Assessment of Recombinants That Arise from the Use of a TMV-Based Transient Expression Vector

Shailaja Rabindran and William O. Dawson[1]

Department of Plant Pathology, University of Florida, Citrus Research and Education Center, Lake Alfred, Florida 33850

A potential use of virus-based transient expression vectors is the large-scale production of commercial specialty products, which would require the inoculation of many acres of plants with the viral vector. However, there are several concerns about the widespread use of virus-based vectors. Among these are the spread of the engineered virus to susceptible plants and the generation and persistence of recombinant viruses in the environment. Using a *Tobacco mosaic virus* (TMV)-based transient gene expression vector, 30B, which expresses the jellyfish green fluorescent protein (30B-GFP), we describe the predominant types of hybrid viruses that developed in plants. In general, the recombinants deleted the foreign gene and repeated sequences, retaining only those sequences required for optimal replication and movement. In pathogenicity studies and challenge experiments designed to make a comparative assessment of the competitiveness of the recombinants with the parent virus, the recombinants had reduced vigor and were less competitive and pathogenic than TMV, a virus which is already present in the areas where tobacco is grown.   © 2001 Academic Press

INTRODUCTION

Virus-based vectors are beginning to have major impact as expression systems for the production of specialty products (Turpen *et al.,* 1997; Turpen, 1999; Yusibov *et al.,* 1999), for the delivery of therapeutics (Berglund *et al.,* 1996; Dubensky *et al.,* 1996; Chu and Dornburg, 1997; Johnson *et al.,* 1997; Tubuleska *et al.,* 1997; Pogue *et al.,* 1998; Shivprasad *et al.,* 1999), and as powerful laboratory tools (Pogue *et al.,* 1998). A major advantage of plant-virus based vectors is that plants provide one of the most economical sources of biomass for the large-scale commercial production of important specialty products. Also, many plant viruses have small genomes that can be easily manipulated, and the viruses accumulate to copious amounts in infected plants in a relatively short period of time. The tobamovirus, *Tobacco mosaic virus* (TMV), can constitute as much as 10% of the dry weight of a plant. The foreign gene product can be harvested for maximal product yield from infected plants within a few days of inoculation. Furthermore, purification of the specialty protein can be simplified by using appropriate leader sequences that target the foreign product to specific locations in the cell such as the endomembrane, the cytosol, or the apoplast (Pogue *et al.,* 1998; Yusibov *et al.,* 1999), or by fusing the foreign protein to the coat protein of the virions (Hamamoto *et al.,* 1993; Turpen *et al.,* 1995; Yusibov *et al.,* 1997; Modelska *et al.,* 1998).

Initially, RNA viruses were considered unsuitable for use as viral vectors because the low fidelity of the viral RNA polymerase would quickly result in mutation of the foreign sequences (van Vloten-Doting *et al.,* 1985). However, Kearney *et al.* (1993) demonstrated that the rate of accumulation of mutations in foreign sequences in a TMV-based vector was low and concluded that sequence drift was not a practical limitation to the use of TMV as a vector. The first generation of TMV-based vectors that contained duplicated sequences were unstable *in planta*, due to rapid recombination between identical sequences (Dawson *et al.,* 1989). A TMV mutant that had an additional homologous coat protein gene was similarly unstable (Beck and Dawson, 1990), whereas a mutant with the extra coat protein gene from a different tobamovirus was remarkably stable (Culver *et al.,* 1993). Based on these observations, the vector was redesigned to include a second subgenomic mRNA promoter and coat protein ORF from *Odontoglossum* ringspot virus (ORSV) (Donson *et al.,* 1991), while foreign gene expression was regulated by the native TMV coat protein subgenomic mRNA promoter. This vector, TB2, has been shown to maintain foreign genes stably in plants during the few weeks of a typical product-manufacturing cycle (Donson *et al.,* 1991; Kearney *et al.,* 1993). Further improvement of TB2 to enhance levels of foreign gene expression resulted in the development of 30B (Shivprasad *et al.,* 1999), which has heterologous sequences from tobacco mild green mosaic virus strain U5 (TMGMVU5). 30B, which has been made available for experimental use worldwide, expresses large amounts of foreign gene product and is sufficiently stable to be

