Functional characterization and subcellular localization of the 16K cysteine-rich suppressor of gene silencing protein of tobacco rattle virus

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The 16 kDa cysteine-rich protein (16K) of tobacco rattle virus (TRV) is known to partially suppress RNA silencing in Drosophila cells. In this study, we show that 16K suppresses RNA silencing in green fluorescent protein (GFP)-transgenic Nicotiana benthamiana plants using an Agrobacterium-mediated transient assay. 16K slightly reduced the accumulation of short interfering RNAs (siRNA) of GFP, suggesting that the protein may interfere with the initiation and/or maintenance of RNA silencing. Deletion of either the N- or C-terminal part of 16K indicated that the entire 16K open reading frame (ORF) is necessary for its silencing suppression function. Pentapeptide insertion scanning mutagenesis (PSM) revealed that only two short regions of 16K tolerated five extra amino acid insertions without considerable reduction in its silencing suppression function. The tolerant regions coincide with sequence variability between tobravirus cysteine-rich proteins, indicating a strong functional and/or structural conservation of TRV 16K. Confocal laser scanning microscopy of transiently expressed 16K fusions to red fluorescent protein (RFP) revealed a predominant cytoplasmic localization and, in addition, a nuclear localization. In contrast, fusions of RFP with the N-terminal region of 16K localized exclusively to the cytoplasm, whereas fusions between RFP and the C-terminal region of 16K displayed an exclusive nuclear localization. Further analysis of 16K-derived peptide fusions demonstrated that the 16K C-terminal region contained at least two functional bipartite nuclear localization signals which were independently capable of nuclear targeting.

INTRODUCTION

RNA silencing in higher plants represents a natural host defence response involving the selective degradation of invading virus RNAs (Voinnet, 2001; Moissiard & Voinnet, 2004). Double-stranded RNAs (dsRNA) (Bass, 2000), formed during the replication of plant RNA viruses, are the key trigger molecules of virus-induced RNA silencing. After processing of dsRNAs into 21–26 nt double-stranded fragments by an RNase III-type Dicer endonuclease (Bernstein et al., 2001), these so-called small interfering RNAs (siRNAs) (Hamilton & Baulcombe, 1999; Elbashir et al., 2001) are integrated into a multicomponent RNase, the RNA-induced silencing complex (RISC), which then mediates specific cleavage of complementary single-stranded RNA (ssRNA) (Hammond et al., 2000). To counteract this resistance mechanism, plant viruses have evolved proteins that suppress RNA silencing at different stages (Moissiard & Voinnet, 2004; Silhavy & Burgán, 2004; Voinnet, 2005). To date, plant virus silencing suppressors of 23 different virus genera have been identified, displaying high genetic diversity (Li & Ding, 2006). The current knowledge on silencing suppressor proteins and the molecular mechanisms of silencing suppression has been reviewed recently (Moissiard & Voinnet, 2004; Silhavy & Burgán, 2004; Li & Ding, 2006). Although the molecular basis for suppressor activity has been described only for selected viral silencing suppressors, siRNA sequestration and dsRNA binding was found to be a common mode of action of several suppressor proteins from different plant virus families (Lakatos et al., 2006; Mériat et al., 2006).

Tobacco rattle virus (TRV), a member of the genus Tobravirus, is a bipartite positive-sense, single-stranded RNA virus which forms rod-shaped particles (MacFarlane,
16K suppressor of gene silencing protein of TRV

In addition, confocal laser scanning microscopy (CLSM) was used to determine the subcellular localization of 16K and selected domains in transient expression assays.

