gene of M1. 28S rRNA, 28S ribosomal RNA used as a loading control.

Fig. 4. Induction of phytoene desaturase (PDS) silencing (photobleaching) by *Tobacco rattle virus* (TRV) vectors in the systemically infected leaves of *Nicotiana benthamiana*. **A**, M1:PDS co-inoculated with RNA2 induced milder PDS silencing (chlorosis, leaf 1) than did co-inoculation with wild-type (wt)RNA1 and RNA2:PDS (leaf 2) at 20 days postinoculation (dpi). Control: uninoculated plant (leaf 3). Bars indicate 2 cm. Samples taken from chlorotic and photo-bleached areas, respectively, after photography of leaves were subjected to quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (right). Relative PDS mRNA expression in leaves systemically infected with (1) M1:PDS + RNA2 and (2) wtRNA1 + RNA2:PDS, and in (3) healthy plants. Error bars indicate standard error ($n = 3$). **B**, Panel i: at 15 dpi, M1 + RNA2:PDS (leaf 1) and 16Kstop + RNA2:PDS (leaf 2) induced less-intensive PDS silencing in the systemically infected leaves than did wtRNA1 + RNA2:PDS (leaf 3). Photos 2' and 3' show the whole plants at 20 dpi. Panel ii: relative PDS mRNA expression levels analyzed by qRT-PCR in the leaves infected with TRV virus-induced gene silencing (VIGS) vectors at 15 dpi were correlated with the visual appearance of photobleaching. Numbers on the graph correspond to the leaves in panel i. Error bars indicate standard error ($n = 3$). Panel iii: Northern blot analysis on PDS mRNA and the corresponding 21- and 24-nucleotide (nt) small interfering (si)RNAs in photobleached areas of leaves infected with VIGS vectors at 15 dpi. Lane numbers correspond to the leaves in panel i. In the PDS siRNA blot, the rightmost lane shows 21- and 24-nt siRNA markers run in the same gel. 28S and 5S ribosomal RNA (rRNA) and tRNA were used as loading controls in mRNA and siRNA analyses, respectively.

Fig. 5. Dysfunctional *16K* increases instability of RNA2:GFP during *Tobacco rattle virus* (TRV) infection in *Nicotiana benthamiana*. **A**, Green fluorescent protein (GFP) fluorescence in the systemically infected leaves at 9 days postinoculation (dpi). Photos in the upper panel were taken under UV light to reveal GFP fluorescence, whereas photos in the lower panel were taken under normal light. Necrotic tissue in the leaf infected with 16Kstop + RNA2:GFP shows autofluorescence under UV light. **B**, Northern blot analysis using 3' untranslated region as a probe revealed only truncated forms of RNA2:GFP (arrows) at 9 dpi in the systemically infected leaves of plants inoculated with M1 + RNA2:GFP and 16Kstop + RNA2:GFP. Shorter sizes of subgenomic (sg)RNA1a and sgRNA1b in M1 are due to the introduced deletion in the *16K* gene. **C**, Reverse-transcription polymerase chain reaction (RT-PCR) analysis with primers flanking the *GFP* insert revealed the PCR product of expected size (approximately 2 kb) in the leaves systemically infected with wtRNA1 + RNA2:GFP. In contrast, truncated forms of RNA2 lacking *GFP* (M1 + RNA2:GFP) or, usually, no amplification products (16Kstop + RNA2:GFP) indicating the frequent loss of RNA2 regions that flank *GFP*, were detected following systemic infection with the RNA1 mutants carrying a dysfunctional *16K* gene. Three lanes per construct represent samples from three different plants.

TMV 30K can substitute for TRV 29K as an MP in TRV.

TRV mutants that were unable to express 16K unexpectedly induced more-severe necrotic symptoms than did the wtRNA1. Because the 29K MP of TRV induces necrotic symptoms in plants (Ghazala and Varrelmann 2007; Ziegler-Graff et al. 1991) and the 30K MP of TMV can functionally complement defective 29K of TRV in trans (Ziegler-Graff et al. 1991), two additional TRV RNA1 mutants were developed by replacing 29K with TMV 30K in wtRNA1 (construct trv30K) and 16Kstop (trv30K16Kstop) (Fig. 1).