[1] To whom correspondence and reprint requests should be addressed. E-mail: wodtmv@lal.ufl.edu.
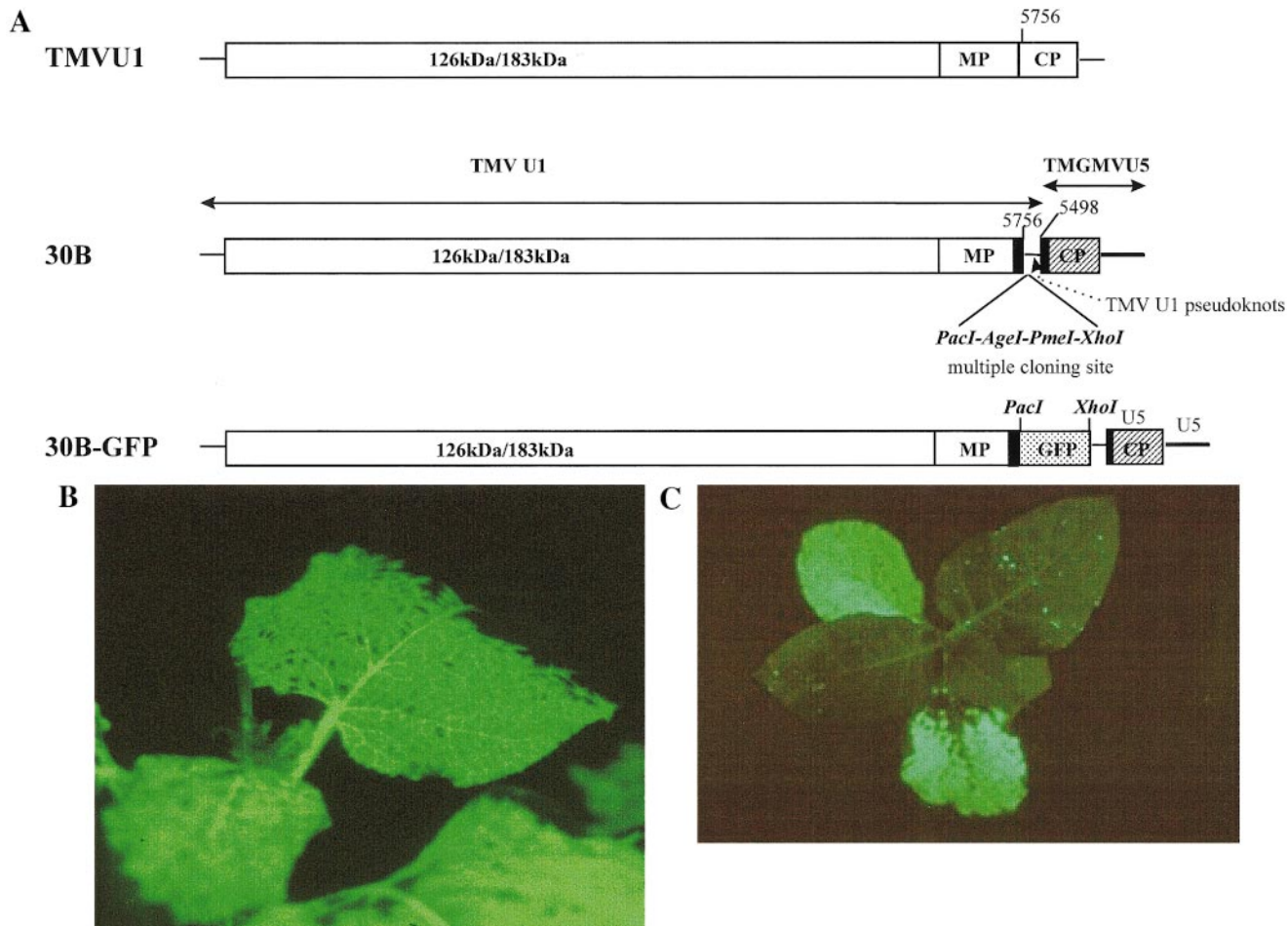
FIG. 1. (A) Schematic diagram of the genomic organization of TMVU1, 30B, and 30B-GFP. As indicated, the 5′ NTR, replicase, and movement protein genes in 30B and 30B-GFP are derived from TMVU1, whereas the 3′ portion of the vector originates from TMGMVU5. The dark boxes correspond to coat protein subgenomic mRNA promoter sequences. Nucleotide numbers in 30B correspond to the position in the respective viral genome. MP, movement protein; CP, coat protein; GFP, green fluorescent protein. (B) Fluorescence emitted under UV illumination (366 nm) of *N. benthamiana* infected with 30B-GFPC3 7 dpi. (C) Fluorescence emitted under UV illumination of *N. tabacum* cv Xanthi infected with 30B-GFPC3 5 dpi.

useful for the production of specialty products in the tobacco field.

Because of their capability of producing large amounts of commercial specialty products utilizing large tracts of land, plant virus-based vectors have opened up new applications for agriculture. But what would be the possible consequences of disseminating large amounts of virus-based vectors into the fields? Ideally with viral vectors, the foreign gene product would initially be produced rapidly, and then the gene would be deleted from the viral genome, ultimately resulting in the development of an innocuous virus. In this way, also, the foreign sequences would not persist in the environment.

In this report we present results from experiments designed to address several potential concerns associated with large-scale dissemination of virus-based vectors. The primary aim of this investigation was to examine the predominant recombinants that developed after inoculation of plants with the TMV-based gene expression vector 30B (Shivprasad *et al.,* 1999), which ex-

presses the green fluorescent protein (GFP; 30B-GFP) from the jellyfish, *Aequorea victoria* (Chalfie *et al.,* 1994), for their virulence and ability to compete with the parental virus, TMV, which already exists in tobacco fields.

RESULTS

Development of recombinants from infection with 30B-GFP

The TMV-based transient expression vector 30B (Fig. 1A) contains heterologous sequences from tobacco mild green mosaic virus U5, including the coat protein subgenomic mRNA promoter (which also corresponds to sequences within the movement protein ORF), the coat protein ORF, and the 3′ nontranslated region. *Nicotiana benthamiana* plants were inoculated with *in vitro* transcripts of 30B-GFP (wild-type GFP; Prasher *et al.,* 1992) and 30B-GFPC3 (enhanced mutant of GFP; Crameri *et al.,* 1996) and the movement of the viruses was monitored by illumination with a hand-held long-wave ultraviolet
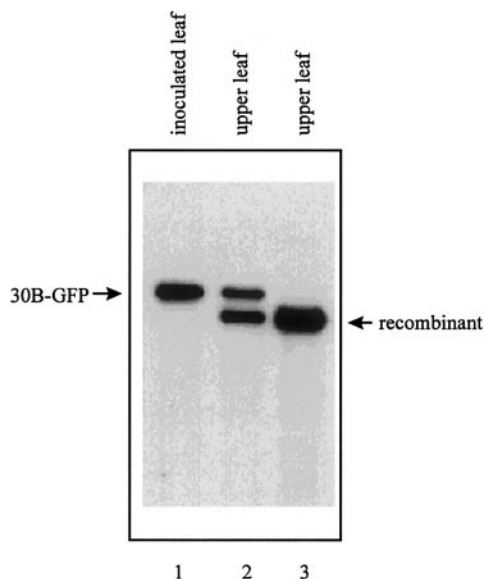
FIG. 2. Northern blot hybridization analysis of RNA extracted from upper leaves of *N. benthamiana*, 7 dpi, after the second passage of 30B-GFP in the plant. Lane 1, inoculated leaf; lanes 2, 3, upper leaves. The membranes were probed with a plus-sense-specific riboprobe which hybridizes to the 5′ 256 nts of the TMV genome.