METHODS

Generation of expression constructs and infectious transcripts. All plasmid manipulations were performed according to standard techniques (Sambrook et al., 1989), unless otherwise specified. Manipulations were verified by sequencing (MWG Biotech). The binary vector pBIN61S (Silhavy et al., 2002), a derivative of pBIN19 containing an enhanced cauliflower mosaic virus 35S-promoter and polyA-terminator cassette, was used for transient expression studies. pBIN61S-GFP (35S–GFP) contains the green fluorescent protein mrgfp4 variant with an endoplasmic reticulum (ER)-targeting signal (Haseloff et al., 1997). pBIN61S-tnos-rev is a derivative of pBIN61S obtained by inserting a PCR-amplified nopaline synthase terminator sequence (Tnos) in antisense orientation into the EcoRI site downstream to the polyA terminator. pBIN61S-GFP-tnos-rev represents the corresponding gfp expressing variant (35S–GFP-tnos-rev). All three plasmids were kindly provided by D. Silhavy (Agricultural Biotechnology Centre, Gödöllő, Hungary). pTRV1, a full-length cDNA clone of TRV RNA-1 isolate Ppk20 (GenBank accession no. AF406990) under control of the enhanced 35S-promoter and Tnos in a binary vector, was kindly supplied by S. P. Dinesh-Kumar (Liu et al., 2002b). The oligonucleotide primers used to construct the different plant expression plasmids are shown in Supplementary Table S1 (available with the online version of this paper). To generate 35S–16K and 35S–16K-tnos-rev, the 16K Ppk20 141 aa ORF (nt 6111–6536) was PCR-amplified from pTRV1 using primers 16K-ORF-5' and -ORF-3', subcloned, sequence-verified and cloned into pBIN61S and pBIN61S-tnos-rev, respectively. In order to express N-terminal aa 1–68 and C-terminal aa 69–141, 16K-deletion mutants under control of the enhanced 35S-promoter, the 5' 204 bp of 16K-ORF and the 3' 219 bp, respectively, were PCR-amplified using primers 16K-N-5' and -N-3', and 16K-C-5' and -C-3', respectively. The 16K fusion constructs for subcellular localization were assembled as follows. Initially, the DsRed-ORF was PCR-amplified from pDsRed-C1 (Clontech) using primers DsRed1-5' and DsRed1-3', and cloned under control of the 35S-promoter into binary vector pBIN61S-tnos-rev to give 35S–DsRed-no-stop. Subsequently, 16K-ORF, 16K-N terminus and 16K-C terminus (mentioned above) were cloned into 35S–DsRed-no-stop resulting in 35S–DsRed-16K, -16K-N and -16K-C, respectively. The coding sequence for three different peptides [16K aa 42–51, aa 92–101 and simian virus 40 (SV40) T antigen nuclear localization signal (NLS) (Kalderon et al., 1984), see Supplementary Table S2] were incorporated into the lower primer for DsRed amplification using the upper primer DsRed1-5' in combination with each of the lower primers DsRed-contr-N-3', -contr-C-3' and -SV40-3', respectively. Subsequently, the mutated DsRed amplification products were cloned into pBIN61S-tnos-rev. In order to fuse the peptides coding for 15–17 aa (16K aa 27–41, aa 75–91 and aa 112–128) in-frame with DsRed, oligonucleotides (NLS1-5' and -3', NLS2-5' and -3', and NLS3-5' and -3') were hybridized, digested and subsequently cloned into pBIN61S-DsRed-no-stop to yield the plasmids named in Supplementary Table S2. Monomeric red fluorescent protein (mRFP) (Campbell et al., 2002) was reamplified from pCB-ER-mRFP (kindly provided by J. Carrington, Oregon State University, OR, USA) using primers mRFP-5' and mRFP-3' and used to replace DsRed in 35S–DsRed, 35S–DsRed-16K, -16K-N and -16-C (Supplementary Table S2).

Subcellular localization studies and CLSM. Agrobacterium tumefaciens C58C1 (pGV2260) was transformed with the 16K fusion constructs generated and used in infiltration studies. For background

1999) transmitted by soil-inhabiting nematodes (Taylor & Brown, 1997). The tobraviral genome consists of two RNA molecules, RNA-1 and RNA-2, in which RNA-1 is conserved in size and gene content between tobraviruses and isolates, RNA-2 displays a high sequence variation (MacFarlane, 1999). TRV RNA-1 encodes four proteins: 134 and 194 kDa replicase proteins at the 5' proximal region of RNA-1, a 29 kDa movement protein (29K) (Ziegler-Graff et al., 1991) and a 16 kDa cysteine-rich protein (16K) at the 3' proximal 'end' of RNA-1. Whereas the replicase proteins are directly translated from genomic RNA, 29K and 16K are expressed from subgenomic RNA species. Due to its cysteine-rich N terminus, 16K was classified as a cysteine-rich protein (CRP). A general feature of plant RNA virus CRPs (the genera Hordeivirus, Furovirus, Pecluvirus, Benyvirus and Tobravirus) is the presence of characteristic sequence motifs containing conserved cysteine residues (Morozov et al., 1989; Koonin et al., 1991; Savenkov et al., 1999; Diao et al., 1999; Te et al., 2005). The two clusters of N-terminal cysteine residues of barley stripe mosaic virus (BSMV) CRP γb have been demonstrated to bind zinc experimentally (Bragg et al., 2004), whereas the TRV 16K cysteine residues have only been suggested to possess zinc-binding properties (MacFarlane et al., 1989). For a number of CRPs, pathogenicity and suppression of gene silencing functions have been demonstrated (Donald & Jackson, 1994; Dunoyer et al., 2002; Liu et al., 2002a; Yelina et al., 2002; Reavy et al., 2004; Yelina et al., 2005). Another indication for common functions is provided by the functional interchangeability of CRPs between different genera: Hordeivirus, Tobravirus and Furovirus (Liu et al., 2002a; Yelina et al., 2002).

