Tomato Bushy Stunt Virus Recombination Guided by Introduced MicroRNA Target Sequences^{▽†}

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Previously we described *Tomato bushy stunt virus* (TBSV) vectors, which retained their capsid protein gene and were engineered with magnesium chelatase (ChlH) and phytoene desaturase (PDS) gene sequences from *Nicotiana benthamiana*. Upon plant infection, these vectors eventually lost the inserted sequences, presumably as a result of recombination. Here, we modified the same vectors to also contain the plant miR171 or miR159 target sequences immediately 3' of the silencing inserts. We inoculated *N. benthamiana* plants and sequenced recombinant RNAs recovered from noninoculated upper leaves. We found that while some of the recombinant RNAs retained the microRNA (miRNA) target sites, most retained only the 3' 10 and 13 nucleotides of the two original plant miRNA target sequences, indicating in planta miRNA-guided RNA-induced silencing complex cleavage of the recombinant TBSV RNAs. In addition, recovered RNAs also contained various fragments of the original sequence (ChlH and PDS) upstream of the miRNA cleavage site, suggesting that the 3' portion of the miRNA-cleaved TBSV RNAs served as a template for negative-strand RNA synthesis by the TBSV RNA-dependent RNA polymerase (RdRp), followed by template switching by the RdRp and continued RNA synthesis resulting in loss of nonessential nucleotides.

Several plant viruses have been developed as tools for various biotechnology applications, including expression platforms for protein production in plants (1, 2, 6) and as gene silencing systems as part of reverse genetics approaches toward understanding host plant gene function (4, 5). For both of these applications, nonviral sequences conferring the desired function are cloned into the virus genome in order to be expressed during replication in plants. One advantage of using viruses engineered with nonviral sequences is flexibility in manipulating these “extra” sequences, which are not essential for viral replication or movement (1). However, recombinant viruses also tend to lose these sequences, causing instability at the insertion site and resulting in loss of function of the recombinant viral vector. The relatively high error rates of viral replicases (7, 14, 24) and the propensity for recombination events (9) contribute to the instability often seen with some viral vector systems.

Recombination events in RNA viruses typically result in joining of two noncontiguous RNA segments (16). These could be sequences from two separate RNA molecules or distant regions of the same molecule. Retention by viruses of favorable sequences is selection driven and eliminates sequences that are unnecessary or negatively affect fitness (11, 31), hence making recombination critical to virus evolution (13, 29). Although phylogenetic analyses predict that recombination events have affected evolution for essentially all groups of RNA viruses (3), some viruses appear to be more prone to recombination than others. For example, plant-infecting supergroup II viruses of the family *Tombusviridae* ap-

pear to undergo frequent recombination, as is supported by the many well-characterized defective-interfering (DI) RNAs of *Tomato bushy stunt virus* (TBSV) (10, 30). The TBSV DI RNAs, derived entirely from the parental viral RNA, are not replication competent alone and depend on the parent virus to replicate them in *trans*. Recent developments of in vitro systems (19, 21) have further enhanced dissection of recombination mechanisms giving rise to TBSV DI RNAs.

Of the proposed mechanisms for viral recombination (12, 20), the copy choice or template-switching mechanism is the most widely reported (8, 16, 18). This occurs when the viral replicase and its attached nascent polynucleotide chain switches viral RNA templates (or jumps locations on the same template) when making cRNA. Some properties for preferred donor/acceptor sites (sequences in the RNA molecule at which viral replicase switches from one template to another) are known for various RNA viruses (3, 20, 27), suggesting that recombination is not entirely random.

The previously described TBSV vectors were efficient silencers of host genes but only while the inserted sequences were retained (23). Thus, optimizing viral vectors requires a better understanding of factors responsible for recombination and consequent loss of insert sequences. In order to address possible recombination mechanisms, we used previously characterized sequence-specific microRNA (miRNA)-guided cleavage determinants as parts of our TBSV vectors. We introduced the miRNA target site sequences for miR171 or miR159 3' to the silencing inserts of our TBSV vectors (23). After plant inoculation, we analyzed recombinant virus sequences, determining specific recombination patterns, and demonstrated miRNA-mediated recombination events in vivo for the recombinant TBSV vectors. We also showed miRNA-guided RNA-induced silencing complex (RISC)-mediated cleavage for recombinant TBSV RNA and evidence supporting the TBSV RNA-dependent RNA polymerase (RdRp) switching templates during cRNA synthesis.