Agroinfiltration of trv30K (without RNA2) into leaves of *N. benthamiana* induced necrosis first in the infiltrated leaf and, subsequently, in the petiole and the base of the stem (Fig. 6A). The expanding apical leaves displayed necrosis in petioles and leaf veins by 16 dpi. This pattern of symptom development in the trv30K-infected plants was consistent with systemic spread of TMV-GFP in *N. benthamiana* (Kreuze et al. 2005). Top necrosis was observed in a few systemically infected plants (Fig. 6B), and all plants were stunted (Fig. 6D). In contrast, agroinfiltration with trv30K16Kstop (without RNA2) caused necrosis only in infiltrated leaves and petioles (Fig. 6C). No spread of symptoms and virus beyond the base of the petiole was observed by 21 dpi (Fig. 6D) and 30 dpi at termination of the experiment, indicating that the virus was unable to pass through the abscission zone (Vuorinen et al. 2011).

Analysis of the inoculated leaves by Northern blot hybridization revealed sgRNA1a and sgRNA1b for trv30K16Kstop and trv30K, which indicated that both viruses were able to replicate, although the RNAs of trv30K16Kstop accumulated at very low titers and were detected only following extended exposure time (Supplementary Fig. S3A). Furthermore, the RNA titers of both of these viruses were lower than those of wtRNA1, 16Kstop, and M1.

Inoculation with trv30K + RNA2 and trv30K16Kstop + RNA2 induced milder symptoms than did infection with trv30K and trv30K16Kstop alone. Leaves systemically infected with trv30K + RNA2 displayed mosaic symptoms and mild epinasty (Fig. 6E), similar to the symptoms described for TMV (Shew and Lucas 1991), and the symptoms appeared 3 to 5 days earlier than after infection with trv30K alone. RT-PCR results showed that RNA1 was present in the apical leaves of plants infected with trv30K + RNA2 but was absent from plants inoculated with trv30K16Kstop either alone or with RNA2.

Co-infiltration of trv30K with RNA2:PDS or RNA2:GFP caused photobleaching (Fig. 6J) or the appearance of GFP fluorescence (Fig. 6G), respectively, in the apical noninoculated *N. benthamiana* leaves at 25 dpi, which was not observed with trv30K16Kstop + RNA2:GFP (Fig. 6H). In plants co-

infiltrated with wtRNA1 + RNA2:GFP, the systemically infected leaves expressed GFP fluorescence by 21 dpi (Fig. 6I).

Similar results were found for three independent experiments. Taken together, they showed that TMV 30K MP was able to substitute for TRV 29K as an MP in *cis* and altered, but did not alleviate, virus symptoms. However, accumulation of TRV was reduced and systemic movement was delayed after substitution of 29K with TMV 30K.

TRV 29K contributes to suppression of RNA silencing.

The ability of TRV 29K, TRV 16K, and TMV 30K to interfere with sense-mediated RNA silencing (co-suppression) was tested using the “silencing on the spot” agroinfiltration assay (Johansen and Carrington 2001) in leaves of *GFP*-transgenic *N. benthamiana* line 16c (Brigneti et al. 1998). TRV 29K and TMV 30K were unable to prevent GFP silencing when expressed in the leaves by agroinfiltration together with a binary