16K is known to be required for efficient replication in Nicotiana benthamiana protoplasts, and was demonstrated to be a pathogenicity determinant enhancing potato virus X symptoms when expressed from an expression vector (Liu et al., 2002a). TRV was shown to suppress transgene silencing in plants in the reversal of silencing assay (Voinnet et al., 1999) and, despite its inability to prevent the accumulation of siRNAs, 16K of TRV was shown to possess a suppressor of gene silencing function in Drosophila cells (Reavy et al., 2004). In an initial expression study, 16K was characterized to accumulate in a high-molecular-mass complex, either as a multimer or in association with host proteins in tobacco protoplasts, probably in a non-membrane-bound state (Angenent et al., 1989). Immunogold labelling of Nicotiana clevelandii ultrathin sections revealed 16K-specific staining of nuclei and, to a minor extent, staining of the cytoplasm of TRV-infected cells (Liu et al., 1991).

Our study aimed to determine the silencing suppression function of TRV 16K in planta. Pentapeptide insertion scanning mutagenesis (PSM) (Haapa et al., 1999; Hayes, 2003), a fast and powerful tool for functional characterization of plant virus proteins (Varrelmann et al., 2007), was carried out to characterize functional domains of 16K.
visualization of the cell, all fusion constructs were infiltrated into leaves of transgenic *N. benthamiana* plants (line 16c) expressing endoplasmic reticulum-targeted GFP (GFP-ER) (Brigneti *et al.*, 1998). Confocal imaging of GFP-, DsRed- and mRFP-expressing leaf tissues was performed using a Leica TCS SP2 confocal imaging system with excitation/emission wavelengths 488/510–515 nm for GFP and 543/600–610 nm for both DsRed and mRFP, respectively.

**Random insertion scanning mutagenesis, mapping of the insertion and sequencing.** Generation of a 16K PSM library was carried out using the ‘Mutation Generation System’ F701 MGS (Finnzymes) consisting of MuA transposase and an artificial entranceposon (MI-KanR) following the manufacturers instructions in a pGEM7Zf(+) (Promega) SacI–XbaI 16K clone, essentially as described by Varrelmann *et al.* (2007). 16K mutants were cloned into pBIN61S-tnos-rev and the exact position of the pentapeptide insertion of each mutant was determined by sequencing. Sequence alignment was carried out using CLUSTAL_X version 1.83 (Thompson *et al.*, 1997).

**Transient silencing suppression assay, GFP visualization and RNA gel blot analysis.** Transient silencing suppression assays and Northern blot analyses of GFP mRNA and GFP-specific siRNA were performed as described previously (Voinnet *et al.*, 1999; Varrelmann *et al.*, 2007). Total area optical density analysis of mRNA and siRNA signals on RNA gel blots relative to the loading control was carried out using Gel-Pro analyser software 6.0 (Media Cybernetics).

**16K antiserum production.** The bacterial expression, chromatography purification and polyclonal antiserum production of histidine-tagged TRV-16K protein was performed as described (Supplementary Methods in JGV Online).

**Protein extraction and immunoblot analysis.** Total plant proteins from agroinfiltrated *N. benthamiana* patches were extracted under denaturing conditions essentially as described previously (Albrecht *et al.*, 1988). Extracted samples were separated by 15 % SDS-PAGE and electroblotted to nitrocellulose membranes as described by Towbin *et al.* (1979). TRV 16K was probed with anti-16K rabbit polyclonal antiserum (2 mg IgG ml⁻¹), which was subsequently detected using a goat anti-rabbit alkaline phosphatase-conjugated polyclonal antibody (Sigma) at a 1 : 10000 (v/v) dilution and chromogenic substrate as described previously (Varrelmann *et al.*, 2007).

