Restoration of Wild-Type Virus by Double Recombination of Tombusvirus Mutants with a Host Transgene

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Nicotiana benthamiana plants transformed with the coat protein gene of tomato bushy stunt virus (TBSV) failed to elicit effective virus resistance when inoculated with wild-type virus. Subsequently, R₁ and R₂ progeny from 13 transgenic lines were inoculated with a TBSV mutant containing a defective coat protein gene. Mild symptoms typical of those elicited in nontransformed plants infected with the TBSV mutant initially appeared. However, within 2 to 4 weeks, up to 20% of the transgenic plants sporadically began to develop the lethal syndrome characteristic of wild-type virus infections. RNA hybridization and immunoblot analyses of these plants and nontransformed *N. benthamiana* inoculated with virus from the transgenic lines indicated that wild-type virus had been regenerated by a double recombination event between the defective virus and the coat protein transgene. Similar results were obtained with a TBSV deletion mutant containing a nucleotide sequence marker, and with a chimeric cucumber necrosis virus (CNV) containing the defective TBSV coat protein gene. In both cases, purified virions contained wild-type TBSV RNA or CNV chimeric RNA derived by recombination with the transgenic coat protein mRNA. These results thus demonstrate that recombinant tombusviruses can arise frequently from viral genes expressed in transgenic plants.

RNA recombination (Lai 1992; Simon and Bujarski 1994; Nagy and Simon 1997) together with the high frequency of mutations associated with the replication of RNA viruses (Holland et al. 1982; Steinhaner and Holland 1986) plays an important role in the genetic variability and repair of virus genomes. Sequence comparisons revealing the relatedness of RNA viruses (Koonin and Dolja 1993; Simon and Bujarski 1994) have provided a generally accepted hypothesis that they evolve by modular recombination between similar

and distantly related progenitors (Strauss and Strauss 1988; Dolja and Carrington 1992), and from host cellular genes (Gorbalenya 1992; Lai 1992). Recently, laboratory reports of recombination between viral genomes and genes expressed in transgenic plants have appeared. The first clearly documented case involved the cauliflower mosaic virus (CaMV) translational transactivator gene VI, which, when expressed as a transgene, recombined with a viral gene VI deletion mutant (Gal et al. 1992). In another case, a CaMV strain (CaMV1841), which was unable to infect solanaceous plants, acquired transgenic sequences that altered the virus symptomatology and extended the host range to members of the Solanaceae (Schoelz and Wintermantel 1993). In both cases, nucleotide sequence analysis of the recombinant viruses indicated that integration of transgene sequences into the viral genome involved a two-step, template switching mechanism during the reverse transcription stage of replication. More recently, recombination has been reported between the viral DNA of a geminivirus with a deletion in the coat protein gene and a functional coat protein transgene (Frischmuth and Stanley 1998). The results in this instance indicated that homologous recombination had occurred in the nucleus between the mutant viral DNA and the integrated coat protein gene to yield wild-type virus.

With RNA viruses, a deletion mutant in the coat protein gene of cowpea chlorotic mottle virus (CCMV) has been shown to be restored by a single recombination event with a transgene expressed in transgenic plants (Greene and Allison 1994). Additionally, restoration of a cell-to-cell movement gene function to an RNA virus, red clover necrotic mosaic virus (RCNMV), by recombination with the movement gene expressed in transgenic plants has been reported (Lommel and Xiong 1991). Some evidence for recombination between potato virus Y strains has also been described in a short research synopsis (Jakab et al. 1997).

In the present study, we investigated whether transgenic *Nicotiana benthamiana* plants expressing the coat protein mRNA of tomato bushy stunt virus (TBSV) were able to resist TBSV infections, and if they were able to recombine with TBSV or cucumber necrosis virus (CNV) derivatives unable to express a functional coat protein. The coat protein mutants were chosen because they can establish systemic infections, yet elicit relatively mild symptoms, whereas plants infected

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with the wild-type TBSV develop a vascular collapse that results in death of *N. benthamiana* (Scholthof et al. 1993a). The mild mutant phenotype and its conversion to the lethal syndrome thus provided a convenient bioassay whereby we were able to visually identify possible recombinants. Analysis of these plants revealed that double recombinations between the mutants and the transgene have the potential to regenerate wild-type virus that can become predominant in the infected plants.

RESULTS

The TBSV coat protein transgene fails to elicit effective disease resistance.

N. benthamiana plants were transformed with the coat protein gene of TBSV to assess the degree of disease resistance to TBSV infections, and 154 R_0 independently transformed plants were selected for resistance to kanamycin. Following self-pollination, seeds were collected and germinating plants were analyzed by Northern (RNA) blot hybridization of total RNA extracts to evaluate the expression and accumulation of the coat protein mRNA (data not shown). Southern blot analysis with genomic DNA isolated from the transgenic plants indicated that single integrations had occurred at different sites in the genomes of 10 different lines analyzed (data not shown). Western blot (immunoblot) analyses were performed to assess the protein expression levels, but we were unable to detect the coat protein in any of these plants in assays capable of detecting less than 100 ng of protein per mg of plant tissue (data not shown).