lamp (366 nm). Green fluorescent spots were observed in locally inoculated leaves at 3 days postinoculation (dpi), and systemically infected leaves had diffuse green fluorescence at approximately 7 dpi (30B-GFPC3; Fig. 1B). To monitor the stability of foreign gene expression, the viruses were serially passaged in *N. benthamiana* at 2-week intervals. After the second passage, systemically infected leaves exhibited diffuse fluorescence, which was interspersed with regions lacking green fluorescence, in contrast to the uniform green observed in the first set of plants. This "mottled" pattern suggested that sequences were being deleted from the virus–vector genome, resulting in the loss of GFP expression. RNA analysis by Northern blot hybridization revealed an additional RNA in the upper leaves which was smaller than the full-length genome of 30B-GFP (Fig. 2).

The vector 30B-GFP does not move efficiently into upper leaves of tobacco plants as it does in *N. benthamiana*. However, the empty vector, 30B, systemically infects tobacco efficiently. When *N. tabacum* cv Xanthi (tobacco) was inoculated with 30B-GFP or 30B-GFPC3 and monitored by UV illumination, diffuse green fluorescence was observed only in the inoculated leaves of the plant (30B-GFPC3; Fig. 1C). Further movement of the virus into the upper leaves was accompanied by the loss of GFP expression and the development of mild mosaic symptoms. The genomic RNAs detected in the upper leaves of the infected plant by Northern blot hybridization analysis were all approximately the size of TMV instead of the size of the original vector (data not shown).

Passaging of 30BGFP, RT-PCR, and sequence analysis

The observation that RNAs smaller than the genome of 30B-GFP were present in leaves that lacked GFP-mediated fluorescence suggested that at least part of the inserted sequences had been deleted. Five independent experiments were performed at different times and from different batches of plants to examine the diversity of the resulting hybrid virus that developed from 30B-GFP when it was passaged in *N. tabacum* cv Xanthi (tobacco) and *N. benthamiana*.

Different series of plants were inoculated with the virus and total nucleic acid was isolated from individual plants of the set after various periods of time. RT-PCR, using primers specific to sequences in the TMV movement protein ORF (5′ primer) and the TMGMVU5 3′ NTR (3′ primer), was performed followed by restriction digestion analysis of the PCR fragments using different enzymes to identify individual patterns. From each experiment, we chose three to five samples for sequence analysis, being sure to select representatives of the diversity of the restriction patterns.

The first experiment was designed to examine the recombinants of 30B-GFP after a relatively long period of multiplication in tobacco. 30B-GFP and the resulting hybrid viruses were serially passaged in tobacco for 4 months. The virus from the uppermost systemically infected nonfluorescent leaves at 1 month postinoculation was used as inoculum for the next set of plants. At each passage total nucleic acid was extracted from each plant for RT-PCR analysis. A single cDNA of ∼1.3 kb (which was smaller than the original 2.5 kb fragment from 30B-GFP) with the identical restriction pattern resulted from each plant. The PCR products were cloned into pUC19 and one clone from each monthly passage was selected for sequencing the insert. The corresponding recombinants were designated RECT1, RECT2, RECT3, and RECT4.

The second experiment was designed to examine the hybrid viruses that resulted from 30B-GFP after different incubation periods of the infected plants. 30B-GFP from systemically infected leaves of an *N. benthamiana* plant was passaged to tobacco plants, and total nucleic acid was extracted for analysis 2 and 3 months postinoculation. At 2 months there was a single RT-PCR product, identical in size (∼1.5 kb), from each of the RNAs. From this set, four samples designated REC1, REC2, REC3, and REC4 were chosen for sequence analysis. The size of the RT-PCR products from the tobacco plants 3 months postinoculation was ∼1.4 kb. Two samples, REC5 and REC6, were chosen for sequence analysis.

