Quantitative Analysis of Efficient Endogenous Gene Silencing in *Nicotiana benthamiana* Plants Using *Tomato bushy stunt virus* Vectors That Retain the Capsid Protein Gene

Daniela Pignatta,¹ Pavan Kumar,² Massimo Turina,³ Abhaya Dandekar,⁴ and Bryce W. Falk⁵

¹Dipartimento di Scienze e Tecnologie Agroambientali, Bologna, Italy; ²Plant Biology Graduate Group, University of California, Davis, U.S.A.; ³Istituto di Virologia Vegetale, CNR-Torino, Italy; ⁴Department of Plant Sciences and ⁵Department of Plant Pathology, University of California, Davis, U.S.A.

Submitted 29 August 2006. Accepted 19 January 2007.

Tomato bushy stunt virus (TBSV) coat protein (CP) replacement vectors have been used previously to silence transgenes (e.g., the *green fluorescent protein* gene) but have not been effective for silencing endogenous plant genes. New TBSV vectors which retained the CP gene were developed by engineering an *XhoI* restriction site in three positions (3f, CEB, and CEA) of the pTBSV-100 infectious clone. *Magnesium chelatase* (*ChlH*) and *phytoene desaturase* (*PDS*) were chosen as targets for endogenous gene silencing. Initial experiments using CP replacement vectors with a 230-bp sense or antisense *ChlH* insert gave a silencing phenotype prominent only in the first new leaves above those inoculated. No silencing phenotype was apparent beyond these leaves whereas, for *PDS*, no silencing phenotype was observed. When plants were inoculated with the *XhoI* insert vectors containing *ChlH* and *PDS* sequences, plants showed a silencing phenotype extensively throughout the challenged plant, indicating an improved ability for virus movement and silencing in *Nicotiana benthamiana* host plants. Silencing efficiencies were quantified using real-time reverse-transcription polymerase chain reaction, indicating specific silencing effects of each individual silencing vector. Only one recombinant vector (pPD-3f5), where the *XhoI* insert was at the 3' end of the CP gene, failed to give effective silencing. Here, we show that our new CP-retaining TBSV vectors (CEA-CEB) form typical TBSV virions, retain silencing inserts of variable lengths (110 to 260 nucleotides), and can systemically silence endogenous genes in *N. benthamiana*.

Virus-induced gene silencing (VIGS) is an RNA-mediated defense mechanism of plants against viruses and is related to post-transcriptional gene silencing (PTGS) (Baulcombe 1999; Vaucheret et al. 2001). The application of VIGS to functional genomics studies in plants involves use of recombinant viruses that carry fragments of host plant genes and that selectively suppress the corresponding endogenous mRNA expression in planta (Baulcombe 1999; Lu et al. 2003). Several VIGS vectors have been developed and used for functional genomics

(Burch-Smith et al. 2004; Robertson 2004). VIGS has proven useful for silencing transgenes like the gene for *green fluorescent protein* (*GFP*) (Naylor et al. 2005; Qiu et al. 2002) and endogenous plant genes like *phytoene desaturase* (*PDS*) (Faivre-Rampant et al. 2004; Ratcliff et al. 2001) by using different recombinant viruses and different plant species. However, new virus vectors are required to optimize and expand the application of VIGS to a wider range of plants (Burch-Smith et al. 2004).

Tomato bushy stunt virus (TBSV) is the type member of the genus *Tombusvirus*, family *Tombusviridae* (Russo et al. 1987). Virions are isometric and contain a single-stranded positive-sense genomic RNA of approximately 4.8 kb that encodes for five major proteins (Fig. 1A) (Hearne et al. 1990). The two 5' proximal open reading frames (ORFs) encode proteins p33 and p92, which are both constituents of the viral RNA replication complex and are translated directly from the genomic RNA (gRNA) (Oster et al. 1998; Scholthof et al. 1995a). P41 represents the coat protein and is translated from sgRNA1 (Hillman et al. 1989). The 3' terminal overlapping p22 and p19 genes encode for the cell-to-cell movement protein (MP) and RNA interference suppressor protein, respectively, and both are translated from sgRNA2 (Chu et al. 2000; Scholthof et al. 1995b). The relatively small TBSV genome together with its broad host range that includes both herbaceous and tree species makes TBSV a good model virus for the study of fundamental virus-plant processes (Yamamura and Scholthof 2005).

Although TBSV is a good model plant virus, it has limitations for VIGS (Qiu et al. 2002). The compact TBSV genome has limited locations for inserting foreign gene sequences and, because TBSV virions are isometric, there is a finite constraint on insert size for the genomic RNA to be encapsidated. However, the coat protein (CP) of TBSV and related tombusviruses is not required for infection of *Nicotiana benthamiana* plants (Scholthof et al. 1993), although its presence enhances efficient systemic spread in most hosts (Qu and Morris 2002; Turina et al. 2003). Therefore, CP replacement TBSV vectors, in which the desired sequence is inserted in place of the CP gene, have been developed and used for reporter gene expression and silencing transgenes such as *GFP* (Qiu et al. 2002), as well as in attempts at silencing endogenous *N. benthamiana* plant genes (Zhong et al. 2005). Unfortunately, rapid recombination and loss of the foreign sequences by these vectors (Scholthof et al. 1996) precluded their use for VIGS at the whole-plant level.

Corresponding author: B. W. Falk; E-mail: bwfalk@ucdavis.edu

D. Pignatta and P. Kumar contributed equally to this work and share senior authorship.

We have developed novel TBSV vectors by engineering an *XhoI* restriction site in three different positions (3f-3' end of the CP ORF and CEB- and CEA-sgRNA2 promoter elements) (Choi and White 2002) of the nucleotide sequence of pTBSV-100 (Fig. 1A, F, and G), a full-length cDNA infectious clone of cherry strain TBSV (Hearne et al. 1990). We also considered possible effects of the p19 suppressor activity on VIGS (Qiu et al. 2002; Voinnet et al. 1999) by using TBSV vectors both active and inactive for p19 protein. Here, we report the silencing of two plant genes, the H subunit of *magnesium chelatase* (*ChlH*) and *PDS*, and we quantify the silencing efficiency for all the different TBSV vectors with SYBR GREEN real-time reverse-transcription polymerase chain reaction (RT-

PCR) analysis. We demonstrated virion integrity for most of the new TBSV vectors by transmission electron microscopy, and confirmed silencing insert retention by RT-PCR and nucleotide sequence analysis.

RESULTS

CP replacement TBSV vectors silence the endogenous host gene *ChlH*.