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FIG. 1. TBSV vector map and recombinant vectors. (A) Map of the TBSV p19 mutant (pTBSV-101) genome, showing sgRNA1 and sgRNA2 transcription initiation sites (bent arrows), 3f and CEA insertion regions (dotted lines), and ORFs (rectangles). The pX and p19 ORFs are not shown. Recombinant vectors are as described in the table below. PDS indicates a 108-nt insertion at 3f, and ChlH indicates a 132-nt insertion at CEA. (B) miR171 and miR159 target sequences inserted 3' of the ChlH and PDS inserts, respectively, to obtain pPD-A3-171 and pPD-3f5-159. *, insert size of 264 nt because it is a double insert of ChlH.

MATERIALS AND METHODS

Recombinant TBSV vectors. Recombinant TBSV vectors (Fig. 1) were constructed following standard protocols (26). pPD-A3 and pPD-3f5 were described previously (23). A 132-nucleotide (nt) fragment was PCR amplified from the *N. benthamiana* magnesium chelatase (ChlH) gene sequence (GenBank accession number AF014052, previously cloned in pGEM-T-Easy vector [23]) with the miR171 sequence added to the reverse primer (underlined) to incorporate the 22-nt target sequence. Both primers had XhoI sites (ctcgag) incorporated at the 5' ends of their sequences and are as follows: forward, 5'AAActcgagAGCCAGAAGACC; reverse, 5'AAActcgagTGATTGAGCCGCGCAATATCGCAAGGTTAAT3'). The resulting product was cloned into CEA-XhoI-engineered nonexpressing p19 TBSV vectors (23) to obtain pPD-A3-171. A 108-nt fragment of the *N. benthamiana* phytoene desaturase (PDS) sequence (GenBank accession number DQ469932, nt 855 to 963) was chemically synthesized (DNA 2.0, Emeryville, CA) with the 22-nt target sequence of miR159 added at the 3' end (TAGAGCTCCCTTCAATCCAAAG) and flanked by XhoI sites for cloning into pPD-3f1 (non-p19-expressing 3f-XhoI-engineered TBSV vector [23]) to obtain pPD-3f5-159.

Transcript inoculation, sample collection, and RNA extraction. Recombinant TBSV vectors were transcribed in vitro, and transcripts were inoculated onto *N. benthamiana* plants as described previously (23). Total RNA was extracted from ~75 mg of upper, noninoculated leaves at 7 days postinoculation (dpi) with the RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. RNA was finally eluted with 100 µl RNase-free water.

Characterization of virion RNA at the insert location and sequencing. One-tube reverse transcription-PCR was initially performed on total RNA using TBSV-specific primers (forward, 5'ATTCCACGGGTTTCCAAGTTAAT3'; reverse, 5'CCAAGACTCTGTGACAGTGAAGCT3') using standard protocols (Promega Corp.). The product was column purified (Zymoresearch, CA), and 1 µl was used as the template for a second PCR using TBSV-specific primers flanking the insertion site (forward, 5'TGATGACAGTTCCACATCTGATCA3'; reverse, 5'TAGCTTGTCCCTAGCGTCGTTT3'). The PCR products were then cloned into TOPO TA cloning vectors (Invitrogen, Carlsbad, CA), and following blue-white selection, white colonies were grown in 96-well plates overnight at 37°C and sequenced at the College of Agriculture and Environmental Science Genomic Facility.

5'RACE. 5' random amplification of cDNA ends (5'RACE) was done as described previously (15). Reverse transcription was performed using the TBSV-specific primer (5'TGGTCTTGTTCAGAGGCCCTCGCA3'), followed by PCR using the adaptor-complementary primer and TBSV-specific primers (forward, 5'CGACTGGAGCAGCAGG-ACACTGA-3'; reverse, 5'TGGTCTTGTTCAGAGGCCCTCGCA3'). PCR products were column purified (Zymoresearch) and sequenced as described above.

RESULTS

We previously engineered TBSV vectors with inserts at the CEA and 3f regions of the TBSV genomic RNA to silence two endogenous genes (the ChlH and PDS genes) in *N. benthamiana* plants (23). The CEA region is immediately upstream of the TBSV subgenomic RNA 2 (sgRNA2) start site (Fig. 1A), while the 3f region extends into the 3'-end open reading frame (ORF) of the capsid protein. To address insert stability and identify potential recombination mechanisms, a 22-nt miRNA171 target sequence was introduced 3' of the 132-nt ChlH insert at the CEA region to obtain pPD-A3-171 (Fig. 1). Similarly, pPD-3f5-159 had a 21-nt miRNA159 target sequence inserted after the 108-nt PDS sequence at the 3f region. These were selected because cleavage sites for both miRNA target sequences have been previously mapped (see Fig. S1 in the supplemental material) (17, 22), and therefore, if miRNA-directed cleavage of the corresponding RNAs occurred during infection, we could then determine whether miRNA target sequences influenced recombination events associated with insert loss.