The third experiment examined the virus 2 months after *N. benthamiana* was inoculated with 30B-GFP. The cDNAs from the *N. benthamiana* plants had identical sizes of ∼1.5 kb. Two samples, REC7 and REC8, were chosen from this set for sequence analysis.
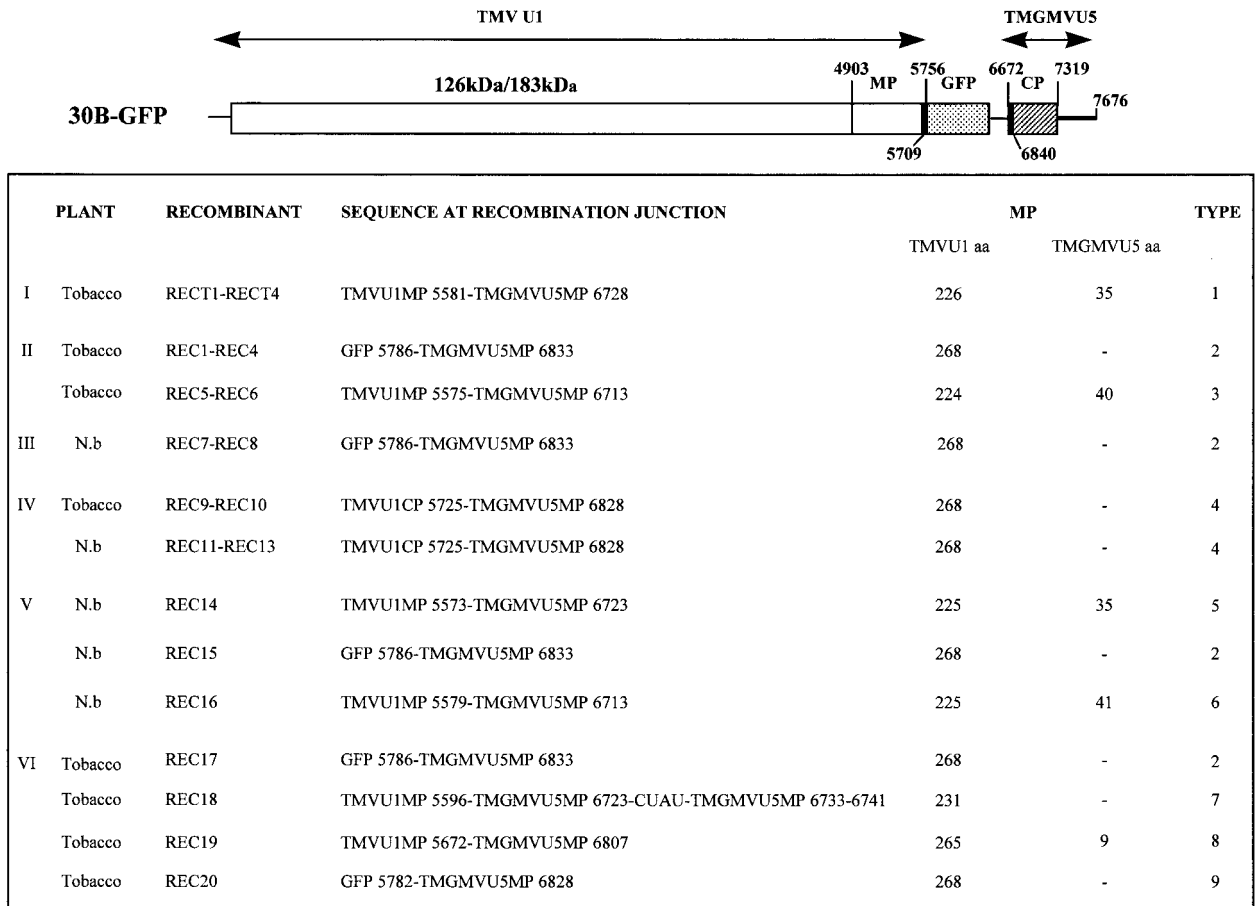
FIG. 3. Description of recombinants that developed from infection of *N. benthamiana* and tobacco plants with 30B-GFP. Schematic diagram of the genomic organization of 30B-GFP is presented. TMV and TMGMVU5 sequences are indicated by upper arrows. Nucleotide numbers correspond to positions in the genome of 30B-GFP. MP, movement protein; CP, coat protein; GFP, green fluorescent protein. Dark boxes correspond to coat protein subgenomic mRNA promoter sequences. The panel to the right describes the composition of the MP ORF. Intact TMVU1 MP has 268 aa, whereas TMGMVU5 MP has 256 aa. The recombinants with chimeric MP ORFs have amino-terminal amino acids from TMVU1 MP and carboxyl-terminal amino acids from TMGMVU5 MP. The numbers 1 through 9 indicate the different types of recombinants which developed in this study.

	PLANT	RECOMBINANT	SEQUENCE AT RECOMBINATION JUNCTION	MP		TYPE
				TMVU1 aa	TMGMVU5 aa	
I	Tobacco	RECT1-RECT4	TMVU1MP 5581-TMGMVU5MP 6728	226	35	1
II	Tobacco	REC1-REC4	GFP 5786-TMGMVU5MP 6833	268	-	2
	Tobacco	REC5-REC6	TMVU1MP 5575-TMGMVU5MP 6713	224	40	3
III	N.b	REC7-REC8	GFP 5786-TMGMVU5MP 6833	268	-	2
IV	Tobacco	REC9-REC10	TMVU1CP 5725-TMGMVU5MP 6828	268	-	4
	N.b	REC11-REC13	TMVU1CP 5725-TMGMVU5MP 6828	268	-	4
V	N.b	REC14	TMVU1MP 5573-TMGMVU5MP 6723	225	35	5
	N.b	REC15	GFP 5786-TMGMVU5MP 6833	268	-	2
	N.b	REC16	TMVU1MP 5579-TMGMVU5MP 6713	225	41	6
VI	Tobacco	REC17	GFP 5786-TMGMVU5MP 6833	268	-	2
	Tobacco	REC18	TMVU1MP 5596-TMGMVU5MP 6723-CUAU-TMGMVU5MP 6733-6741	231	-	7
	Tobacco	REC19	TMVU1MP 5672-TMGMVU5MP 6807	265	9	8
	Tobacco	REC20	GFP 5782-TMGMVU5MP 6828	268	-	9

In the fourth experiment the virus was passaged from systemically infected leaves of *N. benthamiana* infected with 30B-GFP through a series of tobacco and *N. benthamiana* plants in the same experiment. RT-PCR analysis of total nucleic acid from each plant 6 weeks after inoculation revealed the presence of a single RT-PCR product in each of the RNAs. Two samples from the set of tobacco plants, designated REC9 and REC10, and three samples from the *N. benthamiana* plants, REC11, REC12, and REC13, were chosen for sequence analysis.