We first evaluated the TBSV CP replacement vector (Fig. 1A and B) for silencing endogenous *N. benthamiana* genes. A 230-bp fragment of *ChlH* in the sense or antisense orientation was cloned into the CP replacement TBSV vector, pHST2-14

Fig. 1. Genome maps for *Tomato bushy stunt virus* (TBSV) vectors used in this study. **A**, TBSV wild type (pTBSV-100) genome map showing sgRNA1 and 2 transcription initiation sites (indicated by bent arrows). Open reading frames (ORFs) are represented by rectangles (pX ORF is not indicated). **B**, TBSV multiple cloning site (MCS) coat protein (CP) replacement vector with inactive p19. **C**, TBSV *magnesium chelatase* (*ChlH*) sense (pPD-1) and antisense (pPD-3) with active p19. **D**, TBSV-*ChlH* sense (pPD-2) and antisense (pPD-4) with inactive p19. **E**, TBSV *phytoene desaturase* (*PDS*) sense with inactive p19. **F**, Proposed secondary structure of (+) RNA TBSV showing 3f, CEB, and CEA insertion regions and sgRNA2 start site (Choi and White 2002). **G**, *XhoI* restriction site at 3f, CEB, or CEA with active p19. **H**, *XhoI* restriction site at 3f, CEB, or CEA with inactive p19. **I**, *ChlH* insert (132 bp) at 3f, CEB, or CEA with active p19 (pPD-3f2, pPD-B2, or pPD-A2) and *PDS* (108 bp) at 3f, CEB, or CEA with active p19 (pPD-3f4, pPD-B4, pPD-A4). **J**, *ChlH* insert (132 bp) at 3f, CEB, or CEA with inactive p19 (pPD-3f3, pPD-B3, or pPD-A3) and *PDS* (108 bp) at 3f, CEB, or CEA with inactive p19 (pPD-3f5, pPD-B5, or pPD-A5). * Indicates insert size = 264 bp because it is a double insert of *ChlH*.

(Scholthof et al. 1996) and into this vector with an active p19 gene. Inoculation of pPD-1 and 2 (*ChlH* sense, active and inactive for p19, respectively) and pPD-3 and 4 (*ChlH* antisense, active and inactive for p19) (Fig. 1 C and D) in in vitro transcripts effectively silenced the *ChlH* gene in *N. benthamiana* plants. For all the vectors, the silenced phenotype was very obvious, and appeared 3 to 4 days postinoculation (dpi). The first new leaves above the inoculated leaves developed predomi-

nantly yellow-white veins, indicating chlorophyll deficiency (chlorosis) but their lamina often were green (Fig. 2). However, the silencing phenotype was less apparent in the additional upper, uninoculated leaves, which remained green. TBSV was detectable in these green leaves, but not the silencing insert (RT-PCR, data not shown). Furthermore, plants inoculated with pPD-1 and 3 had both the immediate yellow-white symptomatic leaves and the upper nonsymptomatic green leaves

Fig. 2. *Nicotiana benthamiana* plants 9 days postinoculation (dpi) with in vitro transcripts of the *magnesium chelatase* (*ChlH*) *Tomato bushy stunt virus* (TBSV) silencing vectors. Photographs show silencing phenotypes on *N. benthamiana* plants inoculated with the different TBSV silencing vectors. Specific silencing vectors are indicated above each photograph. Names at left indicate the position of the insert (e.g., CEA) and the insert sequence (e.g., *ChlH*). Boxes at top indicate whether or not the given vector expresses TBSV p19. Control indicates plants inoculated with the vector only, containing no silencing insert, except for photograph at lower right, which shows a healthy plant. CP = coat protein and * indicates photographs taken 5 dpi.

curled. As a control, healthy plants (*N. benthamiana* inoculated with only RNA inoculation buffer) and plants inoculated with pHST2-14 were used. As expected, no silencing phenotype was observed (Fig. 2); in fact, pHST2-14-inoculated plants showed mild TBSV symptoms and no chlorosis, indicating the specificity of silencing.

We also tested silencing of the *N. benthamiana* *PDS* gene with the same TBSV vector. A 230-bp *N. benthamiana* *PDS* fragment was cloned in the sense orientation in pHST2-14 (pPD-5) (Fig. 1E). *N. benthamiana* plants inoculated with pPD-5 transcripts had only mild symptoms (Fig. 3), typical of

N. benthamiana plants infected by TBSV p19-inactive mutant (Scholthof et al. 1996), and no silencing phenotype was observed.

***Xho*I engineered TBSV vectors are infectious on *N. benthamiana* plants.**

In order to develop TBSV vectors which could move systemically in various host plants, and retain specific inserts, we constructed new TBSV vectors that express the CP gene and are either active or inactive for p19 gene expression. An *Xho*I restriction site was inserted into the pTBSV-100 nucleotide sequence, separately, at three different positions indicated as CEA (pPD-A), CEB (pPD-B), and 3f (pPD-3f) (Fig. 1F and G) without changing other nucleotides of pTBSV-100 (confirmed by sequencing; data not shown). *N. benthamiana* plants then were inoculated with pPD-A, -B and 3f (p19-active) transcripts and pPD-A1, -B1, and 3f1 (p19-inactive) transcripts (Fig. 1G and H). At 3 to 4 dpi, *N. benthamiana* plants inoculated with pPD-A transcripts began to show mild symptoms, compared with wild-type TBSV infection, in which the plants developed systemic necrosis followed by a vascular collapse 4 to 5 dpi. The pPD-A-infected plants developed necrosis and died by 9 dpi. The pPD-B vector induced symptoms identical to wild-type TBSV, and lethal necrosis was observed after 5 dpi. The pPD-3f vector caused very mild symptoms and no necrosis on *N. benthamiana* plants (data not shown). As expected, when *N. benthamiana* plants were infected with p19-inactive vectors, symptoms were milder than in plants infected with the same recombinant vectors that expressed p19. The expression and lack of expression of p19 protein in *N. benthamiana* plants infected with the p19-active and p19-inactive vectors were confirmed by immunoblot analysis (data not shown). The pPD-A1-infected *N. benthamiana* plants developed mild leaf curling symptoms by 7 dpi, whereas pPD-B1 caused more severe symptoms compared with the pPD-A1-infected plants. The pPD-3f1-infected *N. benthamiana* plants showed either very mild or no symptoms (Fig. 2, control panel). For each construct, we collected samples from upper uninoculated leaves and used RT-PCR followed by *Xho*I restriction digestion and determined that the inserted restriction site was retained (data not shown). Collectively, these results show that our recombinant TBSV vectors can successfully infect *N. benthamiana* plants, move systemically, and retain the 6-nucleotide (nt) *Xho*I insert.

New, *Xho*I-engineered TBSV recombinant vectors silence endogenous host genes *ChlH* and *PDS*.

To examine whether our recombinant TBSV vectors were effective at silencing endogenous genes, *ChlH* and *PDS* were chosen as targets for silencing. A 132-bp *ChlH* fragment and a 118-bp *PDS* insert were cloned into the *Xho*I restriction sites of TBSV vectors (Fig. 1I). For pPD-A2/3, the *ChlH* insert was a 264-bp dimer. All the constructs were used as templates for in vitro transcription and inoculation. Controls were *N. benthamiana* plants inoculated with the pPD-A1, pPD-B1, and 3f1 (*Xho*I as insert, inactive p19) and mock-inoculated plants.