**RESULTS**

**TRV 16K suppresses RNA silencing in the transient silencing suppression assay in GFP-transgenic *N. benthamiana***

To investigate the silencing suppressor activity of 16K *in planta*, the TRV 16K gene was cloned into pBIN61S-tnos-rev, a binary plant expression vector optimized for long-term expression (see comments in the following section). For transient silencing suppression assays, GFP-transgenic *N. benthamiana* (line 16c) were infiltrated with 35S–GFP, 35S–16K-tnos-rev or with both 35S–GFP and 35S–16K-tnos-rev, followed by the monitoring of fluorescence, GFP mRNA levels and GFP-specific siRNAs. Agroinfiltration of 35S–GFP alone led to a visible increase of GFP-derived fluorescence detected at 3 days post infiltration (days p.i.) (Fig. 1a). A reduction of green fluorescence within the infiltrated areas indicative for silencing of the GFP transgene was observed starting from 5 days p.i. and cell-autonomous RNA silencing was represented by a red ring of GFP-silenced cells around co-infiltrated patches (Voinnet & Baulcombe, 1997) (Fig. 1a). At 3 days p.i., GFP-specific siRNAs were detectable and their amount increased to 7 days p.i., while GFP mRNA accumulation in the infiltrated areas was reduced at 5 days p.i. (Fig. 1b). In 35S–16K-tnos-rev + 35S–GFP co-infiltrated patches, GFP fluorescence and mRNA expression remained more or less stable at a high level up to 7 and 5 days p.i., respectively, indicating that 16K prevented GFP mRNA degradation. Northern blot analysis of 21–25 bp GFP-specific siRNAs showed a fivefold reduction of GFP siRNA accumulation in 16K co-expressing patches, compared with 35S–GFP expressing patches at 7 days p.i. (Fig. 1b). Gene silencing of GFP and characteristic red rings around infiltrated patches were observed no earlier than 10 days p.i. (data not shown). Finally, infiltration of 35S–16K-tnos-rev without 35S–GFP, the inducer of silencing, was monitored. The endogenous, transgenic GFP mRNA level was not increased. To demonstrate that the 16K silencing suppression ability was not due to possible enhanced expression from a binary vector containing tnos-rev, the silencing suppression assay was repeated with 35S–16K, resulting in no visible differences in the course of GFP expression (Fig. 1a). To prove and compare expression of 16K from both plasmids, protein samples were extracted at 3, 5 and 7 days p.i., separated by SDS-PAGE and used for 16K detection in immunoblot analysis with 16K specific antiserum (Fig. 1c). The 16K protein was detectable in all treatments in comparable levels over time. Subsequently, we aimed to define regions or domains of TRV 16K protein necessary for silencing suppression; hence 16K-N-terminal and C-terminal deletion mutants were transiently expressed in GFP-transgenic *N. benthamiana* plants, as described above. In both cases, UV-monitoring at 3 and 5 days p.i. revealed cell-autonomous and non-autonomous RNA silencing. Analysis of GFP-mRNA and -siRNA accumulation showed that expression of both the N and the C terminus neither resulted in elevated GFP mRNA levels, nor did it have an influence on the occurrence of siRNAs (data not shown). Therefore, PSM was applied as an alternative mutagenesis approach.

**PSM-based construction of a linker scanning library of TRV 16K**

To characterize 16K domains involved in silencing suppression, we tested the influence of random pentapeptide insertions on the silencing suppressor function of 16K. Insertional mutagenesis was carried out, yielding a library of TRV PpK20 16K gene clones. One hundred transforms were isolated, pooled and cloned into pBIN61S-tnos-rev. This plasmid was chosen to prevent defective silencing suppression pentapeptide scanning (PS) mutant proteins themselves from being targeted by RNA silencing. Kertesz *et al.* (D. Silhavy and colleagues, Agricultural Biotechnology Centre, Gödöllő, Hungary, personal com-
Fig. 1. Effect of TRV 16K on transgene-induced RNA silencing in GFP-transgenic *N. benthamiana* line 16c plants. (a) Leaves were infiltrated with 35S–GFP, co-infiltrated with 35S–GFP + 35S–16K, 35S–16K and 35S–GFP-tnos-rev, respectively. GFP expression under UV illumination is shown at 3, 5 and 7 days p.i. Local silencing of GFP is manifested in reduction of transient additional fluorescence of the patch. Thin red line without fluorescence around the infiltrated area indicates cell-to-cell RNA silencing. (b) Northern blot analysis showing GFP-mRNA and GFP-siRNA (21–25 bp) accumulation in infiltrated leaf areas. Numbers below blots display total area densities relative to loading controls. 35S–16K infiltration shows transgenic GFP-mRNA without induction of local RNA silencing. Equal loading of the gel was verified by ethidium bromide staining of 18S RNA. (c) Immunodetection of TRV 16K protein in infiltrated leaf areas using antiserum raised against bacterially expressed 16K protein [i], and corresponding PAGE Coomassie-stained loading control [ii]. 35S-empty: infiltration with pBIN61S, His-16K: bacterially expressed- and column chromatography-purified TRV 16K N-terminal 6×histidine-tagged (calculated molecular mass of 20.39 kDa).
munication) have shown that agroinfiltration mediated transgene-induced silencing can be prevented if the transgene is expressed from a binary vector which has double-terminator sequences (a sense 35S polyA and an antisense oriented nos terminator). As shown in Fig. 1(a and b), transgene-induced RNA silencing was successfully prevented by the use of pBIN61S-tnos-rev for the transient test-expression of GFP (35S–GFP-tnos-rev), thus demonstrating the suitability of this plasmid to serve as an expression vector of PS library mutants. Evaluation of the entranceposon footprint permitted the selection of 58 clones with insertions randomly spread over the 16K ORF. Sequence determination (Table 1) showed that 30 of the 58 sequenced PSM mutants represented independent isolations with 28 mutants isolated twice from the initial library. Moreover, the 15 nucleotide entranceposon footprint at different nucleotide positions in the 16K gene led to pentapeptide insertions in two cases at the identical amino acid position with varying composition (41::AAATR and CGRTR, 56::CGRII and AAAVI). Since 30 different mutants were selected from the initial 16K mutant library, an average of one insertion per 4.7 aa was generated.