In the fifth experiment a series of *N. benthamiana* plants were inoculated with freshly prepared *in vitro* transcripts of 30B-GFP instead of with inoculum from preinfected tissue. Five weeks after inoculation we sampled the uppermost leaf of each plant, choosing leaf areas with no green fluorescence. Three samples which represented the different groups within the set, REC14, REC15, and REC16, were selected for sequencing.

In a final experiment, virus from the fluorescent regions of inoculated leaves of the *N. benthamiana* plants used in experiment 5 were used as inoculum for a series of tobacco plants. Total nucleic acid was isolated from the uppermost leaves of the tobacco plants at 3 weeks postinoculation. RT-PCR analysis revealed the presence of a single cDNA from each of the RNAs. Four representative samples, REC17, REC18, REC19, and REC20, from each digestion pattern, were selected for sequence analysis.

Sequence analysis of all of the chosen cDNAs revealed a series of hybrid viruses with the TMV replicase and U5 coat protein and 3′ NTR, often with a hybrid MP and the GFP ORF and the extra pseudoknots removed (Fig. 3). However, there were nine individual patterns of the 24 samples. About half of the hybrid viruses were near unit length of their respective parent viruses with recombination junctions within the MP ORFs, resulting in a chimeric MP (Fig. 3). Nine of 24 retained a small amount of the 5′ ORF of GFP, while 14 retained sequences of the TMVU1 coat protein ORF.

Independent plants of the same experiment often con-

TABLE 1

Comparative Host Range and Symptomology of Recombinants and TMV

Host	Virus			
	TMV	RECT4	REC1	REC5
N. tabacum cv Xanthi	vc 3 d	vc 5 d	vc 3 d	vc 5 d
	Systemic mosaic	Systemic mild mosaic	Systemic mosaic	Systemic mosaic
N. tabacum cv Xanthi nc	1.5–2 mm lesion/2 d	1–1.5 mm Lesions/2 d	1 mm lesion/2 d	1 mm lesions/2 d
N. tabacum cv Samsun EN	Mosaic	2 mm lesions/3 d	2 mm lesions/3 d	2–3 mm lesions/3 d
N. sylvestris	Mosaic	<1 mm lesions/2 d	<1 mm lesions/2 d	<1 mm lesions/2 d
N. benthamiana	Necrosis	Mild mosaic	Mild mosaic	Mild mosaic
N. glutinosa	1 mm lesions/2 d	Pinpoint lesions/2 d	Pinpoint lesions/2 d	Pinpoint lesions/2 d
Datura metel	2 mm lesions/4 d	1 mm lesions/4 d	1 mm lesions/4 d	1 mm lesions/4 d
Datura stramonium	1 mm lesions/2 d	1 mm lesions/3 d	1 mm lesions/3 d	1 mm lesions/3 d
Chenopodium quinoa	1 mm lesions/3 d	1 mm lesions/4 d	1 mm lesions/4 d	1 mm lesions/4 d
Lycopersicon esculentum var. cerasiforme	Mild chlorosis, leaf curling/1 wk	Symptomless[a]	Symptomless[a]	Symptomless[a]

Note. vc, vein clearing; d, days; wk, week.

[a] The plant did not show obvious symptoms; however, when infected tissue was ground in GP-celite buffer and transferred onto *N. tabacum* cv Xanthi nc, local lesions were observed.

tained the same hybrid virus. Sequence analysis of the four cDNAs from experiment 1 and the two cDNAs from experiment 3 revealed 100% identity. The other experiments had two to four species of hybrid viruses, with some being identical to those isolated from different experiments (REC1–4, REC7–8, REC15, and REC17). Remarkably, all of the samples from individual plants contained only one major form of deleted hybrid virus resulting in only one cDNA amplified by PCR.

Comparative symptomology of the recombinants and TMV

One concern in the use of virus-based transient expression vectors is the development of new viruses with increased virulence. In general, all the hybrid viruses induced milder symptoms in tobacco and *N. benthamiana* when compared with TMV. We chose RECT4, REC1, and REC5 to further examine whether they induced altered symptomology or modified virulence in a range of different plants compared to TMV. Various species of *Nicotiana*, *Datura,* and *Chenopodium* plus *Lycopersicon esculentum* var. cerasiforme (cherry tomato) were inoculated with TMV and the recombinants, and development of symptoms and their severity were monitored (Table 1). The hybrid viruses induced milder delayed symptoms in all the plants tested than TMV. Neither TMV nor the recombinants induced symptoms in *Chenopodium capitata*.