Upon inoculation of *N. benthamiana* plants with these recombinant viruses, a strong silencing phenotype was observed in plants inoculated with pPD-A3-171, beginning at 4 dpi and remaining stable up to 45 dpi (see Fig. S2 in the supplemental material). This was comparable to silencing achieved by pPD-A3 (containing the identical ChlH insert at the CEA region without the 22-nt miRNA target sequence [reference 23 and data not shown]). In contrast, pPD-3f5-159 showed no visible symptoms of TBSV infection or of photobleaching associated with silencing of the PDS gene (data not shown). This was consistent with what was seen for pPD-3f5 (containing the PDS insert at the 3f region without the 21-nt miRNA target sequence [reference 23 and data not shown]).

Sequence patterns in recovered recombinants indicate miRNA-mediated targeting of pPD-A3-171 and pPD-3f5-159. We next attempted to determine if we could detect hallmark nucleotide

TABLE 1. Distribution of different TBSV recombinants observed at different dpi with pPD-A3-171

dpi	No. of clones sequenced	Recombinants recovered (%) ^a						Sequencing error (%) ^b
		A	B	C	D	E	F	
6	21	38	33		4.5	19		6
14	55	27	13	31	23	2		4
27	21		10		10	24	53	3

^a A to F represent recombinants recovered and are shown in Fig. 2. A, contains the complete 132-nt ChlH insert plus the 22-nt miR171 target sequence; B, contains the 5' 40-nt ChlH insert plus the 3' 13 nt from the miR171 target sequence; C, contains the 5' 15-nt ChlH insert plus the 3' 13-nt miR171 target sequence; D, contains only the 3' 13-nt miR171 target sequence; E, contains only the 5' 40-nt ChlH insert; F, contains only the two XhoI sequences flanking 4 to 20 random nucleotides.

^b Sequencing error or no sequence for the particular clones.

sequence patterns (previously characterized; see Fig. S1 in the supplemental material) (17, 22) indicating miRNA-mediated cleavage at the insert regions (3f and CEA) of corresponding recombinant viruses. We used a PCR/sequencing-based approach to characterize insert regions for the different recombinant TBSV vectors in order to identify recombination events

at different time points. These analyses revealed that at up to 27 dpi neither the pPD-A3-171 nor pPD-A3 recombinants showed deletions in the TBSV genomic RNA, at least in the sequenced regions (data not shown). However, deletions were found in the insert and miRNA target sequences for several recombinants, and by 27 dpi none of the recovered recombinants (from pPD-A3-171 inoculations) contained the complete ChlH and miR171 target sequences (Table 1, recombinant A). A corresponding decrease in the number of recombinants (Table 1, recombinant A) containing the complete insert sequences, recovered at 14 dpi compared to 6 dpi, suggested that recombinant TBSV vectors with deletions of the inserted sequences accumulated over time. Supporting this, no insert-free recombinants were recovered at the 6- or 14-dpi sampling points, while 53% of those recovered at 27 dpi had no insert (Table 1, recombinant F). Analysis of the recombinants recovered at the 6-, 14-, and 27-dpi sampling points showed that they contained various lengths of ChlH insert sequence, but, more interestingly, of the recombinants retaining any of the miR171 target sequence, the majority retained only the 3' 13 nt (Fig. 2; Table 1, recombinants B, C, and D). This sequence represents the 3' region remaining after miRNA cleavage. Recombinants

FIG. 2. Recombination events recovered for pPD-A3-171 in *N. benthamiana* plants. The recombinant TBSV plus-strand RNA at the CEA insertion site indicates ChlH and the miR171 target sequence originally engineered in pPD-A3-171. Shaded lines represent plus-strand TBSV recombinant RNA at the CEA insert locations, showing the fate of the ChlH insert and miR171 target sequence.

TABLE 2. Distribution of different recombinants observed at different dpi with pPD-A3

dpi	No. of clones sequenced	Recombinants recovered (%) ^a					Sequencing error (%) ^b
		A	B	C	D	E	
6	21	24	4.5	4.5	33	28	6
27	21		4.5	28		53	14.5

^a A to E represent recombinants recovered. A, contains the complete 132-nt ChlH insert; B, contains the 5' 80-nt ChlH insert; C, contains the 5' 40-nt ChlH insert; D, contains the 5' 22-nt ChlH insert; E, contains only the two XhoI sequences flanking 4 to 20 random nucleotides.
^b Sequencing error or no sequence for the particular clones.