***ChlH* silencing vectors.** Generally, for all the new recombinant vectors, the inoculated *N. benthamiana* plants developed a yellow-white silencing phenotype 4 to 5 dpi in the first new leaves emerging above those that were inoculated. However, contrary to what was observed for the CP replacement vectors (pPD-1-4), the silencing phenotype was much more widespread, affecting the upper uninoculated leaves (Fig. 2). It appeared first as a yellow-white coloration along the veins and then, as silencing progressed, some yellow spots became visible in the leaves which, in some cases, turned completely yellow (Fig. 2). This progression of silencing phenotype developed upward

Fig. 3. *Nicotiana benthamiana* plants 9 days postinoculation with in vitro transcripts of the *phytoene desaturase* (*PDS*) *Tomato bushy stunt virus* (TBSV) silencing vectors. Photographs show silencing phenotypes on *N. benthamiana* plants inoculated with the different TBSV silencing vectors. Specific silencing vectors are indicated above each photograph. Names at left indicate the position of the insert (e.g., CEA) and the insert sequence (e.g., *ChlH*). Boxes at top indicate whether or not the given vector expresses TBSV p19. CP = coat protein. Photographs of control treatment plants are in Figure 2.

through the canopy of the plant. Furthermore, the recombinant vectors with active p19 caused a severe leaf curling and stunting of the plants, resulting in a bushy appearance along with the yellow-white phenotype for all the upper uninoculated leaves (Fig. 2). In plants inoculated with recombinant vectors not expressing p19, the silencing phenotype still was observed 4 to 5 dpi, together with a mild leaf curling, which also had a minor effect on the plant growth. However, in contrast to the p19-active vectors, the infected plants continued to grow and the new upper leaves above the symptomatic ones were green (15 dpi; *image not shown*). Mock-inoculated (with the inoculation buffer) *N. benthamiana* plants did not show any symptoms, and plants inoculated with pPD-A1, pPD-B1, and pPD-3f1 showed typical TBSV infection symptoms (as described earlier).

PDS silencing vectors. *N. benthamiana* plants inoculated with pPD-A4, -B4, and 3f4 (Fig. 1I and J) developed photo-bleaching in the first upper uninoculated leaves, typical of *PDS* silencing, 4 to 5 dpi. The silencing phenotype also developed in the additional upper, uninoculated leaves as opposed to the phenotype observed for the CP replacement vectors. Leaf discoloration was different than in *ChlH* TBSV vector-inoculated plants: for *PDS*, the symptomatic area was white (Fig. 3), whereas a yellow or yellow-white color was observed for *ChlH* (Fig. 2). Discoloration appeared as a continuous spot covering most parts of the leaf starting from the apex and sometimes

covering it entirely. Plants were bushy and had curly leaves (Fig. 3). *N. benthamiana* plants inoculated with pPD-B5/A5 (p19 inactive) (Fig. 1J) showed a typical silencing phenotype and curled leaves over the whole plant (Fig. 3). Unexpectedly, for pPD-3f5 (p19 inactive)-inoculated *N. benthamiana* plants, no silencing phenotype was observed (Fig. 3), but TBSV (without insert) still was detected in upper uninoculated leaves (RT-PCR; data not shown).

Real-time RT-PCR and statistical analysis of *PDS* and *ChlH* mRNA levels in *N. benthamiana* plants.

To determine whether silencing phenotypes correlated with respective mRNA levels, we used SYBR GREEN real-time RT-PCR to quantify the levels of target mRNAs in *N. benthamiana* plants inoculated with the different recombinant silencing vectors.

ChlH silencing vectors. Statistical analyses showed that *N. benthamiana* plants infected by most of the recombinant silencing vectors had significantly less *ChlH* transcript than did the control group (Table 1, group G). The exceptions were the pPD-B2 and -B3 (CEB-*ChlH* active and inactive for p19, respectively) and pPD-3f3 (3FS-*ChlH* inactive for p19), which were not statistically different from the controls in spite of their consistently reduced *ChlH* mRNA levels (Table 1). In contrast, the CP replacement vectors (pPD-1, pPD-2, pPD-3, and pPD-4) showed the greatest silencing ability, with percentages

Table 1. *Magnesium chelatase (ChlH)* transcript levels for different silencing vectors compared with mock-inoculated plants and controls

TBSV vector	TBSV vector description	Average ^a	SD ^b	Silencing (%) ^c	Group ^d
pPD-2	TBSV <i>ChlH</i> sense inactive p19	0.032	0.075	96.78	A
pPD-1	TBSV <i>ChlH</i> sense active p19	0.017	0.072	98.25	AB
pPD-3	TBSV <i>ChlH</i> antisense active p19	0.016	0.158	98.45	AB
pPD-A2	CEA <i>ChlH</i> active p19	0.076	0.166	92.45	ABC
pPD-4	TBSV <i>ChlH</i> antisense inactive p19	0.106	0.174	89.44	ABCD
pPD-3f2	3f <i>ChlH</i> active p19	0.175	0.247	82.46	BCDE
pPD-B1	CEB inactive p19	0.187	0.047	81.35	CDE
pPD-A3	CEA <i>ChlH</i> inactive p19	0.168	0.136	83.20	CDEF
pPD-B3	CEB <i>ChlH</i> inactive p19	0.251	0.015	74.94	CDEFG
pPD-B2	CEB <i>ChlH</i> active p19	0.333	0.010	66.66	CDEFG
pPD-3f3	3f <i>ChlH</i> inactive p19	0.454	0.158	54.55	DEFG
pPD-3f1	3f inactive p19	1.046	0.445	–	EFG
pHST2-14	TBSV with multiple cloning site, replacing the coat protein, inactive p19	0.767	0.288	23.29	FG
Healthy	Healthy	1.050	0.188	–	G
pPD-A1	CEA inactive p19	1.776	0.292	–	G

^a Numbers indicate *ChlH* transcript amounts detected in *Nicotiana benthamiana* plants (six plants) inoculated with the corresponding TBSV silencing vector, relative to the healthy noninoculated *N. benthamiana* control.

^b Standard deviation.

^c Indicates percent reduction in *ChlH* transcript levels relative to the healthy noninoculated controls; – indicates no silencing effects.

^d Represents different statistical groups based on a 0.05 confidence level.

Table 2. *Phytoene desaturase (PDS)* transcript levels for different silencing vectors compared with mock-inoculated plants and controls

TBSV vector	TBSV vector description	Average ^a	SD ^b	Silencing (%) ^c	Group ^d
pPD-B4	CEB <i>PDS</i> -active p19	0.229	0.117	77.08	A
pPD-B5	CEB <i>PDS</i> -inactive p19	0.367	0.242	63.28	AB
pPD-A5	CEA <i>PDS</i> -inactive p19	0.435	0.255	56.48	ABC
pPD-3f4	3f <i>PDS</i> -active p19	0.420	0.248	58.03	ABC
pPD-A4	CEA <i>PDS</i> -active p19	0.667	0.307	33.35	BCD
pPD-B1	CEB-inactive p19	0.753	0.197	24.72	CD
pHST2-14	TBSV with multiple cloning site, replacing the coat protein, inactive p19	0.910	0.370	9.03	CDE
Healthy	Healthy	0.983	0.077	1.71	DEF
pPD-3f1	3f inactive-p19	1.335	0.410	–	DEF
pPD-A1	CEA-inactive p19	1.385	0.328	–	DEF
pPD-3f5	3f <i>PDS</i> -inactive p19	1.765	0.353	–	EF
pPD-5	TBSV <i>PDS</i> -inactive p19	2.064	0.464	–	F

^a Numbers indicate *PDS* transcript amounts detected in *Nicotiana benthamiana* plants (six plants) inoculated with the corresponding *Tomato bushy stunt virus* (TBSV) silencing vector, relative to the healthy noninoculated *N. benthamiana* control.