Fig. 6. Systemic infection and symptoms induced with *Tobacco rattle virus* (TRV) RNA1 mutants trv30K and trv30K16Kstop in *Nicotiana benthamiana*. All photos were taken 21 days postinoculation (dpi) unless specified differently. **A**, Severe necrosis in the petiole of the leaf inoculated with trv30K and in the base of the stem. **B**, Apical necrosis caused by trv30K at 28 dpi. **C**, Severe necrosis in the petiole of a leaf inoculated with trv30K16Kstop. Necrosis did not expand to the stem base. **D**, Severe stunting of a plant systemically infected with trv30K (left) as compared with a plant inoculated with trv30K16Kstop (right), which shows no systemic symptoms. **E**, Vein clearing and epinasty in a leaf systemically infected with trv30K + RNA2. **F**, Leaf systemically infected with wtRNA1 + RNA2. **G**, Green fluorescent protein (GFP) fluorescence in the systemically infected leaves of plants infected with trv30K + RNA2:GFP. **H**, No GFP fluorescence was observed in the apical noninoculated leaves of plants inoculated with trv30K16Kstop + RNA2:GFP. **I**, GFP fluorescence in a plant infected with wtRNA1 + RNA2:GFP. **J**, Phytoene desaturase (PDS) silencing induced by trv30K + RNA2:PDS at 25 dpi. Bars in E and F = 1 cm; in other panels, bar = 2 cm.

vector expressing *GFP* mRNA to induce GFP co-suppression (Supplementary Fig. S4). In contrast, co-infiltration of *GFP* and the binary vector for expression of TRV 16K (Martínez-Priego et al. 2008) or HC-Pro of *Potato virus A* (Kreuze et al. 2005; Rajamäki and Valkonen 2009) prevented *GFP* silencing and supported strong expression of GFP fluorescence.

Subsequently, the infectious RNA1 mutants and wtRNA1 were tested for their ability to interfere with *GFP* silencing. As expected, GFP fluorescence initially increased in the agroinfiltrated tissue up to 2 to 3 dpi because of GFP overexpression from the introduced binary vector but subsequently diminished by 5 dpi because of co-suppression in leaf tissues lacking an agroinfiltration-introduced silencing suppressor (Fig. 7A, right side of the leaves). wtRNA1 was most efficient in silencing suppression. In addition, M1, M2, 16Kstop, and trv30K showed relatively efficient and similar suppression of *GFP* silencing, in contrast to trv30K16Kstop, which did not prevent *GFP* silencing (Fig. 7A, left side of the leaves). GFP fluorescence from 3 to 7

dpi was positively correlated with GFP mRNA levels and negatively correlated with accumulation of *GFP*-derived siRNAs in the agroinfiltrated leaf tissue (Fig. 7C). Differences in silencing suppression were further highlighted in the leaves systemically infected with RNA1. wtRNA1, and all other RNA1 constructs carrying 29K (M1, M2, and 16Kstop) enhanced GFP fluorescence regardless of whether they contained 16K, and trv30K enhanced GFP fluorescence despite the lack of 29K (Fig. 7B).

Taken together, these data show that all RNA1 mutants devoid of TRV 16K (M1, M2, and 16Kstop) or 29K (trv30K) but not both genes (trv30K16Kstop) could suppress sense-mediated silencing and that silencing suppression by 29K was associated with multiplication of RNA1.

DISCUSSION

This study aimed to develop a VIGS vector based on TRV RNA1 by partial removal of 16K to vacate space for the heter-

Fig. 7. Suppression of sense-mediated silencing of green fluorescent protein (GFP) expression by *Tobacco rattle virus* (TRV) RNA1 and its mutated forms. **A**, Induction of GFP silencing in leaves of the GFP-transgenic *Nicotiana benthamiana* line 16c by overexpression of GFP mRNA “on the spot” from an introduced binary vector pBIN:GFP by agroinfiltration (right and left side of the leaves) and interference with silencing by introducing TRV wild-type (wt)RNA1 or RNA1 mutants by co-infiltration with pBIN:GFP (left side only of the leaves). Suppression of silencing was indicated by GFP fluorescence in the co-infiltrated leaf tissue 5 days postinoculation (dpi). **B**, Effect of systemic infection with TRV wtRNA1 and mutants on the expression level of GFP fluorescence in the systemically infected leaves of the 16c plants at 14 dpi. **C**, Northern blot analysis for detection of GFP mRNA and small interfering (si)RNA in tissues co-infiltrated with pBIN:GFP and the RNA1 construct at 3, 5, and 7 dpi. *GFP* was used as a probe. ‘Agro’ indicates infiltration with *Agrobacterium* carrying a binary vector without an insert (control); 21-24 siRNA represents the 21- and 24-nucleotide siRNAs. 28S and 5S ribosomal RNA (rRNA) and transfer RNA were used as loading controls.