**Transient silencing suppression assay of 16K PS mutants and construction of a functional map of TRV 16K for silencing suppression motifs**

A transient silencing suppression assay was conducted with the independent 30 mutants generated in triplicate using two plants per experiment. GFP fluorescence was monitored under UV light at 3, 5 and 7 days p.i. and compared with parallel infiltrated controls (35S–16K + 35S–GFP and 35S–GFP, respectively). Non-functional 16K PS mutants with no detectable silencing suppression activity, i.e. strong reduction of GFP-fluorescence in the infiltrated area at 5 days p.i., were grouped into class III; mutants with restricted functionality (increased GFP-fluorescence) were assigned to class II, and mutants with strong GFP-fluorescence similar to wild-type fluorescence were grouped into class I (Table 1).

To test if PSM interfered with protein stability, total protein was extracted (5 days p.i.) from co-infiltrated patches for all 30 16K PS mutants (35S–16K-tnos-rev + 35S–GFP) and subjected to immunoblot analysis as described above. All mutants were expressed independently of their silencing suppression activity (Supplementary Fig. S1).

To verify UV monitoring results, 10 PS mutants (13::AAAEV, 16::AAAVL, 20::CAAAAT, 29::LRPQL, 31::AAAQV, 57::AAAAIN, 76::MRPHR, 93::CGRRN, 99::VRPQS and 110::SAAAT) 16K amino acid position followed by pentapeptide insertion) were chosen for a more detailed analysis and tested for their influence on the accumulation of GFP mRNA (displayed in Table 1). Slight differences between the mRNA levels and the visual observations of GFP fluorescence levels were observed (Fig. 2a, b). These were probably due to variation within the assay as shown by independent repetitions (data not shown). Mutants displaying a class II phenotype maintained GFP mRNA levels higher than the control infiltrations with 35S–GFP alone, but in most cases lower than those of the 16K wild-type control at 5 days p.i.. The 16K class III mutants did not influence GFP mRNA levels at 5 days p.i. and were similar to 35S–GFP.

Since visual examination of GFP expression and mRNA levels of the 10 mutants analysed correlated well, UV monitoring results of the remaining 20 mutants were included to map the effect of 16K ORF pentapeptide insertions on suppression of transgene-induced RNA silencing (Fig. 2c; Table 1). Five PS mutants displayed a silencing suppression function similar to wild-type 16K protein (class I), four mutants were assigned to class II and 21 showed no silencing suppression function at all (class III). Remarkably, mutations which did not abolish entirely 16K silencing suppression ability were detected only in two regions of the protein, namely in the region covering aa

<table>
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<th>Pentapeptide insertion position</th>
<th>Inserted pentapeptide</th>
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13–20 (13::MRPQL, 13::AAAEV, 16::AAAVL, 20::CAAAT) and aa 93–110 (93::CGRRN, 99::VRPQS, 100::CGRTA, 102::AAATA, 110::SAAAT), labelled as ‘+’ in Fig. 3. The remaining parts of the protein including cysteine, glycine and histidine residues conserved in CRPs (Savenkov et al., 1998) (aa position 42, 47, 48, 66, 67 and 70, labelled $\_$ in Fig. 3) were functionally intolerant for pentapeptide insertions.

Subcellular localization of TRV 16K

Initially, we carried out a computational prediction of the subcellular localization of TRV 16K using PSORT (http://www.psort.org) (Nakai & Kanehisa, 1991), resulting in the prediction of nuclear localization with an accuracy of 0.880 (Robbins score) and the prediction of two bipartite NLSs, consisting of two basic clusters and a spacer (Robbins et al., 1991) (aa positions 75–91 and 112–128) present within the C-terminal region of 16K protein. To find out whether the two predicted NLSs were functional, 35S promoter-driven binary constructs were generated coding for peptides fused at the C-terminal end of DsRed (35S–DsRed-16K-75-91 and 35S–DsRed-16K-112-128) (Supplementary Table S2).