^b Standard deviation.

^c Indicates percent reduction in *PDS* transcript levels relative to the healthy noninoculated controls; – indicates no silencing effects.

^d Represents different statistical groups based on a 0.05 confidence level.

ranging from 90 to 98% (Table 1). However, these were samples taken only from the first upper symptomatic yellow-white leaves. The most efficient silencing ranged in the approximately 90%+ levels, followed by a second level of silencers in the approximately 80%+ range (Table 1). Of the new vectors, only the CEA recombinants (pPD-A2) showed silencing greater than 90% (Table 1). One of the control vectors, pPD-B1, showed quite a high percentage of silencing (81.35%); however, all plants inoculated with this construct showed typical TBSV symptoms along with visible signs of tissue damage. The decrease in *ChlH* mRNA levels could partly reflect that state. pHST2-14 (multiple cloning site replacing CP) (Fig. 1B) was used to serve as an appropriate control for the *ChlH* vectors. As expected, no silencing phenotype was observed, indicating that the silencing observed for the *ChlH* recombinant vectors was specific.

PDS silencing vectors. Silencing efficiency for *PDS* vectors generally was lower than for *ChlH*. Furthermore, pPD-3f5 and pPD-5 failed to show any silencing (Table 2), confirming the phenotype observed on inoculated plants (Fig. 2). Maximum silencing was achieved for the pPD-B4 and pPD-B5 vectors (CEB-*PDS* active and inactive for p19, respectively). pPD-A5 (CEA-*PDS* inactive for p19) and pPD-3f4 (3f-*PDS* active for p19) also showed good silencing efficiency. Control plants inoculated with pPD-A1 and 3f1 showed a slight increase in *PDS* mRNA levels, but not statistically different from healthy plant levels.

Silencing insert stability.

Unlike CP replacement vectors, which induced a silencing phenotype only in the upper leaves just above inoculated ones, the new recombinant silencing vectors (3f, CEB, and CEA) in-

Fig. 4. Transmission electron micrographs of *Tomato bushy stunt virus* (TBSV) virion particles extracted from upper, noninoculated leaves of *Nicotiana benthamiana* plants inoculated with the vectors as shown. The white bar represents 200 nm. There were no virions observed for the 3f vector-inoculated plants.

Table 3. Insert stability in virion RNAs for the different *Tomato bushy stunt virus* (TBSV) silencing vectors

TBSV vector ^a	TBSV vector description ^b	Complete insert ^c	Partial insert ^d	No insert ^e	Nonspecific sequence ^f
pPD-A2 ^g	CEA <i>Chl</i> -active p19	14/38	15/38	0/38	9/38
pPD-A4	CEA <i>PDS</i> -active p19	16/16	0/16	0/16	0/16
pPD-B2	CEB <i>ChlH</i> -active p19	9/30	2/30	13/30	6/30
pPD-B4	CEB <i>PDS</i> -active p19	38/40	0/40	0/40	2/40
pPD-A3 ^g	CEA <i>ChlH</i> -inactive p19	5/14	1/14	1/14	7/14
pPD-A5	CEA <i>PDS</i> -inactive p19	26/33	1/33	1/33	5/33
pPD-B3	CEB <i>ChlH</i> -inactive p19	13/16	2/16	0/16	1/16
pPD-B5	CEB <i>PDS</i> -inactive p19	11/16	5/16	0/16	0/16

^a *Tomato bushy stunt virus* (TBSV) virions were purified from upper, noninoculated leaves of *Nicotiana benthamiana* plants. Virion RNAs were extracted and used for reverse-transcription polymerase chain reaction (RT-PCR); RT-PCR products were cloned and sequenced.

^b *Chl* = magnesium chelatase and *PDS* = phytylene desaturase.

^c Number of clones showing the presence of complete insert, including the two *Xho*I restriction sites, over total tested.

^d Number of clones showing a partial insert (varying lengths) with one *Xho*I restriction site over total tested.

^e Number of clones showing native TBSV sequence without any insert or restriction site over total tested.

^f Number of clones which did not give any specific results due to sequencing errors.

^g Indicates a double insert of 264 bp for *ChlH*.

duced silencing phenotypes in upper leaves of the plant canopy (Figs. 2 and 3). We reasoned that the difference could be attributed to better mobility of the new vectors in plants, perhaps due to availability of CP (pPD-A2-5/B2-5) and better retention of the silencing insert. Therefore, in order to determine whether the recombinant genome sequences were encapsidated, virions were

Table 4. List of recombinant *Tomato bushy stunt virus* (TBSV) vectors indicating expression of coat protein (CP) and p19, type (*magnesium chelatase* [*ChlH*], *phytoene desaturase* [*PDS*], or *XhoI*), size, and position (CP replacement, 3f, CEB, or CEA) of insert

TBSV vector	CP ^a	p19 Active ^b	Insert ^c	Position
pTBSV-100	Y	Y	–	...
pHST2-14	N	N	Multiple cloning site (≈100 bp)	CP repl.
pPD-1	N	Y	<i>ChlH</i> sense (230 bp)	CP repl.
pPD-2	N	N	<i>ChlH</i> sense (230 bp)	CP repl.
pPD-3	N	Y	<i>ChlH</i> antisense (230 bp)	CP repl.
pPD-4	N	N	<i>ChlH</i> antisense (230 bp)	CP repl.
pPD-5	N	N	<i>PDS</i> sense (230 bp)	CP repl.
pPD-A	Y	Y	<i>XhoI</i> (6 bp)	CEA
pPD-A1	Y	N	<i>XhoI</i> (6 bp)	CEA
pPD-B	Y	Y	<i>XhoI</i> (6 bp)	CEB
pPD-B1	Y	N	<i>XhoI</i> (6 bp)	CEB
pPD-3f	N	Y	<i>XhoI</i> (6 bp)	3 f
pPD-3f1	N	N	<i>XhoI</i> (6 bp)	3 f
pPD-A2	Y	Y	<i>ChlH</i> (264 bp)	CEA
pPD-A3	Y	N	<i>ChlH</i> (264 bp)	CEA
pPD-B2	Y	Y	<i>ChlH</i> sense (132 bp)	CEB
pPD-B3	Y	N	<i>ChlH</i> sense (132 bp)	CEB
pPD-3f2	N	Y	<i>ChlH</i> sense (132 bp)	3 f
pPD-3f3	N	N	<i>ChlH</i> sense (132 bp)	3 f
pPD-A4	Y	Y	<i>PDS</i> sense (118 bp)	CEA
pPD-A5	Y	N	<i>PDS</i> sense (118 bp)	CEA
pPD-B4	Y	Y	<i>PDS</i> sense (118 bp)	CEB
pPD-B5	Y	N	<i>PDS</i> sense (118 bp)	CEB
pPD-3f4	N	Y	<i>PDS</i> sense (118 bp)	3 f
pPD-3f5	N	N	<i>PDS</i> sense (118 bp)	3 f

^a Expression of CP was checked by virion analysis and CP-immunoblot assay of total protein extract (data not shown); Y = yes and N = no.