ologous insert and to reduce the silencing suppression capacity of TRV. The M1 and M2 vectors were infectious and competently spread systemically without 16K, in accordance with two previous studies (Guilford et al. 1991; Martín-Hernández and Baulcombe 2008), and could be used to induce partial silencing of PDS in systemically infected leaves of *N. benthamiana*. However, systemic spread of M1 and M2 was delayed as compared with wtRNA1, and silencing achieved with M1:PDS was not as efficient as with M1 + RNA2:PDS or wtRNA1 + RNA2:PDS. One reason may be that, because TRV RNA1 encodes the proteins needed for replication of RNA1 and RNA2, targeting RNA1 rather than RNA2 to silencing probably reduces the capacity to produce silencing-inducing PDS RNA as compared with when silencing is primarily targeted to RNA2, as in the commonly used TRV-based VIGS vectors (Liu et al. 2002b; Ratcliff et al. 2001). The impaired ability to suppress silencing also impaired invasiveness and accumulation of RNA1 mutants in plant tissues. The balance of silencing and silencing suppression for efficient VIGS seems to be more optimal with wtRNA1 than any of the RNA1 mutants.

Studies on mutant trv30K showed that TMV 30K MP, when used in *cis* to replace TRV 29K in TRV RNA1, maintained cell-to-cell and long-distance movement functions for TRV RNA1 and RNA2, which is supported by a previous study showing that 30K can complement movement of 29K-defective TRV mutants in trans (Ziegler-Graff et al. 1991). However, when expression of 16K was interrupted in the recombinant RNA1 carrying TMV 30K (mutant trv30K16Kstop), virus accumulation was greatly reduced in inoculated leaves, and no systemic infection was observed. Synthesis of sgRNAs provided evidence that trv30K16Kstop was able to replicate in the agroinoculated leaf tissue, but accumulation of viral RNAs remained at a very low level. These results suggest that trv30K16Kstop could not suppress RNA silencing, in contrast to the viral mutants encoding 16K or 29K, which accumulated to high titers, albeit not to titers as high as wtRNA1, which encodes both 16K and 29K. These data, together with the results from silencing-suppression experiments, indicate that TRV 29K contributes to suppression of RNA silencing, which has previously been reported only for 16K of TRV (Ghazala et al. 2008; Martín-Hernández and Baulcombe 2008; Martínez-Priego et al. 2008; Reavy et al. 2004). Because the key experiments on silencing suppression were done using only RNA1, any contribution of RNA2 or CP to silencing suppression observed in this study could be excluded.

The 16K protein suppresses silencing when expressed alone, as shown by our study and previous reports (Ghazala et al. 2008; Martín-Hernández and Baulcombe 2008; Martínez-Priego et al. 2008). In contrast, RNA silencing suppression by 29K was revealed only in the context of RNA1 replication. Hence, 29K seems to protect viral RNA from silencing in an intimate association with the replication complex but does not require 16K for this activity (mutants M1, M2, and 16Kstop). On the other hand, 194K, which is encoded by RNA1 and encompasses the viral replicase, helicase, and methyl transferase functions, was not able to suppress silencing in the absence of 29K (mutant trv30K16Kstop). These results indicate that, whereas 16K suppresses RNA silencing independently, silencing suppression by 29K and 194K are interdependent.