retaining the entire miR171 target site were recovered at the first two sampling times (6 and 14 dpi), and the percentage decreased over time so that no recombinants contained the entire miR171 target sequence by 27 dpi. A few recombinants containing 40 nt of ChlH but lacking the miR171 target sequence were also recovered (Fig. 2; Table 1, recombinant E). Insert analyses on RNA isolated from plants inoculated with the control pPD-A3 (the control recombinant TBSV vector containing the identical ChlH sequence but lacking the miR171 target sequence) also showed more insert-free recombinants (Table 2, recombinant E) progressing from the 6- to 27-dpi time points, consistent with the pPD-A3-171 results, and a corresponding decrease in the percentage of recovered recombinant viruses containing the complete insert over the same time period (Table 2, recombinant A). For both pPD-A3 and pPD-A3-171, 100% (21 of 21 clones for each construct) showed deletions by 27 dpi, and thus we saw no evidence for

differences in recombination frequencies between the constructs with or without the miR171 target site.

Since there was no silencing phenotype for pPD-3f5-159 or pPD-3f5, we limited our analyses to only 4 dpi in order to detect the population of early recombinants before postrecombination selection resulted in a dominant sequence. In contrast to the sequence patterns observed for pPD-A3-171, no recombinants recovered from plants inoculated with pPD-3f5-159 retained the complete miR159 target sequence. However, all did retain 3' portion (10 nt) of the miR159 target sequence (Fig. 3A, panels i to iii). Like for the pPD-A3-171 recombinants, this 3' portion of the miR159 sequence is the 3' region immediately adjacent to the miR159 cleavage site. It is also important to note that 50% of recovered recombinants showed deletions in the TBSV genomic RNA 5' of the 3f insert site (Fig. 3A, panel iii). In contrast, 83% of recombinants recovered from plants inoculated with the control pPD-3f5 (recombinant TBSV containing the identical PDS sequence, but lacking the miR159 target sequence) retained the complete 108-nt PDS gene insert with no other detected deletions (Fig. 3B). Thus, in contrast to pPD-A3-171, a greater frequency of recombination was observed for the pPD-3f5-159 constructs containing the miR159 target sequence (compared to control pPD-3f5), and this was observed as early as 4 dpi.

5'RACE confirmed insert retention features and detected 3' portions of miRNA target sequences at the 5' end of recombinant TBSV RNA. The high percentage of recombinants that retained the 3' portion of the miRNA target sequences for both pPD-A3-171 and pPD-3f5-159 suggested that the miRNA-guided silencing pathway in planta was targeting the recombi-

FIG. 3. Recombination events recovered for pPD-3f5-159 (A) and pPD-3f5 (B) in *N. benthamiana* plants. Recombinant TBSV plus-strand RNA at the 3f region indicates PDS and the miR159 target sequence originally engineered in pPD-3f5-159. Shaded lines represent plus-strand TBSV recombinant RNA at the CEA insert region, showing the fate of the PDS insert and miR159 target sequence. A total of 21 clones were sequenced for both pPD-3f5-159 and pPD-3f5. (i) Contains the 5' 60-nt PDS insert plus the 3' 10-nt miR159 target sequence. (ii) Contains only the 3' 10-nt miR159 target sequence. (iii) Contains 3' 10 nt of the miR159 target sequence accompanied by a 100- to 200-nt deletion in the TBSV genome 5' of the 3f location. (B) Contains the complete 108-nt PDS sequence but no miR159 sequence.

FIG. 4. Recombinants recovered at 6 dpi by 5'RACE analysis. (A) Recombinant virus variants recovered from plants inoculated with pPD-A3-171. The number of clones recovered for each type is indicated. Shaded lines represent plus-strand TBSV recombinant RNA at the CEA insert locations, showing the fate of the ChlH insert and miR171 target sequence. (i) The RNA contains the 5' 40 nt of the ChlH insert plus the 3' 13 nt of the miR171 target sequence. (ii) The RNA contains the 5' 15 nt of the ChlH insert plus the 3' 13 nt of the miR171 target sequence. (iii) The RNA has the 3' 13 nt of the miR171 target sequence at its 5' end. (B) Recombinant virus RNAs recovered from plants inoculated with pPD-3f5-159. Blue lines represent plus-strand TBSV recombinant RNA at the 3f insert locations, showing the fate of the PDS insert and miR159 target sequence. Seven of 12 clones sequenced contained the 3' 10 nt of the miR159 target sequence at the junction of the 3f location and near the 5' end of sgRNA1 (start site, nt 2621).

nant TBSV vectors with the corresponding miRNA-guided RISC. We reasoned that if miRNA-mediated cleavage of the recombinant TBSV vectors occurred in these plants, we should be able to recover TBSV RNA molecules having at their 5' end the specific miRNA target cleavage sequence expected (based on previously identified cleavage patterns for both miR171 and miR159 [see Fig. S1 in the supplemental material]). To test this hypothesis, we performed 5'RACE.