^b Expression of p19 was checked by nucleotide sequence analysis and immunoblot assay of total protein extract (data not shown); Y = yes and N = no.

^c – indicates no insert.

purified from the symptomatic upper uninoculated leaves of *N. benthamiana* plants infected with recombinant silencing TBSV vectors. Transmission electron microscopic analysis showed that all recombinant vectors with inserts at CEA and CEB, and with an active or inactive p19 (pPD-A2-5 and pPD-B2-5), produced virions that appeared indistinguishable from wild-type TBSV virions (Fig. 4). In contrast, the 3f vectors (pPD-3f2-5) showed no detectable virion formation, suggesting that the insertion at the 3' end of the CP ORF affected the ability of the CP to form virion capsids (Fig. 4). Furthermore, the CP was not detected with immunoblot assays on total protein extract (data not shown) for 3f recombinant vector-infected plants.

To assess retention of the silencing inserts in the recombinant TBSV genomes during whole-plant infection, RNAs were extracted from the purified virion preparations and subjected to RT-PCR to amplify the TBSV genomic region flanking and containing the silencing inserts (3f, CEB, and CEA). The resulting RT-PCR products were cloned and approximately 16 recombinant colonies for each construct were sequenced. This approach allowed us to quantitatively and qualitatively assess insert composition. The results represented only encapsidated RNAs, because RNA extraction was performed with purified virions instead of infected plant tissue. Hence, recombinant TBSV vectors with insertions at the 3f site were excluded from this study because no virions were observed.

All of the CEA and CEB recombinants retained silencing inserts. The CEA and CEB *PDS* recombinants were best for insert retention (Table 3), with p19 presence having no observable effect on insert stability. Interestingly, for pPD-A2, which contained a dimer *ChlH* 264-nt insert, many of the vectors retained the dimer (14/38; Table 3) and almost as many had a monomeric insert (15/38; Table 3). Other recombinants showed various amounts of insert retention among progeny virions, but at least 30% retention was observed for all (Table 3).

DISCUSSION

TBSV based silencing vectors for endogenous gene silencing.

TBSV is a very good model virus for studying plant–virus interactions (Yamamura and Scholthof 2005). In previous

Table 5. Oligonucleotide primer sequences used in this study

Serial No.	Primer ^a	Forward (5'–3') ^b	Reverse (5'–3')	Size (bp)
1	<i>ChlH</i> insert	AAActcgagAGCCAGAAGACC	AAAagatctTGCCAACACCAG	230 ^c
			AAActcgagGCAAGGTTAAT	132 ^d
2	<i>PDS</i> insert	ATTActcgagGGCACTTAACCT TCATAAA	GCTagatctCTGCCATTATTCAG	230 ^e
			GCTCtcgagCAGGAGGGTTACC	118 ^f
	Real-time PCR			
3	<i>PDS</i>	TTCTTTTGGCTGAAGACTGGAAA	GAACCTCCCACTAGCTTCTCCAAC	65 ^g
4	<i>ChlH</i>	AGCGTGACCTTGTGGTAGGAA	TGGAGGTTACCAATGATGTGA	94 ^h
5	18s RNA	TACGCCCCGCCCAA	CACTGGCAGTCCTTCGTGAGT	65
	<i>XhoI</i> eng. TBSV vectors			
6	CEA	CTTAagcgccGCTGGGGCCACAGCC	CTTAactcgagCACTAAATTAGAG	1,080
		CTTAactcgagTGTCTGCGAGGGGCC	CTTAgaattcGTCTCATCGTTATGTA	≈200
7	CEB	CTTAagcgccGCTGGGGCCACAGCC	CTTAactcgagACATCATTCTGTCG	≈1,070
		CTTAactcgagTTCTCTAATTATG	CTTAgaattcGTCTCATCGTTATGTA	≈205
8	3f	CTTAagcgccGCTGGGGCCACAGCC	CTTAactcgagCACGCAATGTACTGTG	≈1,050
		CTTAactcgagCGCGCGACACGACAG	CTTAgaattcGTCTCATCGTTATGTA	≈250
	Sequencing insert stability			
9	3f/CEA/CEB	GCCTACGGCAACAACGTTA	CCCTAGCGTCGTTTCTTGT	≈350

^a *ChlH* = *magnesium chelatase*, *PDS* = *phytoene desaturase*, PCR = polymerase chain reaction, TBSV = *Tomato bushy stunt virus*.

^b Lowercase letters indicate restriction sites used for cloning.

^c The silencing insert amplicon corresponds to nucleotides 3,597 to 3,816 (GenBank accession number AF014052).

^d The silencing insert amplicon corresponds to nucleotides 3,597 to 3,729 (GenBank accession number AF014052).

^e The silencing insert amplicon corresponds to nucleotides 855 to 1,103 (GenBank accession number DQ469932).

^f The silencing insert amplicon corresponds to nucleotides 855 to 973 (GenBank accession no. DQ469932).

^g The amplicon corresponds to nucleotides 1,157 to 1,222 (GenBank accession number DQ469932).

^h The amplicon corresponds to nucleotides 2,622 to 2,716 (GenBank accession number AF014052).

VIGS studies using TBSV, p19-inactive TBSV vectors containing sequences of the gene encoding GFP in place of the capsid protein gene silenced GFP in line 16c *N. benthamiana* plants (Qiu et al. 2002). However, a p19-active TBSV CP replacement vector with an approximately 550-nt fragment of the *N. benthamiana ChlH* gene failed to silence this endogenous gene in *N. benthamiana* plants, even in inoculated leaves (Zhong et al. 2005). Here, we used a 230-nt *N. benthamiana ChlH* silencing insert in two TBSV CP replacement vectors, (active and inactive p19), and inoculated plants exhibited a yellow-white phenotype indicative of *ChlH* silencing. Why we obtained silencing whereas previous efforts did not is unknown; however, choice of insert sequence or size might have been important (Burch Smith et al. 2004). It also is possible that the approximately 550-bp sequence used previously was excised from the TBSV genome faster than was our 230-bp insert, allowing silencing in our experiments but not in previous attempts. However, even in our experiments, the silencing phenotype was prominent only in the first new leaves above those inoculated. Subsequent emerging leaves were green and, although they still contained TBSV, the *ChlH* insert was no longer present. These results suggest that the silencing phenotype developed in the plant only where the recombinant TBSV vectors retained the foreign insert and moved effectively, as previously discussed for other *Potato virus X* (PVX) systems (Himber et al. 2003). CP-defective TBSV mutants spread systemically in *N. benthamiana* plants but do so only with delay; thus, they cause milder symptoms than wild-type TBSV (Desvoyes and Scholthof 2002; Scholthof et al. 1993). Furthermore, Qu and Morris (2002) suggested the TBSV CP likely enables efficient viral unloading from the vascular system into leaf mesophyll cells as assembled virions. This could explain the silencing pattern we observed on the symptomatic leaves infected with CP-deficient vectors, where the silencing phenotype was limited to the veins, but not in interveinal leaf lamina.