Challenge experiments between the recombinants and TMV

To investigate the competitiveness of the hybrid viruses with TMV, we first inoculated tobacco plants, in duplicate, with the recombinant viruses RECT4, REC1,

and REC5, and then superinoculated the plants with TMV at different times. Prior to superinoculation with TMV, we examined the extent to which the hybrid viruses moved in the plants by assaying virus from different parts of the plants using the local lesion host *N. tabacum* cv Xanthi nc. Except for the +1 leaf, a photosynthate exporter (Fig. 4), the hybrid viruses moved throughout most of the plant and were detected in the inoculated leaf (leaf 0) and +5
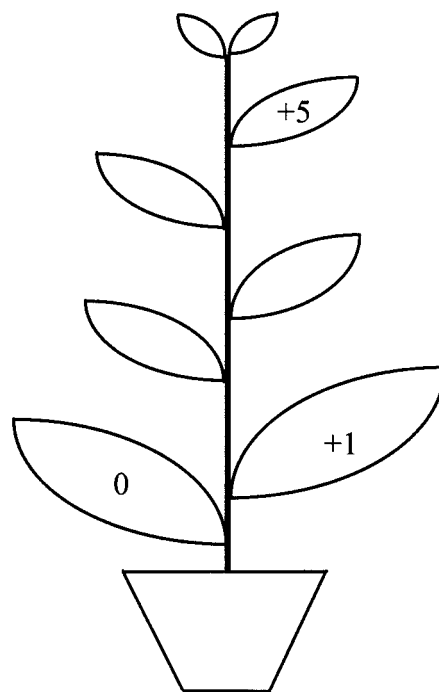

FIG. 4. Diagram representing a tobacco plant depicting the position of the leaves that were inoculated in the challenge experiment between the recombinant viruses and TMV.

leaf (Fig. 4). The recombinants were challenged with TMV at zero time (coinoculation), or at 1 week postinoculation, and by inoculation of different parts of the plants, viz., the same leaf, or the leaf +1 or +5 above the inoculated leaf (Fig. 4). After the challenge, the plants were maintained for 1 month after which virus from the upper infected leaves was analyzed to determine the proportion of TMV and recombinant virus. For this analysis, we used *N. sylvestris*, which produces necrotic local lesions in response to the TMGMVU5 coat protein (recombinants), and Xanthi nc, which produces local lesions to both viruses. TMV produces systemic mosaic symptoms instead of necrotic lesions in *N. sylvestris*.

When tobacco leaves were inoculated at the same time with TMV and any of the recombinants, the upper noninoculated leaves contained virus that produced only systemic mosaic symptoms on *N. sylvestris*. This result indicated that TMV was the prevalent virus. When TMV inoculation was delayed 1 week on the same leaf as RECT4, we could not detect RECT4 in the upper leaves of the challenged plants 1 month after challenge, suggesting that RECT4 was unable to compete with TMV. However, REC1 and REC5 established double infections with TMV under the same conditions, indicated by production of local lesions in *N. sylvestris*, followed by systemic mosaic. Analysis of the same plants 1 month later gave similar results, which indicated that the double infection persisted.

When the +1 leaf of each of the duplicate plant was inoculated with TMV 1 week after the recombinants had been inoculated onto leaf 0 (Fig. 4), RECT4 and REC1 established double infections with TMV. REC5, however, was not detected in the upper leaves of the challenged plants, indicating that it was outcompeted. When the +5 leaf of each plant was challenged with TMV, RECT4 and REC1 were outcompeted, whereas REC5 established a double infection. However, when analyzed a month later, REC5 was not detected in the upper leaves of the challenged plants. Repetition of the experiment gave the same results. When the experiments were performed so that TMV was the first virus to be challenged by the recombinants, in all cases TMV alone was detected in the upper leaves.

To corroborate the results of the infectivity assays, Northern blot hybridization and RT-PCR analyses were performed on total nucleic acid from the upper leaves of the challenged plants. Riboprobes specific to the U5 coat protein sequence and the 3′ NTR of TMV were used to detect the recombinants and TMV, respectively. We did not detect any specific hybridization signal to U5 sequences from RNA isolated from tissue, which was negative in the local lesion assay (data not shown). The same RNAs gave strong hybridization signals with the TMV probe. Only RNA from tissue with virus that produced lesions when inoculated onto *N. sylvestris* gave an RT-PCR product corresponding to U5 sequences. All of the RNAs gave RT-PCR products specific to TMV sequences using primers specific to the TMV genome (data not shown). Thus the recombinants either coexisted with TMV or were outcompeted.

DISCUSSION

Earlier studies showed that duplicated sequences in TMV were unstable (Dawson *et al.,* 1989), whereas heterologous-related sequences were more stable (Donson *et al.,* 1991; Culver *et al.,* 1993). Therefore, vectors were designed with repeated sequences from a different tobamovirus. 30B was chosen as a high expressing vector after extensive examination of the effects of different heterologous sequences (which included the coat protein subgenomic promoter, coat protein ORF, and 3′NTR) from different tobamoviruses (Shivprasad *et al.,* 1999). Vectors containing the U5 sequences were superior in the levels of foreign gene product (GFP) produced. *N. benthamiana* plants infected with 30B-GFP showed lack of GFP expression after two to three passages of the virus through the plant. This result is similar to previous studies with other TMV-based viral vectors harboring neomycin phosphotransferase or dihydrofolate reductase ORFs (Donson *et al.,* 1991; Kearney *et al.,* 1993). Recombination between similar sequences of TMVU1 and TMGMVU5 in 30B-GFP, to remove repeated and foreign sequences, resulted in the development of chimeric tobamoviruses which only maintained sequences required for optimal replication and movement.

Although it is expected that a population of different recombinants should develop from infection with the viral vector, only one recombinant virus was isolated from each plant. This suggests that strong selection quickly produced a homogeneous population of the first recombinants which could move efficiently and establish a systemic infection in each plant. Only nine different types of recombinants were detected from all of the experiments, with some identical recombinants isolated from different host plants in independent experiments. About half the number of recombinant viruses had chimeric MP ORFs (and therefore also chimeric CP subgenomic mRNA promoter sequences).