We recovered and sequenced 12 clones for pPD-A3-171 and 10 for pPD-3f5-159. Sequence analysis of the pPD-A3-171 clones showed that all contained the 3' 13 nt of the miR171 target sequence. Ten of the 12 clones had ChlH gene sequences immediately 5' of the miR171 13 nt, thus giving results similar to those found in our experiments above. However, two of these clones had 5' termini that contained the 13 nt from the 3' end of miR171 target sequence (Fig. 4A; see Fig. S1 in the supplemental material). Thus, these data provided further evidence of miR171 cleaving recombinant TBSV. Our 5'RACE analysis of 10 recovered pPD-3f5-159 clones showed that all contained the 10 nt from the 3' end of the miR159 target sequence. However, for these recombinant RNAs there was also a ~900-nt deletion immediately 5' of the 3f region (nt

2678 to 3779). Interestingly these recombinant TBSV RNA molecules terminated at nt 2621, the natural 5' end of TBSV sgRNA1 (Fig. 4B). We did not recover any clones that terminated at the predicted cleavage site for the miR159 target sequence.

DISCUSSION

miRNA target sequences affect TBSV recombination. Sequencing of reverse transcription-PCR and 5'RACE products from plants inoculated with recombinant TBSV constructs containing miR171 or miR159 target sites showed that the majority of recombinant clones retained only the 3' portion of the respective miRNA target sequence, providing direct evidence for miRNA-mediated cleavage and recombination in planta. For pPD-A3-171, "CGCGGCUCAAUCA" was a consistent part of the insert sequence retained, sometimes in conjunction with fragments of the ChlH insert (Fig. 2). These 13 nt represent the 3' portion of the miR171 target region (or the complementary 5' portion of miR171 [see Fig. S1 in the supplemental material]) (17), the sequence which would result from miR171-mediated cleavage. Only at the earliest sampling

times (6 and 14 dpi) was the entire target site retained in a minority of recovered sequences. Further analysis of the recombinant sequences for retention of ChlH sequence inserts showed that the plant-derived ChlH sequence also was eliminated over time, but in contrast to the miRNA target sequence inserts, there were no apparent sequence-specific determinants.

Interestingly, none of the sequences recovered from plants inoculated with pPD-3f5-159 retained the entire miR159 target sequence, even when samples were analyzed as early as 4 dpi. However, all pPD-3f5-159-derived recombinants sequenced had "UCAAUCCAAA," which again is the 3' portion of the miR159 target region (see Fig. S1 in the supplemental material) (22) resulting from miR159-guided cleavage. This might suggest that miR159 more rapidly and efficiently targets its homologous sequence than does miR171, or this could be partly due to much lower endogenous levels of miR171 present in the *N. benthamiana* leaf tissue compared to miR159 (28).

These miRNA target sequences have been previously used with recombinant *Plum pox virus* (PPV) (28); however, the results differed greatly from ours. Upon inoculating plants with the recombinant PPV, those authors observed reduced infectivity and virus titer in inoculated plants. This was directly attributed to miRNA-guided cleavage of the recombinant PPV. Furthermore, the authors found that the recombinant PPV recovered from infected plants contained point mutations in the seed region of the miRNA target sequences (for both miR171 and miR159), suggesting that these mutations most likely accumulated to allow the recombinant PPV to escape specific miRNA-guided degradation. In contrast, we did not see any reduction in infectivity or silencing efficiency in our miRNA target sequence-containing recombinant TBSVs. This could be directly attributed to template switching of the TBSV RdRp, resulting in a recombinant RNA that was no longer a target for the endogenous miRNA. DI RNA formation is well documented in TBSV infections (10) and has been attributed to the TBSV RdRp switching templates during viral replication (8, 16, 18, 32), though it is known to be strongly favored during plus-strand RNA synthesis (10). Thus, based on previous data for TBSV and our resulting sequence data, template switching may explain how the recombinant TBSV constructs used here by us could be targeted by miRNAs and rapidly lose portions of their miRNA target sequences without showing a detectable loss of infectivity as was observed for PPV.