In order for recombinant TBSV vectors to be movement competent in planta and to retain a sufficiently large silencing insert in the upper uninoculated systemic leaves, we constructed new recombinant TBSV viral vectors. All were developed with the intent to still express TBSV-CP and assemble virions. Variations in the nucleotide sequences of these constructs included position of the silencing insert (3f, CEB, or CEA) (Fig. 1F), expression or inactivation of p19, and size of the silencing insert. The CEA and CEB regions of the TBSV genome are important sequences for sgRNA2 transcription (Lin and White 2004). These sequences interact with other sections of the (+) RNA in extensive secondary structure. Whether altering these regions would affect the ability to infect whole plants was unknown, but our data suggest that the alterations had no significant effects. In contrast, the 3f region is at the very 3' end of the CP ORF, just before the CEA and CEB sgRNA2 transcription elements. It is in the region encoding the P domain of the CP, which protrudes on the exterior of the virion particle (Hsu et al. 2006). We wondered whether the 3f vectors with an additional in-frame insert in this region could still make CP and virions and move normally in plants. However, symptoms of pPD-3f vector-inoculated plants were reminiscent of *N. benthamiana* plants inoculated with the CP-replacement vectors (Scholthof 1999). Because no virions were recovered from the pPD-3f-inoculated plants (Fig. 4), we suggest that the lack of TBSV virion formation is associated with milder symptoms in *N. benthamiana* plants.

Based on overall plant phenotype, the CEA and CEB vectors were better VIGS vectors than the TBSV CP-replacement vectors. The silencing phenotype was obvious in interveinal leaf lamina and even developed well above inoculated leaves. However, p19-expressing vectors affected the plant phenotype,

resulting in stunted plants with very small terminal leaves, but exhibiting a clear silencing phenotype. In contrast, p19-inactive vectors did not stunt plants, but plants did not exhibit the silencing phenotype in the upper, newly emerged leaves. The CEA and CEB recombinant vectors also formed virions in infected *N. benthamiana* plants, and the virion populations retained the silencing inserts. Because we cloned and sequenced virion RT-PCR-derived sequences, each sequence represents a different virion RNA molecule. Our data showed that different recombinant vectors had a higher (e.g., pPD-A4) or lower (e.g., pPD-A3) percentage of intact inserts (Table 3). Furthermore, as these virions were purified from upper uninoculated leaves, this demonstrates that recombinant constructs retaining the ability to form virions could be important for induction of silencing phenotype in upper leaves. This could have important advantages, particularly in plant hosts other than *N. benthamiana* where virion formation could be a prerequisite for systemic movement (Qu and Morris, 2002).

Real-time RT-PCR analysis of *ChlH* and *PDS* silencing in *N. benthamiana* plants.

We developed an accurate quantitative measure of silencing effects for *ChlH* and *PDS* genes in infected plants to determine optimum conditions or vectors for silencing. CP replacement vectors showed the greatest effects, as evident from the high silencing percentages (95 to 98%) for *ChlH* (Table 1). However, the silencing phenotype was observed prominently only in the first upper leaves above the inoculated ones, and these were the leaves analyzed here. In contrast, for the CEA, CEB, and 3f vectors, we analyzed leaves well up into the canopy above the inoculated leaves. Because of the differences in vector construction, size and sequence of silencing insert, and leaves examined, we cannot make generalizations about the significance of the differences for all constructs evaluated. However, significant reductions in target mRNA levels were achieved for both targets with different vectors.

The apparent lack of effect of p19 on silencing efficiencies is interesting. P19 has significant effects on movement or symptom development (Turina et al. 2003) and is a potent gene-silencing suppressor (Voinnet et al. 1999). These properties may have opposing influences on VIGS, because the p19-active vectors may move more efficiently and have higher general fitness in the plant; however, silencing suppression activity from an active p19 could compromise their silencing capability. In contrast, the p19-inactive vectors might be affected for symptoms but, because they lack p19 and its suppressor activity, they might be more effective in gene silencing. These opposing effects of p19 may explain the apparent lack of differences in silencing effects between the p19-active and p19-inactive vectors seen here. Similar results were obtained for the CEB insertion vectors, which showed near-equal silencing effects with active or inactive p19 (Table 1). The CEA (pPD-A2 and -3) insertion vectors were more efficient than their CEB (pPD-B2 and -3) counterparts in *ChlH* silencing.

Different quantitative effects were observed for different target genes. *PDS* silencing levels did not reach the levels achieved by the *ChlH* vectors. Possible reasons for this include choice of silencing insert for each gene in question, or *PDS* may have host paralogs that were not silenced by this particular insert. The 3f vectors (pPD-3f 2 and 3) worked better with active p19 (82.5%) than with inactive (55%) p19 (Table 1), at least for *ChlH*.

Another factor which may affect VIGS is p19 dosage effects on silencing suppression (Qiu et al. 2002). Dimers of p19 bind to siRNAs, preventing formation of an active RNA-induced silencing complex (Lakatos et al. 2004; Omarov et al. 2006), and thereby suppress silencing. The CEA and CEB regions are

important transcription regulatory regions in protoplasts (Lin and White 2004) for sgRNA2, which expresses p22 and p19. Our insertions at the CEA and CEB sites may create differences in silencing capabilities for these different vectors, if the in planta p19 levels for each particular silencing vector were affected. However, we detected comparable amounts of p19 by immunoblot analyses in 3f-, CEB-, and CEA-vector inoculated *N. benthamiana* plants and those inoculated with the pTBSV-100 control (data not shown). However, this result cannot be considered alone for comparison of p19 expression analysis, because the virus titer in the different plants likely varies and must be factored in the analysis before we can comment on the effects of the insertions on p19 levels per se.

Insert integrity.

Insert retention is important because it influences silencing efficiency and may indirectly affect virus fitness and titer. In other studies, PVX vectors carrying the *GFP* gene were targeted and eliminated from a line 16c *N. benthamiana* GFP-expressing plant. But the same PVX vector, when loaded with a host endogenous gene (*RbcS*, small subunit of RUBISCO), was not eliminated from PVX:*RbcS*-silenced plants (Jones et al. 1999; Ruiz et al. 1998). Although this example explored the differences between a transgene and an endogenous gene, another interesting finding was reported on characterizing recombinant forms of the viral vectors (PVX) under RNA-silencing pressure (Barajas et al. 2006). In this case, the viral vector was not eliminated from the infected plant, in spite of retaining its sequences, which were homologous to the silenced transgene sequence. However, recombinant forms of the viral vector were found, which had certain sequences missing from the original insert. This provides an interesting point in that, if the virus retains the insert, it might or might not be targeted by a silencing mechanism of the host and thus be limited in titer, movement, and spread. However, at the same time, as indicated by our studies, when the viral vector loses its insert it also loses its capability to maintain silencing. Thus, there may be a trade-off between silencing capability and overall fitness of the virus, if it is indeed engineered with a host endogenous gene. The stability of foreign inserts demonstrates that our recombinant TBSV vectors may retain and carry inserts up to sizes of approximately 260 bp into the systemic upper leaves, which might make them suitable candidates for viral vectors for VIGS.

MATERIALS AND METHODS

Recombinant TBSV vectors.

Recombinant TBSV vectors (Table 4) were constructed following standard protocols (Sambrook et al. 1989). The sequences of the primers used in this study are reported in Table 5.

pPD-1, pPD-2, pPD-3, pPD-4, and pPD-5 vectors.