In addition, a peptide rich in basic residues (16K aa 27–41), but not fitting to an NLS consensus sequence, was tested for its capability to mediate nuclear targeting. Constructs 35S–DsRed-16K-42-51 and -16K-92-101 served as negative controls, coding for peptides which lack any basic residues typical of nuclear targeting domains. Finally, the NLS of SV40 T-antigen was fused to DsRed (35S–DsRed-NLS-SV40) and used as positive control. For better cell background visualization, constructs were agroinfiltrated into leaves of transgenic N. benthamiana (line 16c), which expresses GFP targeted to the endoplasmic reticulum, followed by CLSM of epidermal cells at 4 days p.i. (Fig. 4a). Unfused DsRed was distributed diffusely throughout the cytoplasm and nucleus, but not the nucleolus of epidermal cells, whereas NLS-SV40 relocalized DsRed exclusively to the nucleus with unequal distribution and stronger probably nucleolar derived fluorescence, proving the suitability of the experimental approach (Fig. 4a). In contrast, the two negative control peptides did not influence the subcellular distribution of DsRed, but both computer-predicted NLSs (16K 75–91 and 112–128) were independently capable of targeting DsRed to the nucleus similarly to NLS-SV40, thus suggesting the functionality of...
the two predicted 16K NLSs. In contrast to these two C-terminal 16K peptides, the N-terminal derived one (16K 27–41) did not affect the general nuclear–cytoplasmic distribution of DsRed in *N. benthamiana* cells, suggesting that this sequence does not function as an NLS. However, in contrast to unfused dsRed which does not localize to the nucleolus within the nucleus, fluorescence was distributed homogeneously throughout the nucleus.

To investigate the influence of the two identified NLSs on the subcellular localization of 16K, the complete 16K ORF, N-terminal and C-terminal parts were translationally fused to DsRed (35S–DsRed-16K, 35S–16K-N and 35S–16K-C, respectively), and used in transient expression assays, as described above. CLSM examination (Fig. 4b) revealed that the C-terminal part mediated nuclear localization of DsRed, supporting the findings obtained with detached

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**Fig. 3.** CLUSTAL _X amino acid sequence alignment of CRPs of members of the genus *Tobravirus*. Different grades of aa conservation are reflected by shading at four levels. Bars below the aa sequence alignment indicate identified NLSs. +, Amino acid positions of 16K PpK20 PS mutants displaying tolerance (class I and II mutants) to pentapeptide insertions in silencing suppression ability; ⨁, 16K residues highly conserved between CRPs derived from different virus genera ([Morozov et al., 1989]; [Savenkov et al., 1998]; [Diao et al., 1999]; [Te et al., 2005]). TRV 16K-PpK20 (GenBank accession no. AAM50511), PEBV 12K (NC_002036), PepRSV 12K (NC_003669).

**Fig. 4.** Intracellular distribution of DsRed and mRFP fusion proteins. The constructs indicated were introduced into GFP-transgenic *N. benthamiana* (line 16c) cells by *Agrobacterium*–mediated gene-transfer, followed by CLSM of epidermal cells. Images in upper rows represent the red detection channel, whereas the lower row represents artificial overlays of red and green fluorescence. Bar, 40 μm. In lower panel, nuclei are labelled by an arrow.
NLSs. In contrast, the N-terminal part unexpectedly directed the DsRed fluorescence to aggregates in the cytoplasm. Similar cytoplasmic aggregation patterns were also observed with the complete 16K ORF (35S–DsRed-16K) in most of the examined cells (not shown); however, in a few cells DsRed was found to be directed to the nucleus, too (Fig. 4b, 35S–DsRed-16K [i] and [ii], respectively). DsRed-1 (Matz et al., 1999) displays obligate tetramerization (Baird et al., 2000) and has been shown by different authors to display the tendency to form intracellular aggregates (Lauf et al., 2001; Mizuno et al., 2001). To exclude the possibility that oligomerization of DsRed-1 might have influenced the subcellular distribution of our target, we cloned mRFP, a variant of DsRed known to form true monomers, under control of the 35S-promoter (35S–mRFP), and subsequently fused the fragments (examined above) to the mRFP C terminus (35S–mRFP-16K, -16K-N and -16K-C in analogy to the DsRed-fusions described above, Supplementary Table S2). Agroinfiltration followed by CLSM analysis (Fig. 4c) showed that 35S–mRFP was uniformly distributed throughout the cell, and freely diffused from cytoplasm to the nucleus like its parental protein DsRed-1. 35S–mRFP-16K-C was only detected in the nucleus and presumably the nucleolus according to the DsRed-1 fusion construct, whereas 35S–mRFP-16K-N was freely distributed within the cytoplasm. Only a few cells displayed a very weak fluorescence in the nucleus in a distribution resembling that of unfused mRFP. Cells expressing mRFP-16K varied in their fluorescence pattern. All cells displayed fluorescence in the cytoplasm, but by contrast in 3 out of 100 cells randomly counted in epifluorescence microscopy, additional strong fluorescence in the nucleus was observed starting from 4 days p.i. (Fig. 4c, 35S–mRFP-16K [i]). Additionally, 34 out of 100 cells displayed moderate to weak nuclear fluorescence [ii]. In the remaining 63 cells displaying only weak fluorescence, the red fluorescence was uniformly distributed in the cytoplasm, and excluded from the nucleus [iii]. Combined, our data suggest that TRV 16K, in addition to the two identified NLSs, possesses a strong signal in the N-terminal 68 aa which may retain the fusion protein in the cytoplasm. Alternatively, the N-terminus might mediate active nuclear export, even though a leucine-rich nuclear export signal (Görlich & Kutay, 1999; Haasen et al., 1999) was not identified within the 16K aa sequence by computer-assisted predictions.