The 29K protein is associated with TRV-induced symptoms (Ghazala and Varrelmann 2007; Ziegler-Graff et al. 1991), and overexpression of 29K under the control of the CaMV 35S promoter induces necrosis in potato cultivars (Ghazala and Varrelmann 2007). The RNA1 mutants carrying a dysfunctional 16K (M1, M2, and 16Kstop) or in which 29K was substituted with TMV 30K (mutant trv30K) caused necrotic symptoms in the inoculated and systemically infected leaves of

N. benthamiana, consistent with results obtained using another TRV RNA1 mutant in which the expression of 16K was prevented (Martín-Hernández and Baulcombe 2008). Hence, 29K and 16K of TRV appear to have coordinated functions during the infection cycle of TRV, which alleviate host responses that are deleterious to the virus and which are also important for successful use of TRV as a VIGS vector. The TMV 30K MP gene was not competent to substitute for TRV 29K in this respect, as indicated by the severe necrotic symptoms caused by trv30K that carried 16K. Necrotic symptoms are also induced following overexpression of 29K (Ziegler-Graff et al. 1991) or 16K (H. Liu et al. 2002a) from an additional copy of the gene cloned in place of the 16K ORF in RNA1 or into RNA2, respectively, further supporting the requirement of balanced production of 16K and 29K to maintain favorable conditions for the infection cycle of TRV.

It was unexpected to find the most severe necrotic symptoms in *N. benthamiana* induced by the mutant 16Kstop that contains only a few nucleotide substitutions in the beginning of the 16K ORF that prevent 16K expression. According to MacFarlane (2010), the ORF for 16K contains another, putative ORF for a 13K protein in a different reading frame but little is known about whether the putative 13K protein is produced during the infection cycle of TRV. The 13K ORF would remain unaffected in 16Kstop, unlike the mutants M1 and M2 lacking most of the 16K ORF. Overlapping ORF are common in RNA viruses (Chirico et al. 2010; Chung et al. 2008; McFadden et al. 2011), and the proteins expressed from overlapping ORF typically play a role in viral pathogenicity and spread (McFadden et al. 2011; Rancurel et al. 2009). At present, the existence of 13K and its role in the severe necrosis induced by 16Kstop remain enigmatic.

Coordinated functions of RNA1 and RNA2 were evident in the experiments using the RNA1 mutants. In the absence of a functional 16K (mutants M1, M2, and 16Kstop), necrotic symptoms were induced in *N. benthamiana* infected with TRV RNA1. Co-infection with wtRNA2 (or RNA2:PDS) did not alleviate necrosis but enhanced systemic infection. Infection with trv30K also caused severe necrosis in inoculated leaves as well as necrosis in apical leaves following systemic infection but these severe necrotic symptoms were not observed in the leaves systemically infected with trv30K + RNA2:GFP or trv30K + RNA2:PDS. Therefore, whereas TMV 30K was not able to complement the silencing-suppression functions of 29K in TRV infection, it could complement the putative functions of 29K that are needed to inhibit the harmful host responses and that involve RNA2, the CP encoded by RNA2, or both. This model gains support from previous studies on transgenic tobacco plants that express the TMV 30K and do not develop necrotic symptoms following infection with a TRV 29K mutant that causes necrosis in wild-type tobacco plants (Ziegler-Graff et al. 1991).

The coordinated functions of RNA1 and RNA2 were also evident with the increased instability of the GFP insertion in RNA2 in infections involving RNA1 that carried a dysfunctional form of 16K. This result is potentially significant because it suggests that the silencing suppression may be functionally linked to suppression of recombination during virus replication. Variability in size and gene content of RNA2 is characteristic of isolates of TRV and other tobamoviruses, including recombination of 3'-proximal sequences from RNA1 to RNA2 (MacFarlane 2010), and the role of 16K (or the putative 13K) might be to control these events. The 16K protein is believed to interfere with RNA silencing after the initial step of recognition and cleavage of long dsRNA molecules by DCL (Martínez-Priego et al. 2008). Next, siRNA molecules bind to and guide the RNA silencing complex containing an ssRNA-specific ribonuclease (AGO1 or AGO2) to cleave and inactivate the homologous ssRNA mole-