Transgenic plants from 72 lines that expressed the coat protein mRNA were tested for their ability to resist wild-type TBSV infections derived from pTBSV-100 (Hearne et al. 1990) transcripts (Fig. 1). In these experiments, R_1 progeny (20 plants per line) were inoculated with a 1/5,000 dilution (approximately 1 ng/ml) of TBSV-infected sap. Plants in most of the infected lines developed a lethal response that was indistinguishable from that elicited in the nontransformed *N. benthamiana* plants shown in Figure 2A. This response was characterized by appearance of a mild mosaic interspersed with faint yellow spots by 3 days post inoculation (dpi), which subsequently progressed into necrotic local lesions. A top necrosis and vascular collapse appeared after 1 week, and the plants usually died by 9 dpi. However, plants from 13 independent lines (Table 1, showing six of these lines), developed symptoms that were delayed from 1 to 2 weeks post inoculation (wpi) when dilute inoculum was used, but, within 2 to 4 wpi, the remaining plants also developed a progressive necrosis and died. In plants of these same lines, a more extreme infection occurred after inoculation with a higher concentration (approximately 10 ng/ml) of purified TBSV virions (data not shown). Less than 20% of the plants survived past 10 dpi, and all of the plants died between 2 and 4 wpi. At the highest virus concentration (1 mg/ml), the inoculated plants quickly developed the necrotic syndrome and died by 2 wpi. In addition, inoculation of transgenic plants with TBSV in vitro transcripts resulted in rapid appearance of TBSV symptoms followed by death of the plants (data not shown). These results suggest that plants of these particular transgenic lines were unable to elicit effective coat protein-mediated resistance to either wild-type virus or to RNA infections.

A TBSV coat protein deletion mutant and the coat protein transgenic mRNA recombine to generate wild-type virus.

To determine whether resistance was expressed against a TBSV derivative that elicited milder symptoms than wild-type TBSV, plants were inoculated with T- Δ cp RNA (Fig. 1), which has a small deletion in the coat protein gene and fails to elicit the lethal disease syndrome (Fig. 2B; and Scholthof et al. 1993a). This mutant has a 46 nucleotide (nt) deletion that produces a downstream frameshift and a premature termination nine codons after the deletion. The deletion encompasses a region of the coat protein sequence encoding most of the positively charged R domain, which is thought to interact with RNA in the virion. Systemic symptoms elicited by the deletion mutant were less severe than the lethal syndrome produced by wild-type virus infections. These symptoms included a mild mosaic and leaf distortion, localized necrosis, and stunting (Fig. 2C). However, within 2 wpi with T- Δ cp, some of the plants from transgenic R_1 and R_2 progeny lines began to exhibit a vascular collapse and died (Fig. 2D). Interestingly, the times at which the plants began to die were quite variable and extended throughout the next 2 weeks of observation.

Fig. 1. Schematic representation of tomato bushy stunt virus (TBSV) and cucumber necrosis virus (CNV) derivatives used. Designation T-100 illustrates genome organization of the TBSV clone pTBSV-100 (Hearne et al. 1990). The T- Δ cp construct (pHS7A; Scholthof et al. 1993a) has a 46-nucleotide (nt) deletion within the coat protein coding region, resulting in a frameshift that terminates translation nine codons after the deletion. The T-CAT variant (pHS82; Scholthof et al. 1993a) has 56% of the coat protein sequence substituted by the chloramphenicol acetyl transferase (CAT) reporter gene, which was inserted in frame with the 5' terminus of the coat protein open reading frame. The TSpe construct contains a 3-nt mutation downstream of the TBSV p92 gene that generates a *SpeI* restriction site recombination marker at positions 2624 to 2627. The derivative TSpe- Δ cp contains the same *SpeI* restriction site marker, and the 46-nt deletion present in T- Δ cp. The Ch construct represents the TBSV-CNv chimera, where all the sequences are derived from CNV, except for the coat protein gene from TBSV. An *SpeI* site was also introduced at the 3' end of the CNV p92 gene to facilitate the exchange of the coat protein gene between both viruses and also for use as a recombination marker. Its derivative, Ch- Δ cp, contains the *SpeI* marker and the same 46-nt deletion as in T- Δ cp. CNV illustrates the genome organization of CNV. C- Δ cp was constructed by deleting 53 nt from the hinge domain of the CNV coat protein gene.

Eventually, 12 to 20% of the plants in six of the most extensively tested lines exhibited the lethal syndrome (Table 2). In marked contrast, all of the nontransformed control plants inoculated with T- Δ cp RNA maintained the milder systemic phenotype through flowering.

Leaf tissue of plants from the six lines displaying the delayed lethal response following inoculation with T- Δ cp RNA were tested for the presence of virus particles. Several other T- Δ cp-infected plants from the same or different lines that developed only the mild phenotype were used as controls. After agarose gel separation, virus particles could be detected by immunoblot analyses (Fig. 3). Virions were recovered from the nontransformed control plants infected with wild-type TBSV (Fig. 3, lane 6), and from transgenic plants that developed the lethal syndrome after inoculation with T- Δ cp (Fig. 3, lane 14). Virus particles were not evident in extracts from uninfected plants (Fig. 3, lane 1), from nontransformed plants infected with T- Δ cp (Fig. 3, lane 2), or from transgenic plants expressing mild symptoms after inoculation with T- Δ cp (Fig. 3, lane 10).

Accumulation of virus particles after inoculation of transgenic plants with T- Δ cp could have been due either to complementation between the mutant and the coat protein expressed in the transgenic plants, or to recombination between the mRNA transgene and the coat protein defective mutant. To distinguish between these two alternatives, purified virions isolated from plants developing the necrotic syndrome were used to inoculate nontransformed *N. benthamiana* plants. We expected that infections resulting from transencapsidation of mutant RNA would develop the milder infection phenotype and would produce no virus particles. In contrast, a recombinant virus resulting from recombination between T- Δ cp and the coat protein mRNA transgene would result in death of the plants concomitant with accumulation of large amounts of virus particles. Each of the nontransformed *N. benthamiana* plants infected with purified virions from the transgenic plants undergoing the lethal response developed wild-type virus symptoms, and virus particles could be purified from these plants (data not shown). This result suggested that functional virus was generated via a recombination event between the coat protein transgene and the defective virus.