**Sequence comparison of 16K with related CRPs and those from other tobravirus members**

The amino acid sequence of several CRPs, possessing silencing suppression ability [i.e. BSMV and Poa semilatent virus (PSLV) yb, beet necrotic yellow vein virus (BNYVV) P14, peanut clump virus (PCV) P15, soil-borne wheat mosaic virus (SBWMV) 19K, PEBV and PepRSV 12K, and TRV 16K], were used for CLUSTAL_X alignment. The observed sequence homology of TRV 16K to non-tobraviral CRPs was relatively low, ranging from only 8.5 to 10.6% (data not shown). In contrast, CRPs from closely related tobravirus members, PEBV and PepRSV 12K proteins (MacFarlane, 1999), possess sequence homology of 64.5% to each other and display 27.7% (PEBV 12K) and 31.9% (PepRSV 12K) homology to TRV 16K, respectively. This is mainly caused by the smaller size of the 12K proteins, leading to a gap in the alignment between 16K aa positions 83 and 117 (Fig. 3). When these additional 16K 35 aa are not considered in the alignment, the homology increases to 41.3 and 47.7%, respectively. Since this gap affected both NLSs identified in 16K, additional computational prediction of nuclear localization was carried out for both 12K proteins and statistical analysis did not predict the presence of a NLS in the PEBV and PepRSV 12K proteins. PSORT only predicted one monopartite NLS at amino acid position 74 (PKRK) of PEBV 12K. Interestingly, the variable N-terminal region and the 35 aa gap in this alignment coincide with the regions in 16K displaying tolerance to pentapeptide insertions.

**DISCUSSION**

In this study, we have found evidence that TRV 16K suppresses transgene-induced RNA silencing. This finding represents the first in planta proof of function and supports previous descriptions of this protein as a virus pathogenicity factor in *N. tabacum* (Liu et al., 2002a). Our results on the noticeable effect of 16K on the accumulation of GFP-specific siRNAs in *N. benthamiana* tissue is in contrast to a previous report in *Drosophila* cells (Reavy et al., 2004), where a reduction of *lacZ*-specific siRNAs was not found. Thus, it can be concluded that the 16K suppression of gene silencing is organism-specific, depending on the assay applied. It is well-known from several previous studies that CRPs from different plant RNA viruses possess only distant sequence homology relationships (Koonin et al., 1991; Savenkov et al., 1998; Diao et al., 1999; Te et al., 2005). This homology is mainly restricted to a conserved motif (Cys-Gly...Cys-Gly-X-X-His, see the tobravirus CRP alignment in Fig. 3), which is supposed to have some functional significance. However, homology of the TRV 16K with the 12K protein of PEBV and PepRSV was much more extensive, sharing several short amino acids stretches of 100% sequence homology, indicative as well for functional homology.

The average PS mutant density in the 141 aa 16K protein at every 4.7 aa is high. Unfortunately, an unexplained gap of 25 aa in the C-terminus of the protein (between aa 110 and 135) not covered by PS mutants had to be excluded from the functional analysis. The results of the PSM study carried out with 30 independent mutants show that randomly inserted 5 aa do not significantly interfere with 16K protein stability. This is in agreement with other PSM studies (Poussu et al., 2004; Fransen et al., 2005; Varrelmann et al., 2007). The finding that only two short
aa regions tolerated the pentapeptide insertion and maintained silencing suppression is however remarkable. It might indicate a dysfunction resulting from an alteration of the 3D structure of the protein or modification of a functional domain. Interestingly, the sequence variability between TRV 16K and the 12K proteins of tobravirus PEBV and PepRSV correlate well with the tolerance of the silencing suppression function of 16K to pentapeptide insertions. It provides evidence that these two parts of 16K are dispensable for the silencing suppression function. Previous PSM studies of proteins with known 3D structure (Hallet et al., 1997; Petyuk et al., 2004; Poussu et al., 2004) have shown that pentapeptide insertions in proximity to an active site of a protein or insertions that disrupt the secondary protein structure have stronger effects on the functionality of the protein than insertions in inter-domain regions or surface loops. The high functional sensitivity in most parts of the protein to PSM proposes strong functional and structural conservation, suggesting the complete protein is required for silencing suppression.