cules (Harvey et al. 2011). A secondary pool of virus-derived siRNA is generated by the cellular RdRps RDR1 and RDR6, which recognize cleaved ends of ssRNA and synthesize a complementary strand, hence producing new substrates for DCL (Qu 2010). Whether i) the viral ssRNA cut by AGO can be used as a template by the viral RdRps and ii) there is competition for viral ssRNA by the viral and host RdRps need to be determined in the context of template switching reported for RdRps of many viruses (Sztuba-Solinska et al. 2011). Such potential competition may explain the acceleration of recombination in the absence of 16K, which might suppress recombination by interfering with AGO and the cleavage of viral RNAs. Many unrelated viral silencing suppressors interfere with AGOs or host RdRps (Baumberger et al. 2007; Bortolamiol et al. 2007; Du et al. 2011; Glick et al. 2008). Nuclear localization (Liu et al. 1991) may also have a role in the putative mechanism of silencing suppression of 16K (Ghazala et al. 2008). The silencing-suppressor proteins of a few other plant-infecting RNA viruses accumulate in the nucleus and nucleolus (Rajamäki and Valkonen 2009; Taliansky et al. 2010). According to some studies, the 2b protein of *Cucumber mosaic virus* interacts with AGOs in the nucleolus to facilitate silencing suppression functions (Duan et al. 2012), whereas other studies suggest that nuclear localization of the 2b protein is not required for silencing suppression (Gonzalez et al. 2012). Many questions related to control of recombination by the 16K ORF remain to be addressed in future studies, but the findings reported here provide another perspective of the factors playing a role in stability of heterologous gene insertion in viral genomes and VIGS vectors.

Taken together, this study showed that it is possible, with some limitations, to use RNA1 of TRV as a VIGS vector in *N. benthamiana*, in addition to its previous use as a vector for expression of heterologous proteins such as TMV CP (Guilford et al. 1991) or an extra copy of TRV 29K (Ziegler-Graff et al. 1991) in tobacco leaves. Results also suggested coordinated functions between 29K and 16K, and TRV RNA1 and RNA2, which alleviate host responses harmful to the virus. Most importantly, suppression of RNA silencing by 29K was detected and found to work only in association with viral replication, which was different from 16K that also suppressed silencing independently. These findings provide new insights into silencing suppression by TRV and suggest different mechanisms used by 29K and 16K. Altogether, the results shed new light on virulence factors of TRV.

MATERIALS AND METHODS

Plasmid constructs.

The binary vectors pBINTRA6 and pTV00 containing the cDNA of TRV RNA1 and RNA2, respectively, under the CaMV 35S promoter have been described (Ratcliff et al. 2001) and were obtained from the Sainsbury Laboratory, Norwich, U.K. (courtesy of D. C. Baulcombe). pTV00 contained a multiple cloning site. Details of these binary vectors and the five TRV RNA1 mutants made in this study are provided in Figure 1. The primers used for mutagenesis, cloning, and PCR are listed in Supplementary Table S1.

An intermediate plasmid, pJK1, was constructed by digesting pBINTRA6 with *SalI* and *SmaI* and introducing the digested fragment (nucleotides 3,786 to 7,653; GenBank accession number AX391686) into the pBluescript II SK(+) plasmid (Fermentas, St. Leon Rot, Germany). This fragment of TRV RNA1 contains the 29K and 16K genes and part of the *RdRp* gene and was the target of mutations introduced using the Site-Directed Mutagenesis Kit (Finnzymes, Espoo, Finland). Subsequently, mutated viral genome fragments were digested and introduced back into pBINTRA6 using *SalI* and *SmaI* sites.