In an additional series of experiments, we coinoculated nontransformed plants with 120 μ g of the T- Δ cp transcript per ml and concentrations of wild-type TBSV ranging from 200 ng to 120 μ g per ml. In all cases, the plants developed the wild-type lethal syndrome, and contained virus particles (data not shown). These results thus indicate that the wild-type virus is able to compete more effectively for systemic movement than the T- Δ cp mutant, and they suggest that the putative recombinants that appear in T- Δ cp infections rapidly become predominant in the infected plants.

A TBSV coat protein mutant containing a marker and a CNV chimeric coat protein deletion mutant recombine with the plant transgene.

To verify that recombination rather than contamination with wild-type virus had occurred, we conducted several control experiments. Two marker constructs were also designed to specifically identify the recombinant progeny. The first was a TBSV coat protein deletion mutant, TSpe- Δ cp, containing an *Spe*I restriction site marker, which is located just upstream of

the coat protein coding region (Fig. 1). We also constructed a related tombusvirus chimera, designated Ch- Δ cp (Fig. 1), in which we replaced the CNV coat protein with a defective TBSV coat protein gene identical to that present in T- Δ cp. Ch- Δ cp also contains the *Spe*I restriction site as a marker.

As a first step, we wanted to confirm the phenotype caused by the marker constructs. When RNAs from the TSpe- Δ cp and Ch- Δ cp mutants were used to inoculate nontransformed plants, only the mild phenotype was observed (Fig. 2B). In contrast, the equivalent wild-type viruses (TSpe, Ch, and CNV; Fig. 1) elicited the severe lethal phenotype (Fig. 2A). Western blot analyses from these plants demonstrated that viral particles were present only when the wild type constructs were used as inoculum (Fig. 3, lanes 7, 8, and 9), but not in nontransformed plants infected with the TSpe- Δ cp and Ch- Δ cp mutants (Fig. 3, lanes 4 and 5).

When RNAs from TSpe- Δ cp and Ch- Δ cp were used to inoculate transgenic plants, most of the plants developed mild symptoms similar to those resulting from the TBSV deletion mutant in nontransformed plants (Fig. 2C). However, an average of 14% of the plants from one line infected with TSpe- Δ cp, and 16% of the plants from six of the lines infected with the coat protein deletion chimera, Ch- Δ cp (Table 2), developed wild-type, TBSV-like lethal symptoms (Fig. 2D). These results indicated that TSpe- Δ cp and Ch- Δ cp were also able to undergo recombination with the transgene. Additional serological analyses following separation of viruses in agarose gels revealed the presence of viral particles in transgenic plants exhibiting the lethal syndrome (Fig. 3, lanes 16 and 17), but not in transgenic plants that survived (Fig. 3, lanes 12 and 13).

In order to provide a more detailed analysis of the viral RNA in the transgenic plants developing the lethal necrosis, we conducted Northern blot and sequence experiments. For the Northern blot analyses, total RNA was isolated from nontransformed plants inoculated with T- Δ cp, TSpe- Δ cp, and Ch- Δ cp as a control, or from nontransformed plants inoculated with the wild-type derivatives TBSV, TSpe, and Ch. RNA was also isolated from transgenic plants with the mild and severe phenotypes after infection with the coat protein deletion mutants (T- Δ cp, TSpe- Δ cp, and Ch- Δ cp). As a hybridization probe, we used the oligonucleotide cpNB (5' CCCTGCAAG GCTGTGGCCC 3'), whose sequence is absent in the T- Δ cp, TSpe- Δ cp, and Ch- Δ cp mutants used in the infections. The results shown in Figure 4 indicate that RNA isolated from healthy tissue (Fig. 4, lane 1) or nontransformed plants infected with T- Δ cp, TSpe- Δ cp, and Ch- Δ cp failed to develop a signal after hybridization with the cpNB oligo (Fig. 4, lanes 2, 4, and 5, respectively). In contrast, RNA from nontransformed plants infected with the wild-type versions of these mutants (no coat protein deletions) TBSV, TSpe, and Ch hybridized to the oligo (Fig. 4, lanes 6, 7, and 8, respectively). However, wild-type CNV showed weaker hybridization to the cpNB oligo (Fig. 4, lane 9), which we attribute to mismatched hybridization because CNV contains only 10 of the 19 nt in the cpNB sequence. Only RNA isolated from transgenic plants that developed the lethal syndrome following infection with T- Δ cp, TSpe- Δ cp, and Ch- Δ cp transcripts had a strong hybridization signal with the cpNB oligonucleotide (Fig. 4, lanes 15, 17, 18, respectively), and no signal was detected with RNA isolated from transgenic plants expressing the milder symptoms (Fig. 4, lanes 11, 12, and 14, respectively). These results

indicated that the transgenic plants undergoing the lethal syndrome contained recombinants in which the deleted sequences had been restored.

Sequence analyses verify double recombination events.