Template switching in TBSV directed by miRNA target sequences. Previous reports have detailed template-switching mechanisms, especially for tombusviruses (8). Based on the miRNA target sequences in our recombinant TBSV vectors and the sequences of the resulting recombinants after infection, we propose a possible model for generation of the TBSV recombinants observed here. Recombinant TBSV RNA retaining the full-length ChlH gene insert and miR171 target sequence (shown as an example) undergoes complementary base pairing with the miR171-loaded RISC in infected cells (Fig. 5A). The recombinant TBSV plus-sense RNA is cleaved at the miRNA171-determined cleavage sequence, resulting in two RNAs (Fig. 5B). This miRNA-mediated cleavage could potentially occur before or after the recombinant TBSV RNA is recruited in the replication complex. The observation that some clones retained the entire miR171 target sequence at

earlier sampling times, though, suggests that there was at least some miR171-directed cleavage after replication. After miR171-directed cleavage, the RNA fragment containing the 3' region of the miR171 target sequence also contains the promoter for TBSV minus-strand synthesis (gPR) and the replication silencer element at its 3' end (25) (Fig. 5C). Both of these elements are critical for TBSV replication (25); thus, the miR171-cleaved TBSV recombinant RNA can likely serve as a template for minus-strand TBSV RNA synthesis. The TBSV RdRp then synthesizes the minus-strand RNA, using as template the cleaved plus-strand recombinant RNA, now also including 13 nt of the miR171 target sequence at its 5' end (Fig. 5D). On reaching the 5' end of the miR171-cleaved plus-strand RNA template, the RdRp can either fall off the template, ending the minus-strand RNA synthesis, or disassociate from the template and attach to another plus-strand RNA, taking along its synthesized minus strand.

Based on the recombinants we recovered, our evidence suggests that both of the events described above might occur. However, we provide direct evidence that the TBSV RdRp can switch templates when it comes to the 5' end of the plus-strand RNA donor molecule (containing the 3' 13 nt of the miRNA171 target sequence) and then can land internally on another recombinant TBSV plus-strand RNA molecule (Fig. 5E). A recombinant TBSV RNA carrying a full-length insert is shown as an example of an acceptor template RNA. There is also the possibility of the 5' fragment of the recombinant TBSV (originating from the initial cleavage by miR171) or sgRNAs serving as an acceptor template for the switching RdRp. Irrespective of the choice of acceptor template RNA, the RdRp on restarting minus-strand RNA synthesis can extend to the end of the acceptor template RNA (Fig. 5F), resulting in a recombinant minus-strand TBSV molecule with the 3' 13 nt of the miRNA171 target sequence present at the junction of the donor and acceptor TBSV sequences (Fig. 5G). Following this, natural selection then plays a role favoring only those recombinants that enhance (or diminish least) viral fitness for critical properties such as replication competence, movement, or protein expression.

This model is well supported by previous reports where template switching was shown to originate most frequently from the 5' end of the donor RNA molecule, land internally on a acceptor template RNA, and restart synthesis (8), thus not being limited to end-to-end switching between two RNA templates. An AU-rich region (27) along with as few as 1 to 4 nt base pairing (10) are additional requirements for efficient internal landing and continued synthesis for tombusvirus RdRps (25). This is consistent with our data, where sequences immediately 3' of the proposed landing site for the TBSV RdRp in the acceptor RNA (Fig. 2, clones B,C,D), were A rich (~70%) (see Table S1 in the supplemental material). There was also no significant (>2-nt) base pairing between the 3' end of the nascent minus-strand RNA synthesized by the switching TBSV RdRp and the acceptor template RNA.

This mechanism is further supported by the recombination events detected at 6 dpi in pPD-3f5-159-inoculated plants. The recombinants exhibited massive deletions 5' of the 3f insertion site (Fig. 4B) in the TBSV genome (nt 2678 to 3779). In this particular recombination event, the TBSV RdRp along with its nascent minus RNA strand lands on sgRNA1 (at nt 2678),

FIG. 5. Proposed mechanism for recombinant TBSV synthesis. (A) Recombinant TBSV plus-strand RNA (blue) with the insertion region magnified to show the complete 132-nt ChlH plus the 22-nt miR171 target sequence. The 3' end TBSV recruitment site for TBSV RdRp (orange rectangle) is also shown. Mir171-guided RISC targets the recombinant plus-strand RNA by complementary base pairing (solid lines). (B) Cleavage of targeted recombinant plus-strand TBSV RNA at the miR171 cleavage site, resulting in two RNAs. (C) Recruitment of TBSV RdRp (gray oval) for minus-strand RNA synthesis (dashed arrow) to the 3' end of the plus-strand RNA template molecule containing the natural 3' end of TBSV (orange rectangle). (D) Synthesis of minus-strand RNA (black line) using the plus-strand RNA as template, resulting in a sequence complementary to the miRNA target sequence. The synthesized minus-strand RNA (a) and template plus-strand RNA (donor template RNA) are shown. (E) The TBSV RdRp with the synthesized minus-strand RNA (a) switches templates after coming to the end of the donor plus-strand RNA template, landing internally on another plus-strand TBSV RNA (acceptor template RNA). Here, a recombinant TBSV plus-strand RNA with the full ChlH insert and miRNA target sequence is shown as an example. The ChlH insert region is magnified to show the potential internal landing point (AU rich) for TBSV RdRp. (F) TBSV RdRp restarts minus-strand RNA synthesis (dotted black line), using the acceptor plus-strand RNA as a template. (G) Formation of recombinant minus-strand RNA synthesized as outlined, containing the 3' segment of miRNA target sequence at the junction of the sequence complementary to the donor (black line) and acceptor (black dotted line) template molecules.