Large-scale production of specialty products using plant virus-based transient expression vectors would require the inoculation of plants on extensive tracts of land. Campbell (1999) expressed concern that engineered TMV could spread to susceptible plants growing near experimental plants during this large-scale production phase. In most cases, insertion of sequences into TMV to produce a transient expression vector results in a virus with substantially lower replicative capacity than the parent. Since these TMV-based viral vectors with heterologous repeated sequences ultimately lose the extra sequences inserted and result in the development of recombinants, it would be the hybrid viruses, not the

original vector nor TMV, which would persist in the environment. When examined alone on various host plants, the hybrid viruses displayed milder symptoms than did TMV, and the time of onset of symptom development was delayed. Further, the hybrids that were generated from 30B-GFP were either outcompeted by the wild-type virus or persisted in a double infection when challenged by TMV, depending on how well the recombinant virus was established in infected plants prior to challenge. Double infection of hybrid and wild-type TMV was no more severe in symptomology than wild-type alone. Thus, the recombinants appeared to be less competitive and less pathogenic than the parent virus.

In the design of virus-based expression vectors for plant systems, different approaches will result in the addition of different types of viral inoculum to the environment when used in the field. For an autonomous replicon capable of moving a foreign sequence throughout the plant, the "add-a-gene" vectors were first shown to be effective. These vectors, which contain insertions of a repeated subgenomic mRNA promoter plus a foreign sequence upon recombination and selection, result in a unit-length virus. If homologous repeated viral sequences are used, the original wild-type virus is produced and the result of large-scale use in the field would only be an increase in wild-type inoculum (Dawson *et al.,* 1989; Chapman *et al.,* 1992). If heterologous repeated sequences (from a related virus) are used, which stabilizes TMV-based expression vectors (Donson *et al.,* 1991; Casper and Holt, 1996; Shivprasad *et al.,* 1999), the ultimate recombinant will be a chimera of two viruses. Large-scale application of this type of vector will result in the introduction of new hybrid viruses. In the case of 30B-GFP, the resulting chimeric viruses appear to be less virulent than the wild-type virus. New virus-based transient expression vectors could be designed to reduce the development of any new virus inoculum in the field by dividing the viral function into separate units (Lewandowski and Dawson, 2000).

MATERIALS AND METHODS

Plasmids and plant inoculations

30B, 30B-GFP, and 30B-GFPC3 have been described previously (Shivprasad *et al.,* 1999). *In vitro* transcripts of 30B-GFP and 30B-GFPC3 using T7 RNA polymerase were synthesized by the procedure described previously (Lewandowski and Dawson, 1998) and were used to infect *N. benthamiana*. The presence of GFP in plants was monitored by illuminating the plant with a hand-held long-wave UV lamp (366 nm).

Analysis of RNA and isolation of recombinants

Northern blot hybridization analysis of RNA extracted from leaves of *N. benthamiana* and tobacco was carried out as described previously (Lewandowski and Dawson, 1998). The digoxigenin-labeled probes used were a positive strand-specific riboprobe to the 5′ 256 nucleotides (nts) of the TMV genome, the 3′ NTR of TMV, and the coat protein and 3′ NTR of TMGMVU5.

For isolation of recombinants from infected plants, total nucleic acid was extracted from *N. benthamiana* and tobacco, and reverse transcription was carried out using a primer specific to the 3′ 21 nts of the genome of 30B-GFP (nts 7656–7676) and Superscript II (Life Technologies). Polymerase chain reaction (PCR) was carried out using Vent DNA polymerase (New England Biolabs) with 5′ primer which hybridized to a specific region within the MP ORF of TMVU1 (nts 5154–5175) and a 3′ primer specific to the 3′ NTR of TMGMVU5 (nts 7656–7676 in 30B-GFP; nts 6482–6502 in TMGMVU5). The PCR fragments from RECT1-RECT4 were gel purified, cloned into *Sma*I-digested pUC19, and then sequenced. The PCR fragments from REC1–REC20 were gene cleaned and sequenced. Sequencing was carried out at the ICBR Core Facility at the University of Florida. Cloning procedures and restriction enzyme digestion were carried out essentially as described by Sambrook *et al.* (1989). Nucleotide numbering of TMV is essentially as described by Goelet *et al.* (1982). The sequence of TMGMVU5 MP ORF is extrapolated from that of TMGMVU2 (Garcia-Arenal, 1988).

REFERENCES

Beck, D. L., and Dawson, W. O. (1990). Deletion of repeated sequences from tobacco mosaic virus mutants with two coat protein genes. *Virology* **177,** 462–469.

Berglund, P., Tubulekas, I., and Liljestrom, P. (1996). Alphaviruses as vectors for gene delivery. *TIBTECH* **14,** 130–134.

Campbell, R. N. (1999). Assessing the spread of engineered TMV. *Science* **286,** 903.

Casper, S. J., and Holt, C. A. (1996). Expression of the green fluorescent protein-encoding gene from a tobacco mosaic virus-based vector. *Gene* **173,** 69–73.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263,** 802–805.

Chapman, S., Kavanagh, T., and Baulcombe, D. (1992). Potato virus X as a vector for gene expression in plants. *Plant J.* **2,** 549–557.

Chu, T-H. T., and Dornburg, R. (1997). Toward highly efficient cell-type specific gene transfer with retroviral vectors displaying single-chain antibodies. *J. Virol.* **71,** 720–725.

Crameri, A., Whitehorn, E. A., Tate, E., and Stemmer, W. P. (1996). Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. BioTechnnol.* **14,** 315–319.

Culver, J. N., Lehto, K., Close, S. M., Hilf, M. E., and Dawson, W. O. (1993). Genomic position affects the expression of tobacco mosaic virus movement and coat protein genes. *Proc. Natl. Acad. Sci. USA* **90,** 2055–2059.