To analyze the sequence of the putative recombinant RNAs from transgenic plants that developed the severe phenotype after infection with T-Δcp, TSpe-Δcp, and Ch-Δcp transcripts, virions were purified and RNA was recovered from the virus particles. Reverse transcription-polymerase chain reaction (RT-PCR) was then performed to amplify the coat protein gene and surrounding sequences with oligonucleotides (see Materials and Methods) specific for each putative recombinant. The oligonucleotide pairs 5'cp-3'cp or TF-Tr were used to amplify the T-Δcp, TSpe-Δcp, and Ch-Δcp sequences (Fig. 5A and B). We should emphasize that the primer sets used in the amplification reactions for Ch-Δcp included one primer specific for TBSV and a second primer specific for the flanking CNV sequences. The primer pairs CF-3'cp or 5'cp-Cr (Fig. 5A) permitted amplification of the chimeric CNV virus and did not amplify the TBSV recombinants (Fig. 5B). Sequence analysis of the amplified products with the 5'cp oligo (to obtain sequence upstream, downstream, and within the deletion), and the cpNB oligo (to sequence upstream of the coat protein gene) verified the presence of the *SpeI* site marker in the TSpe-Δcp and Ch-Δcp clones. In these cases, an intact coat protein gene was present in 18 clones derived from six plants (three clones per plant; one or two plants per line) from

four independent lines infected with T-Δcp, in nine clones from three plant representatives of three lines infected with Ch-Δcp, and in nine clones from three plants of a single line infected with TSpe-Δcp. These results indicated that double homologous recombination events had regenerated the wild-type coat protein genes. However, two additional sequenced clones recovered from plants infected with T-Δcp and TSpe-Δcp contained a compensatory guanine residue inserted after the original 46-nt deletion to restore the coat protein open reading frame. These derivatives will be analyzed further in a subsequent study.

Table 1. Survival of coat protein transgene plants after inoculation with tomato bushy stunt virus

Plant line	9 dpi ^a	28 dpi
29 ^c	18/20 ^b	1/20
24b	19/20	0/20
63	20/20	2/20
65	20/20	1/20
39	17/20	1/20
16	19/20	0/20
81	0/20	0/20
69	0/20	0/20
CT ^c	0/20	0/20

^a Days post inoculation.
^b Numerator: Number of plants surviving. Denominator: Number of plants inoculated with a 1/5,000 dilution of sap containing tomato bushy stunt virus.
^c Nontransgenic plants.

Fig. 2. Symptoms of nontransformed and transgenic plants 4 weeks after inoculation. A healthy *Nicotiana benthamiana* plant is on the left in all panels. **A and B**, Nontransformed plants. **C and D**, Coat protein transgenic plants. **A**, Nontransformed plants infected with RNA transcripts from tomato bushy stunt virus (TBSV), Ch, TSpe, and cucumber necrosis virus (CNV) illustrating the severe phenotype caused by viral infection. **B**, Nontransformed plants infected with transcripts from the coat protein deletion derivatives T-Δcp, T-CAT, Ch-Δcp, and TSpe-Δcp, showing the mild phenotype. **C**, Coat protein transgenic plants infected with transcripts from the same coat protein deletion derivatives as in **B** that developed only a mild phenotype. **D**, Coat protein transgenic plants infected with transcripts from the same coat protein deletion derivatives as in **B** and **C** that progressed into the lethal phenotype.

The length and extent of the region of homology affect the recovery of recombinants.

In order to determine if the recovery of recombinants could be correlated with the proportion of sequence shared between the transgene and the defective virus, transgenic plants were inoculated with transcripts from the construct T-CAT (Fig. 1). This derivative contained the chloramphenicol acetyl transferase (CAT) reporter gene cloned in frame with the 5' end of the TBSV coat protein gene, so that 655 nt, or 56% of the coat

Table 2. Recovery of viral particles from different lines of infected transgenic plants

Plant line	T-Δcp	T-CAT	Ch-Δcp	TSe-Δcp	C-Δcp
29 ^c	6/40 ^a	2/40	6/40	11/80	0/120
24b	6/40	2/40	7/40	ND ^b	0/40
63	5/40	1/20	4/20	ND	ND
65	8/40	0/20	5/20	ND	ND
39	3/20	1/20	4/20	ND	ND
16	4/20	1/20	0/20	0/20	ND
CT ^c	0/20	0/20	0/20	0/20	0/20
Total rec ^d	35	7	26	11	0
Rec (%) ^e	16	4	16	14	0

^a Numerator: Number of plants developing lethal symptoms and from which virus could be recovered. Denominator: Number of plants inoculated.

^b Not determined.

^c Nontransgenic plants.

^d Total number of plants containing viral particles.

^e Average percentage of plants containing viral particles.

Fig. 3. Immunodetection of virus particles isolated from nontransformed and transgenic plants. Virus particles were extracted 2 weeks post inoculation from nontransformed and transgenic plants after infection with transcripts derived from derivatives indicated above each lane. Virus particles were separated in a Tris-glycine agarose gel and subsequently transferred to a nitrocellulose membrane. Polyclonal primary antibody used for detection was derived from mice injected with whole tomato bushy stunt virus (TBSV) particles. Healthy plants or nontransformed plants inoculated with transcripts from the coat protein deletion derivatives, T-Δcp, T-CAT, TSpe-Δcp, and Ch-Δcp, yielded no detectable virus band (lanes 1 to 5, respectively). Nontransformed plants inoculated with transcripts from TBSV, TSpe, Ch, and cucumber necrosis virus (CNV) had positive reaction for virus particles (lanes 6 to 9, respectively). The TBSV antibody against whole viral particles cross-reacted with CNV particles (lane 9). Extracts from transgenic plants inoculated with the deletion derivatives T-Δcp, T-CAT, TSpe-Δcp, and Ch-Δcp that developed the mild phenotype lacked virus particles (lanes 10 to 13), whereas transgenic plants that progressed to the severe phenotype yielded virus particles (lanes 14 to 17).