For mutant M1, the 3'-proximal deletion and a stop codon were introduced simultaneously into 16K using PCR (primers 16K_dM1F and 16K_dM1R). For mutant 16Kstop, one premature stop codon was introduced by PCR (primers 16K_StopF and 16K_StopR), and the fourth codon of 16K was mutated from GTA to TGA. Mutant M2 was generated from 16Kstop by PCR with primers 16K_dM2F and 16K_dM2R. Two restriction sites, *PacI* and *AscI*, were introduced into M1 and M2 by PCR. Mutants 16Kstop and M1 were similar to those used by Guilford and associates (1991).

To construct trv30K, the whole 29K gene was first removed from the intermediate plasmid pJK1 (primers 29K_dF and 29K_dR) and replaced with TMV 30K that had been amplified by PCR (primers trv30K_F and trv30K_R) from the plasmid TMV p30B-GFPC3 (courtesy of W. O. Dawson, Citrus Research and Education Center, Florida) (Kreuze et al. 2005; Shivprasad et al. 1999). Mutant trv30K16Kstop was generated from trv30K essentially as described for 16Kstop.

A fragment of *PDS* from *N. benthamiana* (GenBank accession number EU165355.1) corresponding to nucleotides 1,094 to 1,295 was amplified by PCR (primers PDS_F and PDS_R) and cloned into M1 and M2 using *AscI*, resulting in constructs M1:PDS and M2:PDS, respectively. RNA2:PDS was constructed by cloning the same *PDS* fragment into pTV00.

RNA2:GFP was designed as described (MacFarlane and Popovich 2000). The complete *GFP* gene was amplified by PCR from *Potato virus A* (PVA):GFPuv (primers GFPuv_F and Gateway_GFP_R) (Rajamäki et al. 2005). The CP promoter (subgenomic RNA promoter [sgP], 237 nt) of *Pea early-browning virus* (MacFarlane and Popovich 2000) was constructed from synthesized oligonucleotides by PCR. sgP and *GFP* sequences were fused and cloned to the gateway plasmid pYL179 (provided by S. P. Dinesh-Kumar, University of California, Davis) (Liu et al. 2002c) according to the manufacturer's instructions to obtain RNA2:GFP.

The binary vectors pBIN:GFP (Haseloff et al. 1997; Voinnet et al. 1998) and pA:HcPro (Savenkov and Valkonen 2001) have been described. pA:16K, pA:29K, and pA:30K were developed based on pA:HcPro by replacing the PVA HC-Pro sequence for PCR-amplified 16K, 29K, and 30K, respectively (primers 16K_F and 16K_R, 29K_F and 29K_R, and 30K_F and 30K_R, respectively).

Plant material.

Seed of the GFP-transgenic *N. benthamiana* line 16c (Brigneti et al. 1998) (supplied by D. Baulcombe) and wild-type *N. benthamiana* seed were planted in soil prepared by mixing peat (Kekkilä Horticulture Peat, Kekkilä Oyj, Finland) and washed sand at a 5:1 ratio (vol/vol). Plants were grown in a growth room under controlled conditions (21 and 19°C day and night temperatures, respectively; 16-h photoperiod; 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity; and 40% relative humidity). The plants were watered when needed and fertilized weekly with a 1.0% solution of N/P/K (8:4:6 or 16:9:22) fertilizer (Yara, Espoo, Finland).

Virus infection and agroinfiltration.

Binary vectors were introduced into *Agrobacterium tumefaciens* C58Ci(pGV2260) (Deblaere et al. 1985). *Agrobacterium* cells were grown and collected as described (Liu et al. 2002c) and resuspended in infiltration medium containing 10 mM 2-(*N*-morpholino) ethanesulfonic acid, 200 μM acetosyringone, and 10 mM MgCl_2 . For virus inoculations, the *Agrobacterium* suspension was diluted to an optical density at 600 nm (OD_{600}) = 1 and, for silencing suppression assays, to OD_{600} = 0.4. The diluted *Agrobacterium* suspensions were incubated at room temperature for 3 h. For co-infiltration with two plasmids,

the two respective *Agrobacterium* cultures were mixed 1:1 (vol/vol) before infiltration. *Agrobacteria* were syringe infiltrated into the lower side of leaves using 1-ml syringes lacking a needle. For virus infection and VIGS, 4-week-old seedlings were agroinfiltrated at the four-leaf stage. For the transient *Agrobacterium*-mediated expression assay, approximately 6-week-old plants were agroinfiltrated at the eight-leaf stage.