restarting synthesis and continuing to the end of the sgRNA1 acceptor template (nt 2621, the natural 5' end of sgRNA1). The region 3' of nt 2678 is again A rich and thus a potential hot spot for the viral replicase to land and reinitiate minus-strand RNA synthesis.

Thus, using engineered miRNA target sites, we provide evidence supporting sequence-specific cleavage of recombinant TBSV RNAs in planta. The sequence patterns recovered were identical to previously mapped patterns of natural mRNA targets for miR171 and miR159, suggesting that the engineered miRNA target sites containing recombinant TBSV could be targeted by the host miRNA silencing machinery. These data are consistent with a recent publication (33) which demonstrated the role of a host Dicer in promoting recombination and negatively affecting insert stability for a hypovirus, CHV1-EP713, in the fungal host *Cryphonectria parasitica*. In both cases, there is evidence of viral RNA being susceptible to the host RNA interference machinery, which in turn has an effect on the recombination, probably by creating new 5' and 3' ends for the viral replicase to switch templates, thus promoting recombination. Combined with the advantage of known cleavage patterns of miRNA-mediated targeting, this approach could be developed for further dissection of previously unchar-

acterized recombination events in vivo for different viral vector systems to better analyze and predict insert stability at different sites of the viral genome.

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REFERENCES

- Barajas, D., F. Tenllado, and J. R. Díaz-Ruiz. 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.
- Benedito, V. A., P. B. Visser, G. C. Angenent, and F. A. Krens. 2004. The potential of virus-induced gene silencing for speeding up functional characterization of plant genes. *Genet. Mol. Res.* **3**:323–341.
- Bujarski, J. J., P. D. Nagy, and S. Flasinski. 1994. Molecular studies of genetic RNA-RNA recombination in brome mosaic virus. *Adv. Virus Res.* **43**:275–302.
- Burch-Smith, T. M., J. C. Anderson, G. B. Martin, and S. P. Dinesh-Kumar. 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant J.* **39**:734–746.
- Burch-Smith, T. M., M. Schiff, Y. Liu, and S. P. Dinesh-Kumar. 2006. Efficient virus-induced gene silencing in Arabidopsis. *Plant Physiol.* **142**: 21–27.
- Canizares, M. C., L. Liu, Y. Perrin, E. Tsakiris, and G. P. Lomonosoff. 2006. A bipartite system for the constitutive and inducible expression of high levels of foreign proteins in plants. *Plant Biotechnol. J.* **4**:183–193.