protein gene, were deleted. When T-CAT transcripts were used to inoculate nontransformed plants, all the plants developed the mild phenotype (Fig. 2B), and the proportion of transgenic plants with the severe phenotype (Fig. 2D) was reduced to about 4%, compared with 14 to 16% in plants inoculated with the Δcp derivatives (Table 2). Again, immunological analyses of virus gels revealed that nontransformed or transgenic plants infected with T-CAT that developed the mild phenotype lacked virus particles (Fig. 3, lanes 3 and 11). In contrast, transgenic plants showing the lethal phenotype contained virus particles (Fig. 3, lane 15). Northern blot analyses also showed that nontransformed or transgenic plants with the mild phenotype failed to hybridize to the cpNB oligo (Fig. 4, lanes 3 and 12), whereas transgenic plants showing the severe phenotype hybridized to the cpNB oligo (Fig. 4, lane 16). Sequencing of the coat protein-CAT junctions also demonstrated that a double recombination had restored the deleted coat protein sequences (data not shown).

To determine if the region of homology between the transgene and a defective virus was important for recombination, experiments were also conducted with a CNV coat protein deletion mutant, C-Δcp, that contains a 53-nt deletion from

Fig. 4. Northern (RNA) blot analyses of inoculated nontransformed and transgenic plants. Northern blot analysis of total RNA isolated 2 weeks post inoculation from nontransformed and transgenic *Nicotiana benthamiana* plants that were infected with 5 μg of transcripts from the derivatives indicated above each lane. RNA was probed with a ³²P-labeled oligonucleotide (cpNB) complementary to 19 nucleotides (nt) of the genomic RNA that are absent in the deletion derivatives, so that only RNAs containing the deleted sequence hybridized to the oligonucleotide. The positions of the genomic RNA (gRNA) and the coat protein subgenomic RNA (sgRNA1) are shown along the side of the photograph; estimated positions of the subgenomic RNAs for the movement genes (sgRNA2) are indicated by an asterisk (*). Sequences of sgRNA2 are not complementary to the cpNB oligonucleotide, and hence failed to form a hybridizing band. RNA from healthy or nontransformed plants inoculated with the deletion derivatives T-Δcp, T-CAT, TSpe-Δcp, and Ch-Δcp did not hybridize to the cpNB oligo (lanes 1 to 5). RNA from nontransformed plants inoculated with TBSV, TSpe, and Ch hybridized to the oligo (lanes 6, 7, and 8). Cucumber necrosis virus (CNV) contains 10 of 19 bases that are complementary to those in the cpNB oligo; consequently, only faint cross-hybridizing bands correspond to the CNV genomic RNA and sgRNA1 positions (lane 9). RNA from healthy or from transgenic plants inoculated with the deletion derivatives T-Δcp, T-CAT, TSpe-Δcp, and Ch-Δcp with the mild phenotype, developed no signal upon hybridization with the cpNB oligo (lanes 10 to 14). In contrast, RNA from transgenic plants inoculated with the same derivatives that developed the severe phenotype hybridized to the cpNB oligo (lanes 15 to 18).

positions 3388 to 3441. This region encompasses the hinge domain of the coat protein gene and the deletion produces a downstream frameshift and an early stop codon after the deleted sequence (Fig. 1). In this case, 160 plants were tested, but recombinants that could have resulted from nonhomologous recombination were not recovered following Western blot analysis. Moreover, C-Δcp produced very severe symptoms, which created difficulties in screening by phenotype, and the lethal effects of the mutant also constrained the length of time available for possible nonhomologous recombinants to emerge.

DISCUSSION

TBSV recombination with a host transgene is a frequent event.

The most important finding from these experiments is that restoration of wild-type virus by a double recombination event

between a coat protein mRNA transgene expressed in plants and tombusvirus mutants with defects in the coat protein gene is a reproducible phenomenon that can easily be detected. Our initial observations of wild-type virus regeneration were obtained during experiments designed to evaluate the resistance of transgenic *N. benthamiana* plants. However, none of these plants exhibited the pronounced coat protein-mediated protection of the type described for numerous other viruses (Baulcombe 1996; Beachy 1993; Lomonossoff 1995; Scholthof et al. 1993b; Wilson 1993), and all of the plants died within 4 wpi. These results contrasted somewhat with those reported for Cymbidium ringspot virus (CymRSV), a closely related tombusvirus that elicits a milder infection phenotype (Rubino et al. 1993). The CymRSV transgenic plants were resistant to infection with low concentrations of purified virions, but were susceptible to higher virus concentrations, and to RNA transcripts. Therefore, the discrepancy between our results and those of Rubino et al. (1993) could be related to the severity of the TBSV infection syndrome in *N. benthamiana* plants. Another major difference was that CymRSV coat protein was present in the transgenic plants, whereas we were unable to detect coat protein accumulation in our experiments.

To circumvent the rapid death associated with wild-type TBSV infections, we inoculated plants with a TBSV coat protein mutant (T-Δcp) that causes a milder phenotype than that of wild-type TBSV (Scholthof et al. 1993a). The inoculated transgenic plants initially developed the milder T-Δcp symptoms and no virus particles were evident. However, within 2 wpi, the easily recognized wild-type lethal syndrome began to appear sporadically in individual plants of the transformed lines, and these plants contained large numbers of virus particles. Different assays, including Western blots, RNA hybridization analyses, and sequencing of the RT-PCR products from RNA isolated from virus particles, all indicated that virions with functional coat protein had been regenerated by homologous recombination with the coat protein transgene. Control experiments with a TBSV coat protein mutant containing a restriction enzyme polymorphism for identification and a CNV chimera containing the 46-nt TBSV coat protein deletion provided additional evidence for restoration of the coat protein gene. From these experiments, we conclude that double recombination events involving the coat protein mRNA expressed in transgenic plants and coat protein defective genomes are common during infection of individual plants. With the TBSV system, the appearance of the lethal syndrome in association with restoration of wild-type TBSV and the CNV chimera provides an easily recognizable phenotypic marker for identification of recombinants.