A 230-bp fragment of the gene encoding the H subunit of *ChlH* (GenBank accession no. AF014052) was PCR amplified from *N. benthamiana* DNA and cloned into pGEM-T-Easy Vector System I (Promega Corp., Madison, WI, U.S.A.). The *ChlH* fragment was removed by digestion with *XhoI* and *BglII* and cloned in the sense (pPD-2) and antisense (pPD-4) orientations in place of the multiple cloning site insert in pHST2-14 (Fig. 1B), as previously described (Scholthof et al. 1996). The same procedure was followed to clone a 230-bp fragment of the *N. benthamiana PDS* (GenBank accession no. DQ469932) gene in the sense orientation and to obtain pPD-5. pPD-2 and pPD-4 were modified by replacing the inactive p19 gene with the active p19 gene from pTBSV-100, by exchanging the *EcoRI* and *SmaI* fragment, to obtain pPD-1 and pPD-3.

pPD-A, pPD-A1, pPD-B, pPD-B1, pPD-3f, and pPD-3f1 vectors.

A 6-bp *XhoI* restriction site was engineered by PCR into three different positions of pTBSV-100 to construct pPD-A, pPD-B, and pPD-3f (Fig. 1G). The two TBSV PCR products flanked by *EcoRI/XhoI* and *XhoI/NotI*, respectively (Table 5), were ligated together as part of a tripartite ligation with pTBSV-100 digested with *EcoRI* and *NotI*, resulting in the incorporation of the *XhoI* site in the desired locations. PD-A, -B, and 3f were modified by replacing the active p19 gene with the inactive p19 gene from pHST2-14 to obtain pPD-A1, -B1, and 3f1.

pPD-A, pPD-B, and pPD-3f vectors.

A 132-bp fragment was PCR amplified from the pGEM-T-Easy Vector containing the *N. benthamiana ChlH* sequence and cloned into all the *XhoI*-engineered TBSV vectors to obtain pPD-A2*, pPD-A3* (* = double insert, 264 bp), pPD-B2, pPD-B3, pPD-3f2 and pPD-3f3. Similarly, pPD-A4, pPD-A5, pPD-B4, pPD-B5, pPD-3f4, and pPD-3f5 were obtained by cloning a 118-bp fragment of the *N. benthamiana PDS* gene.

Transcript inoculation and sample collection.

All TBSV vectors were digested with *SmaI* and used for in vitro transcription (MEGAscript Kit, Ambion, Inc., Austin, TX, U.S.A.). Two leaves of 3-week-old *N. benthamiana* plants were inoculated with transcripts and FES inoculation buffer (1% sodium pyrophosphate, 1% bentonite, 1% celite in 0.1 M glycine, 0.06 M dibasic potassium phosphate). Six plants were inoculated for each transcript. *N. benthamiana* plants were sampled 9 dpi; upper, uninoculated leaves were collected and processed for RNA and virion analysis.

mRNA quantification

using real-time RT-PCR and statistical analysis.

SYBR GREEN real-time RT-PCR was used to quantify *ChlH* and *PDS* mRNA expression by the comparative threshold cycle method (User Bulletin #2; Applied Biosystems, Foster City, CA, U.S.A.). 18s rRNA was the endogenous control. Primers for *ChlH* and *PDS* mRNA quantification were designed outside the silencing region, using the Primer Express software (Applied Biosystems). A six-point dilution series (in duplicate) of an *N. benthamiana* RNA sample was used to obtain a standard curve for all primer pairs. Amplification efficiencies of each primer pair were calculated from the slope of the standard curve using the formula $E = 10^{1/\text{slope}} - 1$.

Total RNA was extracted from approximately 75 mg of leaf tissue with the RNeasy kit (Qiagen Inc., Valencia, CA, U.S.A.) according to the manufacturer's instructions. RNA was finally eluted with 100 μ l of RNase free water and DNase (Fermentas, Hanover, MD, U.S.A.) treated as per the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from DNase-digested total RNA using standard protocols (Promega Corp.).

Real-time PCR reactions (25 μ l) were done in 96-well reaction plates using 2 $\times$ SYBR GREEN PCR Master Mix (Applied Biosystems), 5 μ l of cDNA, and 400 nM gene-specific primers. Real-time PCR was performed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems) under standard amplification conditions. Melting curve analyses was performed for each gene (*PDS*, *ChlH*, 18s).

Statistical analyses was carried out using the Tukey-Kramer test with $P = 0.05$.

Virion RNA extraction, silencing insert stability, and sequence.

Virions were extracted from upper uninoculated leaves of *N. benthamiana* plants. Leaf tissue (100 mg) was ground in liquid

nitrogen and 1 ml of ice cold Na-Acetate (0.2 M, pH 5.0). After overnight incubation at 4°C, samples were centrifuged (12,000 rpm, 4°C, 10 min) and the supernatant adjusted to 8% polyethylene glycol (MW 8,000) and 1% NaCl and incubated at 4°C for 1 h. After centrifugation (10,000 rpm, 4°C, 15 min), the pellet (virions) was redissolved in 50 µl of ice-cold Na-Acetate (0.2 M, pH 5.0). The partially purified TBSV virions first were fixed with 3.4% formaldehyde at room temperature for 5 min. Formvar or carbon-coated copper grids were allowed to float on 5 µl of partially purified virions for 2 min. Grids then were transferred to 10 µl of 2% phosphotungstate (pH 7.2) and stained for 2 min. Grids then were examined under a transmission electron microscope.

Virion preparations (11 µl) were used for RNA extraction (20 mM Tris-HCl, pH 8, 1% sodium dodecyl sulfate, 200 mM NaCl, and 5 mM EDTA, followed by a phenol/chloroform extraction). The RNA pellet was dissolved in 15 µl of diethylpyrocyanate-treated water. One-tube RT-PCR (25 µl) was performed using standard protocols (Promega Corp.) with TBSV-specific primers. The PCR products were cloned into TOPO TA Cloning vectors (Invitrogen, Carlsbad, CA, U.S.A.) and, following blue-white selection, 16 white colonies were grown in a 96-well plate at 37°C overnight and sequenced.

Insert stability for CP replacement vectors (pPDI-4).

Total RNA was extracted as described for real-time PCR analysis. A one-tube RT-PCR (25 µl) was performed as described before. PCR products were sequenced.

ACKNOWLEDGMENTS

The authors would like to thank H. B. Scholthof and G. Bruening for helpful discussions and for providing TBSV-related clones and antibody, C. Leutenegger for helping with real-time PCR, and T. Tian for the transmission electron micrographs of the TBSV virions. This research was supported in part by the University of California, the Jastro Shields Graduate Research Scholarship Award (P. Kumar, Plant Biology Graduate Group; 2005-2007), and the Ministry of Education, University and Research, Italy.