7. **Castro, C., J. J. Arnold, and C. E. Cameron.** 2005. Incorporation fidelity of the viral RNA-dependent RNA polymerase: a kinetic, thermodynamic and structural perspective. *Virus Res.* **107**:141–149.
8. **Cheng, C. P., and P. D. Nagy.** 2003. Mechanism of RNA recombination in carmo- and tombusviruses: evidence for template switching by the RNA-dependent RNA polymerase in vitro. *J. Virol.* **77**:12033–12047.
9. **Cheng, C. P., T. Panavas, G. Luo, and P. D. Nagy.** 2005. Heterologous RNA replication enhancer stimulates in vitro RNA synthesis and template-switching by the carmovirus, but not by the tombusvirus, RNA-dependent RNA polymerase: implication for modular evolution of RNA viruses. *Virology* **341**:107–121.
10. **Cheng, C. P., J. Pogany, and P. D. Nagy.** 2002. Mechanism of DI RNA formation in tombusviruses: dissecting the requirement for primer extension by the tombusvirus RNA dependent RNA polymerase in vitro. *Virology* **304**:460–473.
11. **Fernandez-Cuartero, B., J. Burguan, M. A. Aranda, K. Salanki, E. Moriones, and F. Garcia-Arenal.** 1994. Increase in the relative fitness of a plant virus RNA associated with its recombinant nature. *Virology* **203**:373–377.
12. **Garcia-Andres, S., D. M. Tomas, S. Sanchez-Campos, J. Navas-Castillo, and E. Moriones.** 2007. Frequent occurrence of recombinants in mixed infections of tomato yellow leaf curl disease-associated begomoviruses. *Virology* **365**: 210–219.
13. **Hill, K. R., M. Hajjou, J. Y. Hu, and R. Raju.** 1997. RNA-RNA recombination in Sindbis virus: roles of the 3' conserved motif, poly(A) tail, and nonviral sequences of template RNAs in polymerase recognition and template switching. *J. Virol.* **71**:2693–2704.
14. **Holland, J. J.** 2006. Transitions in understanding of RNA viruses: a historical perspective. *Curr. Top. Microbiol. Immunol.* **299**:371–401.
15. **Kasschau, K. D., Z. Xie, E. Allen, C. Llave, E. J. Chapman, K. A. Krizan, and J. C. Carrington.** 2003. P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. *Dev. Cell* **4**:205–217.
16. **Lai, M. M.** 1992. RNA recombination in animal and plant viruses. *Microbiol. Rev.* **56**:61–79.
17. **Llave, C., Z. Xie, K. D. Kasschau, and J. C. Carrington.** 2002. Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. *Science* **297**:2053–2056.
18. **Nagy, P. D., and J. J. Bujarski.** 1993. Targeting the site of RNA-RNA recombination in brome mosaic virus with antisense sequences. *Proc. Natl. Acad. Sci. USA* **90**:6390–6394.
19. **Nagy, P. D., J. Pogany, and A. E. Simon.** 2001. In vivo and in vitro characterization of an RNA replication enhancer in a satellite RNA associated with turnip crinkle virus. *Virology* **288**:315–324.
20. **Nagy, P. D., and A. E. Simon.** 1997. New insights into the mechanisms of RNA recombination. *Virology* **235**:1–9.
21. **Nagy, P. D., C. Zhang, and A. E. Simon.** 1998. Dissecting RNA recombination in vitro: role of RNA sequences and the viral replicase. *EMBO J.* **17**:2392–2403.
22. **Palatnik, J. F., H. Wollmann, C. Schommer, R. Schwab, J. Boissbouvier, R. Rodriguez, N. Warthmann, E. Allen, T. Dezulian, D. Huson, J. C. Carrington, and D. Weigel.** 2007. Sequence and expression differences underlie functional specialization of Arabidopsis microRNAs miR159 and miR319. *Dev. Cell* **13**:115–125.
23. **Pignatta, D., P. Kumar, M. Turina, A. Dandekar, and B. W. Falk.** 2007. Quantitative analysis of efficient endogenous gene silencing in Nicotiana benthamiana plants using tomato bushy stunt virus vectors that retain the capsid protein gene. *Mol. Plant-Microbe Interact.* **20**:609–618.
24. **Pita, J. S., J. R. de Miranda, W. L. Schneider, and M. J. Roossinck.** 2007. Environment determines fidelity for an RNA virus replicase. *J. Virol.* **81**: 9072–9077.
25. **Pogany, J., M. R. Fabian, K. A. White, and P. D. Nagy.** 2003. A replication silencer element in a plus-strand RNA virus. *EMBO J.* **22**:5602–5611.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY.
27. **Shapka, N., and P. D. Nagy.** 2004. The AU-rich RNA recombination hot spot sequence of brome mosaic virus is functional in tombusviruses: implications for the mechanism of RNA recombination. *J. Virol.* **78**:2288–2300.
28. **Simón-Mateo, C., and J. A. García.** 2006. MicroRNA-guided processing impairs plum pox virus replication, but the virus readily evolves to escape this silencing mechanism. *J. Virol.* **80**:2429–2436.
29. **Strauss, E. G., and J. H. Strauss.** 1991. RNA viruses: genome structure and evolution. *Curr. Opin. Genet. Dev.* **1**:485–493.
30. **White, K. A., and T. J. Morris.** 1999. Defective and defective interfering RNAs of monopartite plus-strand RNA plant viruses. *Curr. Top. Microbiol. Immunol.* **239**:1–17.
31. **White, K. A., and T. J. Morris.** 1994. Enhanced competitiveness of tomato bushy stunt virus defective interfering RNAs by segment duplication or nucleotide insertion. *J. Virol.* **68**:6092–6096.
32. **White, K. A., and T. J. Morris.** 1994. Recombination between defective tombusvirus RNAs generates functional hybrid genomes. *Proc. Natl. Acad. Sci. USA* **91**:3642–3646.
33. **Zhang, X., and D. L. Nuss.** 2008. A host dicer is required for defective viral RNA production and recombinant virus vector RNA instability for a positive sense RNA virus. *Proc. Natl. Acad. Sci. USA* **105**:16749–16754.