A diverse array of biological factors may influence generation of wild-type virus.

Several factors may have contributed to the large numbers of recombinants recovered in our study. A major factor is that tombusviruses generally appear to lack high levels of transcriptional fidelity. One of the hallmarks of tombusvirus infections is frequent generation of DI (defective interfering) RNAs that arise via recombination (Burgyn et al. 1989; Finnen and Rochon 1993; Hillman et al. 1987; Rubino et al. 1995). Efficient recombination has even been observed in protoplasts co-infected with replication-defective TBSV and CNV (White and Morris 1994). In those experiments, several

Fig. 5. Amplification strategy, and hybridization of reverse transcription-polymerase chain reaction (RT-PCR) products from recombinant viruses. **A**, Schematic representation of the coat protein gene and surrounding regions in the recombinant RNAs. Primers used in the RT-PCR reactions are indicated for each class of construct. **B**, Southern blot of the PCR products performed with viral RNA extracted from virions recovered from nontransformed plants inoculated with wild-type constructs, tomato bushy stunt virus (TBSV), or Ch (left); or from transgenic plants inoculated with the deletion derivatives T-Δcp and Ch-Δcp (right). Templates and primers from A used in the PCR are indicated above each lane: Tp (TBSV-specific primers: TF-Tr), Cp (cucumber necrosis virus [CNV]-specific primers: CF-Cr); when other primers are used, they are indicated above each lane. The Tp primers amplify TBSV sequences (lane 1) but not Ch sequences (lane 3); conversely, Cp primers amplify Ch sequences (lane 4) but not TBSV sequences (lane 2). Tp (lane 5) and Cp (lane 6) represent PCRs performed in the absence of template. Lanes 7 to 18 show primer-specific amplifications of selected recombinants.

defective chimeric RNAs evolved as a consequence of non-homologous recombination, and autonomously replicating hybrid RNA genomes were also generated that were able to infect plants and induce wild-type symptoms. Those results thus demonstrate that tombusvirus RNA recombination events occur frequently in single-cell infections and that functional chimeric genomes as well as DI RNAs can evolve via recombination. In contrast, DI RNAs were only infrequently observed during replication of the bromoviruses (Romero et al. 1993), or cucumoviruses (Fraile et al. 1997). The propensity to undergo high levels of recombination and DI generation could be a consequence of the lack of a helicase domain in the replicases of the tombusviruses. The lack of this domain may have profound effects on faithful processivity and template switching during synthesis of complementary strands. This hypothesis is supported by studies with brome mosaic virus that show that mutations within the helicase domain of the 1a replicase subunit affect the location of recombination sites (Nagy et al. 1995). However, other replicase domains also appear to affect template switching, because a more recent study (Figlerowicz et al. 1997) has shown that mutations in the brome mosaic virus polymerase gene affect nonhomologous but not homologous recombination. Therefore, it is plausible that several specific regions within tombusvirus replicases may have evolved to facilitate increased evolution via recombination.

Another substantial factor that may have influenced the observed levels of recombination is that the TBSV coat protein defective mutants are able to spread systemically (Scholthof et al. 1993a). Consequently, in plants inoculated with the tombusvirus mutants a large number of cells eventually supported virus replication. As recombination events occur in the developing infections, the wild-type recombinants quickly dominate the infections and elicit the lethal syndrome. In contrast, in plants infected with CCMV the infection foci of the mutant viruses were constrained to the initially infected cells until a movement-competent recombinant appeared. Therefore, the high abundance of replicating TBSV RNAs, coupled with the large number of infected cells developing systemic infections, probably contributed to the relatively high probability of regeneration of wild-type virus in individually inoculated plants.

A third factor facilitating regeneration of functional TBSV coat protein was the large amount of homology existing between the transgene and the mutated coat protein gene. Our analyses with the CAT derivatives suggest that the proportion of common sequence affects the recombinant recovery, which probably relates directly to an increased frequency of recombination permitted in the infected cells. In T-CAT, which contains a more substantial deletion (655 nt; 56%) of the coat protein gene than T- Δ cp, the length of the region of homology between the transgenic RNA and the mutant viral coat protein gene appeared to influence the frequency of generation of recombinants. Less than 4% of the inoculated plants developed the lethal syndrome, compared with approximately 15% when plants from the same lines were inoculated with the smaller (46 nt; approximately 4%) T- Δ cp deletion. In both cases, the proportion of inoculated plants regenerating wild-type TBSV was somewhat higher than that observed by Greene and Allison (1994), where about 3% of the transgenic plants inoculated with a CCMV coat protein deletion mutant regenerated wild-type virus. Nevertheless, it is difficult to compare the

recovery of recombinants directly because of the large number of differences between the TBSV and CCMV experimental systems.