Northern blot analysis.

Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.). Low molecular weight (LMW) and high molecular weight (HMW) RNAs were separated in the presence of 2 M lithium chloride as described (Llave et al. 2000) and used for siRNA and mRNA detection, respectively. For mRNA analysis, 10 µg of HMW RNA was separated on a 1.2% (wt/vol) formaldehyde agarose gel by electrophoresis, transferred to a nylon membrane (Hybond-N; Amersham, Bucks, U.K.), and cross-linked with UV light. To analyze siRNA, 15 µg of LMW RNA was mixed with an equal volume of deionized formamide and separated by electrophoresis in a denaturing polyacrylamide gel (15%, wt/vol). RNA was transferred to a nylon membrane (Hybond NX; Amersham) and cross-linked with EDC cross-linking solution (Pall and Hamilton 2008). Prehybridization, hybridization, and washing were done as described (Hamilton and Baulcombe 1999). A DNA probe labeled with Dig-11-dUTP (Roche, Mannheim, Germany) corresponding to the 3' untranslated region of TRV RNA1 was PCR amplified and used to detect TRV RNA2, TRV RNA1, and the sgRNAs (Fig. 1). For detection of mRNA and siRNA of *PDS* and *GFP*, antisense *PDS* and *GFP* RNA probes were synthesized and randomly labeled with [α -³²P]rUTP (Perkin Elmer, Boston) through in vitro transcription (T3 RNA polymerase; Promega, Charbonnières, France) using the following templates: *Apa*I-digested pGEM-T (Promega Corp., Madison, WI, U.S.A.) carrying an 801-nt fragment of *N. benthamiana* *PDS*, or *Xba*I-digested pGFPuv (GenBank accession number U62636) carrying the full-length GFP ORF (717 nt) (Kreuze et al. 2005). For hybridization with siRNAs, the probe was cleaved by alkaline hydrolysis to approximately 50-nt fragments, as described (Hamilton and Baulcombe 1999). After final washing, the blots were wrapped in polyethylene plastic and exposed to film (Kodak, Rochester, NY, U.S.A.).

qRT-PCR.

First-strand cDNA was synthesized from 1 µg of total RNA using SuperScript III Reverse Transcriptase Kit (Invitrogen). cDNA (20 µl) was diluted 10-fold to a final volume of 200 µl. qPCR was performed using the LightCycler 480 Real-Time PCR System (Roche). The PCR master mix (15 µl) contained 4.5 µl of cDNA, 7.5 µl of LightCycler 480 SYBR Green I Master mix, and 1.5 µl of (5 µM) each primer. The thermal cycler program was an initial cycle of denaturation (95°C for 10 min) followed by 45 cycles of amplification (95°C for 10 s, 62°C for 10 s, and 72°C for 10 s). The *N. benthamiana* elongation factor 1 α (*EF1 α*) gene (GenBank accession number AY206004.1) was used as an internal control for normalization of data (primers qPCR_EF1 α _F and qPCR_EF1 α _R). qPCR efficiencies were calculated with the LightCycler 480 program by analyzing a dilution series of cDNA. The relative expression ratios of each mRNA were calculated as described (Pfaffl 2001), with normalization based on *EF1 α* .

GFP imaging.

GFP fluorescence was monitored with a hand-held UV source (Black-Ray, Upland, CA, U.S.A.). Images were captured with a digital camera (Canon EOS 40D; Canon, Tokyo) equipped with a Canon EFS 17- to 85-mm objective lens. The

images were further analyzed with the CorelDRAW X5 program (Corel Corp., Ottawa, ON, Canada).

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