LITERATURE CITED

- Barajas, D., Tenllado, F., and Diaz-Ruiz, J. R. 2006. Characterization of the recombinant forms arising from a *Potato virus X* chimeric virus infection under RNA silencing pressure. *Mol. Plant-Microbe Interact.* 19:904-913.
- Baulcombe, D. C. 1999. Gene silencing: RNA makes RNA makes no protein. *Curr. Biol.* 9:R599-601.
- Burch-Smith, T. M., Anderson, J. C., Martin, G. B., and Dinesh-Kumar, S. P. 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant J.* 39:734-746.
- Choi, I.-R., and White, K. A. 2002. An RNA Activator of subgenomic mRNA1 transcription in *Tomato bushy stunt virus*. *J. Biol. Chem.* 277:3760-3766.
- Chu, M., Desvoyes, B., Turina, M., Noad, R., and Scholthof, H. B. 2000. Genetic dissection of *Tomato bushy stunt virus* p19-protein-mediated host-dependent symptom induction and systemic invasion. *Virology* 266:79-87.
- Desvoyes, B., and Scholthof, H. B. 2002. Host-dependent recombination of a *Tomato bushy stunt virus* coat protein mutant yields truncated capsid subunits that form virus-like complexes which benefit systemic spread. *Virology* 304:434-442.
- Faivre-Rampant, O., Gilroy, E. M., Hrubikova, K., Hein, I., Millam, S., Loake, G. J., Birch, P., Taylor, M., and Lacomme, C. 2004. *Potato virus X*-induced gene silencing in leaves and tubers of potato. *Plant Physiol.* 134:1308-1316.
- Hearne, P. Q., Knorr, D. A., Hillman, B. I., and Morris, T. J. 1990. The complete genome structure and synthesis of infectious RNA from clones of tomato bushy stunt virus. *Virology* 177:141-151.
- Hillman, B. I., Hearne, P., Rochon, D., and Morris, T. J. 1989. Organization of tomato bushy stunt virus genome: Characterization of the coat protein gene and the 3' terminus. *Virology* 169:42-50.
- Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C., and Voinnet, O. 2003. Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO (Eur. Mol. Biol. Organ.) J.* 22:4523-4533.
- Hsu, C., Singh, P., Ochoa, W., Manayani, D. J., Manchester, M., Scheemann, A., and Reddy, V. S. 2006. Characterization of polymorphism displayed by the coat protein mutants of *Tomato bushy stunt virus*. *Virology* 349:222-229.
- Jones, L., Hamilton, A. J., Voinnet, O., Thomas, C. L., Maule, A. J., and Baulcombe, D. C. 1999. RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell* 11:2291-2301.
- Lakatos, L., Szittyá, G., Silhavy, D., and Burgan, J. 2004. Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *EMBO (Eur. Mol. Biol. Organ.) J.* 23:876-884.
- Lin, H.-X., and White, K. A. 2004. A complex network of RNA-RNA interactions controls subgenomic mRNA transcription in a tombusvirus. *EMBO (Eur. Mol. Biol. Organ.) J.* 23:3365-3374.
- Lu, R., Malcuit, I., Moffett, P., Ruiz, M. T., Peart, J., Wu, A. J., Rathjen, J. P., Bendahmane, A., Day, L., and Baulcombe, D. C. 2003. High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. *EMBO (Eur. Mol. Biol. Organ.) J.* 22:5690-5699.
- Naylor, M., Reeves, J., Cooper, J. I., Edwards, M. L., and Wang, H. 2005. Construction and properties of a gene-silencing vector based on *Poplar mosaic virus* (genus *Carlavirus*). *J. Virol. Methods* 124:27-36.
- Omarov, R., Sparks, K., Smith, L., Zindovic, J., and Scholthof, H. B. 2006. Biological relevance of a stable biochemical interaction between the tombusvirus-encoded P19 and short interfering RNAs. *J. Virol.* 80:3000-3008.
- Oster, S. K., Wu, B., and White, K. A. 1998. Uncoupled expression of p33 and p92 permits amplification of tomato bushy stunt virus RNAs. *J. Virol.* 72:5845-5851.
- Qiu, W., Park, J. W., and Scholthof, H. B. 2002. Tombusvirus P19-mediated suppression of virus-induced gene silencing is controlled by genetic and dosage features that influence pathogenicity. *Mol. Plant-Microbe Interact.* 15:269-280.
- Qu, F., and Morris, T. J. 2002. Efficient infection of *Nicotiana benthamiana* by *Tomato bushy stunt virus* is facilitated by the coat protein and maintained by p19 through suppression of gene silencing. *Mol. Plant-Microbe Interact.* 15:193-202.
- Ratcliff, F., Martin-Hernandez, A. M., and Baulcombe, D. C. 2001. Technical advance. *Tobacco rattle virus* as a vector for analysis of gene function by silencing. *Plant J.* 25:237-245.
- Robertson, D. 2004. VIGS vectors for gene silencing: Many targets, many tools. *Annu. Rev. Plant Biol.* 55:495-519.
- Ruiz, M. T., Voinnet, O., and Baulcombe, D. C. 1998. Initiation and maintenance of virus-induced gene silencing. *Plant Cell* 10:937-946.
- Russo, M., Di Franco, A., and Martelli, G. P. 1987. Cytopathology in the identification and classification of tombusviruses. *Intervirology* 28:134-143.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Scholthof, H. B. 1999. Rapid delivery of foreign genes into plants by direct rub-inoculation with intact plasmid DNA of a tomato bushy stunt virus gene vector. *J. Virol.* 73:7823-7829.
- Scholthof, H. B., Morris, T. J., and Jackson, A. O. 1993. The capsid protein gene of tomato bushy stunt virus is dispensable for systemic movement and can be replaced for localized expression of foreign genes. *Mol. Plant-Microbe Interact.* 6:309-322.
- Scholthof, K.-B. G., Scholthof, H. B., and Jackson, A. O. 1995a. The tomato bushy stunt virus replicase proteins are coordinately expressed and membrane associated. *Virology* 208:365-369.
- Scholthof, H. B., Scholthof, K.-B. G., Kikkert, M., and Jackson, A. O. 1995b. Tomato bushy stunt virus spread is regulated by two nested genes that function in cell-to-cell movement and host-dependent systemic invasion. *Virology* 213:425-438.
- Scholthof, H. B., Scholthof, K.-B. G., and Jackson, A. O. 1996. Plant virus gene vectors for transient expression of foreign proteins in plants. *Annu. Rev. Phytopathol.* 34:299-323.
- Turina, M., Omarov, R., Murphy, J. F., Bazaldua-Hernandez, C., Desvoyes, B., and Scholthof, H. B. 2003. A newly identified role for *Tomato bushy stunt virus* p19 in short distance spread. *Mol. Plant Pathol.* 4:67-72.
- Vaucheret, H., Beclin, C., and Fagard, M. 2001. Post-transcriptional gene silencing in plants. *J. Cell Sci.* 114:3083-3091.
- Voinnet, O., Pinto, Y. M., and Baulcombe, D. C. 1999. Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants. *Proc. Natl. Acad. Sci. U.S.A.* 96:14147-14152.
- Yamamura, Y., and Scholthof, H. B. 2005. *Tomato bushy stunt virus*: A resilient model system to study virus-plant interactions. *Mol. Plant Pathol.* 6:491-502.
- Zhong, X., Hou, H., and Qiu, W. 2005. Integrity of nonviral fragments in recombinant *Tomato bushy stunt virus* and defective interfering RNA is influenced by silencing and the type of inserts. *Mol. Plant-Microbe Interact.* 18:800-807.