In our limited experiments, we were unable to demonstrate nonhomologous recombination between the defective CNV coat protein and the TBSV transgene. Nevertheless, the protoplast experiments of White and Morris (1994) involving coinoculations of replication-defective TBSV and CNV transcripts show that nonhomologous recombination events can occur in single cells. A low level of nonhomologous recombination between RCNMV and a transgene expressed in a host plant has also been described in an abstract (Lommel and Xiong 1991). In this case, recombination occurred between a transgene with a noncoding deletion within the 5' leader sequence of RCNMV RNA2 and a functional RNA 1 transcript that was inoculated onto the transgenic plants. Two RNA 2 recombinants, which had 10 and 17 nt derived from the 5' terminus of RNA 1, were recovered from these plants. However, the time required for appearance of the recombinants was considerably longer than with either TBSV or CCMV, presumably because regeneration of a movement-competent derivative required a nonhomologous recombination event in the initially infected cell (S. A. Lommel, *personal communication*).

One factor in our study that undoubtedly restrained restoration of wild-type virus was that the expressed TBSV transgenic RNA required a double recombination between TBSV and the coat protein transgene. In contrast, regeneration of wild-type CCMV (Greene and Allison 1994), and RCNMV (Lommel and Xiong 1991) required only a single recombination event. When double recombination events were required for CCMV, no recombinants were recovered (Greene and Allison 1996). Therefore, the recovery of recombinants in the systems that have been described has varied depending on the virus, on whether homologous or nonhomologous recombination events were required for regeneration of function, and on whether virus multiplication was restricted to the initially infected cells or could occur in a much larger population of infected cells in systemically invaded plants (Rao and Hall 1993). Thus, a large number of factors, including the polymerase error frequency, the nature of the recombination event, the phenotype of the virus mutant, the ability of mutants to establish systemic infections, and the stringency of selection pressures required for emergence of recombinants, are all important determinants that could affect evolution of recombinant virus derivatives.

Will use of transgenic plants lead to emergence of new virus strains?

Our experiments and those of others (Greene and Allison 1994, 1996; Lommel and Xiong 1991; Gal et al. 1992; Schoelz and Wintermantel 1993; Wintermantel and Schoelz 1996) raise questions about whether or not widespread use of transgenic crops will exacerbate evolution of new virus strains with shifts in host range and vector specificity. Although recombination to repair defective genes has been observed in each of these systems, we should stress that none of the plants used in these experiments were resistant to infection, and that the conditions used in the experiments were optimal for detection of recombinants. There are a number of indications that the lower virus load in highly resistant plants, and the

mechanisms contributing to transgenic resistance, will reduce emergence of recombinant viruses. In fact, benefits associated with the use of transgenic resistance may actually reduce the probability of evolution of recombinant viruses over the levels currently existing in nature. We have elaborated more extensively on this in a related commentary (Rubio et al. 1999).

MATERIALS AND METHODS

Recombinant plasmids and plant transformation.

Standard molecular biology techniques were employed throughout this investigation (Sambrook et al. 1989). The plasmids used in the study were derived from a cDNA clone of TBSV, designated pTBSV-100 (Hearne et al. 1990). Scholthof et al. (1993a) previously described the construction of pHS7A, designated T-Δcp throughout this study, which has a 46-nt deletion within the gene that results in a downstream frameshift and produces a premature termination site nine codons after the deletion, and T-CAT (pHS82), which contains the CAT reporter gene cloned in frame with the 5' end of TBSV coat protein gene, so that 655 nt of the TBSV coat protein gene were deleted. These derivatives are illustrated in Figure 1.

An *SpeI* site was engineered into TBSV to create TSpe as a marker for the recombination studies (Fig. 1). The *SpeI* site was introduced 7 nt downstream of the stop codon of the replicase gene by site-directed mutagenesis, with the oligonucleotide T2 (5' CCGCCGTAGCTTGACTAGTAATACACACACGCAG 3'), corresponding to positions 2609 to 2642 of TBSV (Chang 1993). The wild-type TBSV sequences at positions 2624, 2626, and 2627 (noted in lowercase) change from CCAAGAA to CtAgtAA. A subsequent deletion in the coat protein of TSpe encompassing the region between *NotI* and *BalI* was produced to generate TSpe-Δcp (Fig. 1). The plasmid was first digested with *NotI* and then treated with Klenow polymerase. The linearized DNA subsequently was digested with *BalI* and ligated.

Similar modifications of a biologically active CNV cDNA clone (Rochon and Johnston 1991) were used to construct the chimeric derivatives Ch and Ch-Δcp (Fig. 1). For introduction of an *SpeI* site just downstream of the stop codon of the CNV replicase gene by site-directed mutagenesis, the oligonucleotide C2 (5' CCGCCGTAGCATGACTAGT CAAACA-CAAACACAA 3') from positions 2598 to 2631 was used, to generate CSpe (Chang 1993). The wild-type CNV sequences at positions 2613, 2615, and 2616 (noted in lowercase) change from CCAAGCA to CtAgtCA. To generate pCh (Fig. 1), the fragment from *SpeI* to *NcoI* from CSpe was substituted for the *SpeI*-*NcoI* fragment from TSpe. To create the chimeric coat protein deletion mutant (Ch-Δcp), the fragments flanked by the *SpeI* and *NcoI* sites were exchanged between TSpe-Δcp and CSpe (Fig. 1).

The CNV coat protein deletion mutant C-Δcp (Fig. 1) was constructed by deleting 53 nt from *BglII* site to the *Eco47III* site at positions 3384 to 3439 to eliminate the hinge domain from the coat protein. All constructions used in this study were confirmed by sequence analysis with oligonucleotide primers that flanked the sites of the gene fusions.