The DsRed/mRFP subcellular distribution and exclusive nuclear targeting of DsRed-SV40 following agroinfiltration proved the suitability of the experimental approach. Applying this method, we were able to show that 16K possesses at least two independent bipartite NLSs (aa 75–91 and 112–128) responsible for nuclear targeting in context of the C-terminal half of the protein, as well as in the minimal consensus sequence fused to DsRed. The nuclear targeting phenotype, including nucleolar fluorescence produced by the two bipartite signals, was comparable to that induced by the SV40 monopartite NLS. This is supported by other studies, demonstrating that regions rich in basic residues, lysine and arginine from other plant viral proteins can be responsible for nuclear as well as nucleolar localization (Kim et al., 2004; Ryabov et al., 2004; Haupt et al., 2005). The finding that 16K, despite containing two NLSs, is also present in the cytoplasm is consistent with previous findings (Liu et al., 1991), but its homogeneous cytoplasmic distribution detected in our study is in contrast to the observations of Angenent et al. (1989), who suggested 16K incorporation in a high molecular mass complex. In our study, we observed a uniform distribution of the fluorescence when the whole 16K was fused to mRFP; however, we cannot exclude organelle targeting of 16K. On the other hand, the exclusion of the 16K N terminus–mRFP fusion from the nucleus and the observation that mRFP-16K nuclear targeting was incomplete, suggest the presence of a nuclear export signal in the N terminus, its targeting to or retention in the cytoplasm, possibly by cytoplasmic protein interaction, or organelle targeting. The observation of a slightly stronger nuclear fluorescent signal when the detached N-terminal peptide 27–41 was fused to DsRed remains unexplained. However, additional analysis is necessary to identify the exact domain and the underlying mechanism responsible for this nuclear exclusion. Whether C-terminal-mediated nuclear targeting or N-terminal-driven export from the nucleus of this small protein is involved in silencing suppression cannot be confirmed, since both were non-functional in this respect.

The results of the sequence analysis and prediction of subcellular localization of tobravirus CRPs, showing absence of the two 16K NLSs in PepRSV and PEBV 12K due to the C-terminal gap in these two proteins, is remarkable. On the assumption that tobravirus CRP is functionally analogous, it is tempting to speculate that the two bipartite NLSs of TRV 16K are dispensable for silencing suppression and belong to a secondary 16K function. These 16K NLS are unique among plant RNA virus CRPs. PSLV γb (Yelina et al., 2002) and PCV P15 (Dunoyer et al., 2002) CRPs are targeted to peroxisomes via a C-terminal SKL motif shown to be dispensable for silencing suppression. This SKL motif is absent in some isolates of BSMV γb (Gustafson et al., 1987; Kozlov et al., 1989), SBWMV 19K, TRV 16K and BNYVV P14 and indicates a functional diversity of RNA virus CRPs.

The components of the silencing machinery targeted by TRV 16K, however, remain to be determined. Comparing our and other (Voinnet et al., 1999) findings on TRV 16K with those of different CRPs possessing silencing suppression ability supports functional diversity even in this group of related proteins. The observed effect of 16K on the accumulation of GFP-specific siRNAs indicates a protein activity upstream of siRNA production and possibly for an interference with the initiation step of RNA silencing. The reversion of an established RNA silencing with TRV found in earlier studies (Voinnet et al., 1999) supports our conclusion that 16K suppresses the initiation and maintenance of transgene-induced RNA silencing. In contrast to this finding and supporting the functional diversity of CRPs, BSMV γb and PCV P15 CRPs have both been identified recently to bind small-size dsRNA selectively (Mérai et al., 2006). In addition, PSLV γb CRP does not reduce siRNAs levels (Yelina et al., 2005), but PCV P15 does (Dunoyer et al., 2002). Another indication for a completely different mode of action of these proteins is given by the finding that, in contrast with 16K, both γb and P15 CRPs possess a coiled-coil sequence in the C-terminal part which is responsible for dimerization and indispensable for silencing suppression (Dunoyer et al., 2002; Bragg & Jackson, 2004). The plant RNA silencing/dsRNA processing machinery is localized in the cytoplasm and the nucleus (Moissiard & Voinnet, 2004; Ding and Voinnet, 2007). A previous study demonstrated that Arabidopsis thaliana Dicer-like protein DCL1 processes siRNA precursors within the nucleus (Papp et al., 2003), and Brosnan et al. (2007) gave evidence for the involvement of nuclear silencing in the perception of long-distance mRNA silencing. Finally, the connection between nuclear and/or nucleolar targeting of 16K and its other biological functions (e.g. pathogenicity) remains unknown. Therefore, further experiments are necessary to correlate 16K subcellular distribution to its silencing suppression...
function, preferably including studies on the protein’s 3D structure and its dsRNA-binding abilities.

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