Dawson, W. O., Lewandowski, D. J., Hilf, M. E., Bubrick, P., Raffo, A. J., Shaw, J. J., Grantham, G. L., and Desjardins, P. R. (1989). A tobacco mosaic virus-hybrid expresses and loses an added gene. *Virology* **173,** 285–292.

Donson, J., Kearney, C. M., Hilf, M. E., and Dawson, W. O. (1991). Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. *Proc. Natl. Acad. Sci. USA* **88,** 7204–7208.

Dubensky, T. W., Jr., Driver, D. A., Polo, J. M., Belli, B. A., Latham, E. M., Ibanez, C. E., Chada, S., Brumm, D., Banks, T. A., Mento, S. J., Jolly, D. J., and Chang, S. M. W. (1996). Sindbis virus DNA-based expression vectors: Utility for *in vitro* and *in vivo* gene transfer. *J. Virol.* **70,** 508–519.

Garcia-Arenal, F. (1988). Sequence and structure at the genome 3′ end of the U2-strain of tobacco mosaic virus, a histidine-accepting tobamovirus. *Virology* **167,** 201–206.

Goelet, P., Lomonosoff, G. P., Butler, P. J. G., Akam, M. E., Gair, M. J., and Karn, J. (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proc. Natl. Acad. Sci. USA* **79,** 5818–5822.

Hamamoto, H., Sugiyama, Y., Nakagawa, N., Hashida, E., Matsunaga, Y., Takemoto, S., Watanabe, Y., and Okada, Y. (1993). A new tobacco mosaic virus vector and its use for the systemic production of angiotensin-I-converting enzyme inhibitor in transgenic tobacco and tomato. *Biotechnology* **11,** 930–932.

Johnson, J., Lin, T., and Lomonosoff, G. (1997). Presentation of heterologous peptides on plant viruses: Genetics, structure, and function. *Annu. Rev. Phytopathol.* **35,** 67–86.

Kearney, C. M., Donson, J., Jones, G. E., and Dawson, W. O. (1993). Low level of genetic drift in foreign sequences replicating in an RNA virus in plants. *Virology* **192,** 11–17.

Lewandowski, D. J., and Dawson, W. O. (1998). Deletion of internal sequences results in tobacco mosaic virus defective RNAs that accumulate to high levels without interfering with replication of the helper virus. *Virology* **251,** 427–437.

Lewandowski, D. J., and Dawson, W. O. (2000). Functions of the 126- and 183-kDa proteins of Tobacco Mosaic Virus. *Virology* **271,** 90–98.

Modelska, A., Dietzschold, B., Fleysh, N., Fu, Z. F., Steplewski, K., Hooper, D. C., Koprowski, H., Yusibov, V. (1998). Immunization against rabies with plant-derived antigen. *Proc. Natl. Acad. Sci. USA* **95,** 2481–2485.

Pogue, G. P., Lindbo, J. A., Dawson, W. O., and Turpen, T. (1998). Tobamovirus transient expression vectors: Tools for plant biology and high-level expression of foreign proteins in plants. *In* "Plant Molecular Biology Manual" (S. B. Gelvin and R. A. Schilperoort, Eds.), L4, pp. 1–27. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Prasher, D., Eckenrode, V., Ward, W., Prendergast, F., and Cornier, M. (1992). Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* **111,** 229–233.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning," 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shivprasad, S., Pogue, G. P., Lewandowski, D. J., Hidalgo, J., Donson, J., Grill, L. K., and Dawson, W. O. (1999). Heterologous sequences greatly affect foreign gene expression in tobacco mosaic virus-based vectors. *Virology* **255,** 312–323.

Tubuleska, I., Berglund, P., Fleeton, M., and Liljestrom, P. (1997). Alpha-virus expression vectors and their use as recombinant vaccines: A minireview. *Gene* **190,** 191–195.

Turpen, T. H., Reinl, S. J., Charoenvit, Y., Hoffman, S. L., Fallarme, V., and Grill, L. K. (1995). Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. *Biotechnology* **13,** 53–57.

Turpen, T. H., Cameron, T. I., Reinl, S. J., Pogue, G. P., Garger, S. J., McCulloch, M. J., Holtz, R. B., and Grill, L. K. (1997). Production of recombinant proteins in plants: Pharmaceutical applications. The Soc. Exper. Biol., Canterbury, U.K. *J. Exp. Botany* **48**(Suppl.)**,** 12.

Turpen, T. H. (1999). Tobacco mosaic virus and the virescence of biotechnology. *Phil. Trans. R. Soc. Lond. Bio. Sci.* **354,** 665–673.

van Vloten-Doting, L., Bol, J. F., and Cornelissen, B. (1985). Plant-virus-based vectors for gene transfer will be of limited use because of the high error frequency during viral RNA synthesis. *Plant. Mol. Biol.* **4,** 323–326.

Yusibov, V., Modelska, A., Steplewski, K., Agadjanyan, M., Weiner, D., Hooper, D. C., and Koprowski, H. (1997). Antigens produced in plants by infection with chimeric plant viruses immunize against rabies virus and HIV-1. *Proc. Natl. Acad. Sci. USA* **94,** 5784–5788.

Yusibov, V., Shivprasad, S., Turpen, T. H., Dawson, W. O., and Koprowski, H. (1999). Plant viral vectors based on tobamoviruses. *In* "Plant Bio/Technology" (J. Hammond, P. McGarvey, and V. Yusibov, Eds.), pp. 81–94. Springer-Verlag, Berlin/New York.