For creation of transgenic plants, the coat protein gene was amplified from pTBSV-100 by PCR with the oligonucleotide primers 5'cp (5' CGCAGGATAGACCCATGGC 3') and cp3'

(5' CGGAGCTCGCAGGACACAC 3'). These primers generated an *NcoI* site at the 5' end of the coat protein gene and an *SacI* site at the 3' end to facilitate subsequent cloning steps. The PCR-amplified DNA was precipitated with ethanol and digested with *NcoI*, and the recessed end was filled in with Klenow polymerase. The fragment was subsequently cleaved with *SacI* and cloned into *HincII*/*SacI*-digested Bluescript SK⁺ (Stratagene, La Jolla, CA) to create the plasmid pBCP. In order to excise the coat protein gene, pBCP DNA was digested with *XhoI* and *SacI*, and the resulting fragment was cloned into the *XhoI* and *SacI* sites of the plant gene expression vector pKYLX71:35S2 (Schardl et al. 1987). This manipulation placed the coat protein gene under the control of a modified CaMV 35S promoter containing a duplicated enhancer region (Kay et al. 1987). The resulting transformation vector, pKLCP, was mobilized into *Agrobacterium tumefaciens* (strain LBA4404) (Hoekema et al. 1983), and the transconjugants were used to transform *N. benthamiana*, as previously described (Rogers et al. 1986), except that the shoot-inducing media contained 50 μg of kanamycin, 500 μg of carbenicillin, and 2 μg of benzylaminopurine per ml.

Analysis of transgenic plants for resistance and RNA recombination.

Transgenic seeds were germinated on 0.8% agar plates containing MS (Murashige and Skoog) medium—MS salt mixture (Gibco BRL, Gaithersburg, MD), 0.8% sucrose, vitamins, MES (2-[N-morpholino] ethanesulfonic acid), pH 5.7—and 50 μg of kanamycin per ml to select for transformants. Prior to virus infection, plants from different lines were evaluated for expression of the transgene by RNA blot (Verwoerd et al. 1989) and Western blot (Donald et al. 1992) analyses.

After linearization of the plasmids with *SmaI*, infectious transcripts were generated from pTBSV-100, T-Δcp, T-CAT, TSpe-Δcp, Ch-Δcp, and C-Δcp with T7 polymerase (New England Biolabs, Beverly, MA), and 2 to 5 μg of transcribed RNA in 50 ml of GKP buffer (50 mM glycine, 30 mM KH₂PO₄, pH 7.4, 1% Celite, 1% bentonite) was inoculated onto *N. benthamiana* plants (Hearne et al. 1990). To screen for TBSV resistance, transgenic plants with four to six leaves were mechanically inoculated with sap from infected leaf tissue diluted 1:500, 1:1,000, or 1:5,000 (wt/vol) in 100 mM sodium phosphate buffer (pH 7.4), or with 1 ng, 10 ng, or 1 μg of purified virions in a total of 50 ml per plant. Throughout the experiments several precautions were taken to avoid contamination, including frequent steaming of benches that contained plants, separation of healthy and inoculated transgenic plants from infections with wild-type virus, and maintenance of transgenic and nontransgenic plants in different areas of the greenhouse room.

RNA for hybridization was isolated from 200 mg of infected tissue as described by Verwoerd et al. (1989) and 10-μg samples of total RNA were electrophoresed through a 1% formaldehyde denaturing agarose gel (Sambrook et al. 1989). The gel was then rinsed twice with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 20 min, and the RNA was transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) and subjected to RNA blot analysis at 42°C (Jones et al. 1990) with ³²P-labeled oligonucleotide cpNB (5' CCCTGCAAGGCTGTGGCCC 3') as a probe. This probe was derived from the region between the *NotI* and *BalI*

sites of TBSV-100, and the sequences complementary to the probe were deleted from the T-Δcp, T-CAT, and Ch-Δcp derivatives used for inoculation.

Virus purification experiments were performed with infected transgenic plants. Extracts from these plants were separated in 1% Tris-glycine agarose gels (10 mM Tris, 75 mM glycine, pH 8), and transferred to nitrocellulose membranes. The presence of virus particles was assessed by Western blot analysis (Donald et al. 1992) with polyclonal antisera to intact virus particles.

Viral RNA was extracted (Jones et al. 1990) and denatured in the presence of 10 mM hydroxymethyl-mercury, and the coat protein gene and surrounding sequences were amplified by RT-PCR. First-strand cDNA synthesis was performed with SuperScript II reverse transcriptase (Gibco, BRL) for 2 h at 42°C, and specific oligonucleotides for each presumptive recombinant. Second-strand synthesis and amplification of the products were achieved by PCR with the same antisense oligonucleotides Tr (5' TATCTTGATTCTGAATTCGTC 3') or 3'cp, in combination with the specific sense oligonucleotide TF (5' GGGATCCGGTGGGAGT 3') for T-Δcp, T-CAT, and TSpe-Δcp putative recombinants. For Ch-Δcp the antisense oligo Cr (5' GATCTTTATCCTCACTCTCC 3') was used in combination with the 5'cp sense oligo, or the antisense 3'cp oligo in combination with the sense oligo CF (5' CCCTTCTGGCGGTGAC 3').

Fragments resulting from PCR amplification were cloned into TA-cloning (Promega, Madison, WI/Invitrogen, Carlsbad, CA) or PCR-Script (Stratagene) vectors. In the latter case, the PCR fragments were treated with T4 DNA polymerase (New England Biolabs) before cloning. Sequence analyses were performed on double-stranded plasmid DNA with Sequenase (U.S. Biochemicals, Cleveland, OH) with oligonucleotide primers used for RT-PCR. An additional antisense oligonucleotide designated cpNdeIrev (5' GGGCCA-GATGCTGTGAC 3') was designed for sequence analysis of the 3' end junction of the CAT gene with the coat protein of TBSV, and the T7 or T3 oligonucleotides (Promega).

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