Unusual Events Involved in *Banana bunchy top virus* Strain Evolution

Hui-Chuan Fu, Jer-Ming Hu, Ting-Hsuan Hung, Hong-Ji Su, and Hsin-Hung Yeh

First, third, fourth, and fifth authors; Department of Plant Pathology and Microbiology, second author: Institute of Ecology and Evolutionary Biology, and fifth author: Research Center for Plant Medicine, College of Agriculture, National Taiwan University, 1, Sec 4, Roosevelt Road, Taipei 106, Taiwan.

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ABSTRACT

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Banana bunchy top virus (BBTV) can be transmitted by aphids and consists of at least six integral components (DNA-R, -U3, -S, -M, -C, and -N). Several additional replication-competent components (additional Reps) are associated with some BBTV isolates. A collected BBTV strain (TW3) that causes mild symptoms was selected to study the processes in BBTV evolution. Southern blot hybridization, polymerase chain reaction (PCR), and real-time PCR did not detect DNA-N in TW3. Real-time PCR quantification of BBTV components revealed that, except for the copy number of TW3 DNA-U3, each detected integral component of BBTV TW3 was at least two orders lower than that of the severe strains. No infection was observed in plants inoculated with aphids, which were first given acquisition access to the TW3-infected banana leaves. Recombination analysis revealed recombination between the integral component TW3 DNA-U3 and the additional Rep DNA-Y. All BBTV integral components contain a replication initiation region (stem-loop common region) that share high sequence identity. Sequence alignment revealed that TW3 DNA-R, -S, -M, and -C all have a stem-loop common region containing a characteristic 9-nucleotide deletion found only in all reported DNA-N. Our data suggest that the additional Rep DNAs can serve as sources of additional genetic diversity for integral BBTV components.

Banana bunchy top disease (BBTD) is considered one of the most economically important plant viral diseases worldwide (12,43). The causal agent of BBTD is *Banana bunchy top virus* (BBTV), belonging to the genus *Babuvirus* in the family *Nanoviridae*, with ≈18- to 20-nm isometric virions (19,57,63). BBTV is transmitted by vegetative planting materials or by an aphid vector, *Pentalonia nigronervosa* (37). BBTV infection is restricted to phloem and causes symptoms of dwarfing, leaf atrophy, leaf chlorosis, and vein clearing.

The genome of BBTV consists of six integral components originally named DNA 1 to 6 and recently renamed DNA-R, -U3, -S, -M, -C, and -N, respectively, according to their putative functions (16,63). The integral components were found in all isolates collected around the world (28). Each BBTV-associated component is ≈1.1 kb, and the transcripts have been mapped (2,3, 8,16). DNA-R encodes a replication initiation protein (Rep) (20), which later was shown to support the replication of other non-Rep-encoding components (23). The open reading frame (ORF) of DNA-U3 is not consistently present among characterized isolates, and the function of DNA-U3 in BBTV infection is currently unknown. DNA-S encodes a capsid protein (65), DNA-M and DNA-N encode possible movement proteins (64), and DNA-C encodes a protein that can bind retinoblastoma and may be involved in host-cell-cycle manipulation (1,63,64).

Of interest, in addition to the integral genome components, some viruses within the family *Nanoviridae* contain several additional components (4,5,7,16,23,24,29,48,53,66,67). The ORFs of these components encode proteins with similarity to replication initiation factors and are known as "additional Reps." Additional Rep-encoded components in *Faba bean necrotic yellows virus* (FBNYV), *Milk vetch dwarf virus*, *Subterranean clover stunt virus*, and BBTV are replication competent but they cannot *trans* replicate the non-Rep-encoding components. For viruses within the nanoviridae, only DNA-R can *trans* replicate the non-Rep-encoding components. This concept is known as the "master Rep" in nanovirus replication (23,60). Interestingly, some nanovirus-like components are also associated with geminiviruses (6,7,39, 49–51,53). Several additional Reps have been reported in BBTV but are confined to banana cultivated in Asia (4,24,66,67). The existence of additional Reps may suggest opportunities for nanovirus evolution (60); however, solid data is needed to support this speculation.

All BBTV integral components and associated additional Reps contain a stem-loop (SL) structure with a conserved sequence, TA(G/T)TATTAC, in the loop region. The SL can be identified in all single-stranded plant DNA viruses, including geminiviruses and nanoviruses. The SL contains the origin of replication for single-stranded DNA viruses (18,22,31,46,48,52,61). Interestingly, among BBTV integral components, the conserved loop sequence is TATTATTAC; however, in additional Reps, the sequence is TAGTATTAC. All BBTV integral components share at least 62% sequence identity in a 69-nucleotide (nt) sequence stretch surrounding and including the SL structure and defined as the SL common region (9). As well, all BBTV integral components share at least 76% sequence identity in another stretch of

Corresponding author: H.-H. Yeh; E-mail address: hyeh@ntu.edu.tw

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* The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains two tables showing the sequences and primers used in this study, respectively. Figure 5 appears in color online.

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66 to 92 nt located at the 5′ end of the common region SL (CR-SL) and identified as the major common region (CR-M) (9). Previous studies indicated that DNA primers associated with BBTV virions can bind to the CR-M region and can initiate the synthesis of complementary-strand DNA in vitro (17). An iterated sequence in the intergenic region of each BBTV component has also been identified and is essential for efficient replication of BBTV (21).

The multipartite genome organization and the unusual association of several replication-competent components with viruses in the family *Nanoviridae* or *Geminiviridae* suggest complicated processes involved in nanovirus evolution. More interestingly, nanoviruses have also been suggested to have switched hosts from plants to a vertebrate and then, on recombination with a vertebrate-infecting virus, give rise to circoviruses (15).

Previously, we used phylogenetic analysis to show recombination and reassortment in BBTV evolution (25). From codon usage analysis, we also revealed that BBTV DNA-R and -S may be alien components resulting from an ancient co-infection and reassortment event in the formation of the current genome of BBTV (25). The analysis was based on worldwide reported BBTV sequences; however, the evolutionary processes involved in BBTV strain evolution remain to be solved.

To study the processes contributing to the evolution of BBTV, we took advantage of the collected BBTV strains that cause atypical symptoms (54). We characterized the genome organization of a stable BBTV strain causing mild symptoms, BBTV type IV (TW3; originally named BBTV type IV) (55), by use of molecular biology approaches and phylogenetic analysis. DNA-N was not detected in BBTV TW3 on Southern blot hybridization, polymerase chain reaction (PCR), or real-time PCR. DNA-N may be not associated with BBTV TW3 or does exist but at below detection levels. Surprisingly, sequence alignment revealed that TW3 DNA-R, -S, -M, and -C all have a CR-SL containing a characteristic 9-nt deletion found only in all reported DNA-N. In addition, we show recombination between DNA-U3 and additional Reps. Our findings support that the additional Rep DNAs may have contributed to the evolution of BBTV.

MATERIALS AND METHODS

Plants. The symptom-attenuating strain BBTV TW3 has been maintained in banana through newly grown suckers or tissue culture for more than 10 years. Plants are kept in an insect-proof greenhouse at the Department of Plant Pathology and Microbiology, National Taiwan University. The identity of BBTV TW3 was confirmed by its field symptoms and by comparing PCR results of C1, CR-SL, and TS primer pairs with those of previous studies (55). One sample of the maintained BBTV TW3 was used for detailed genome characterization.

Cloning and sequencing of BBTV TW3 genomic DNA of Taiwanese isolates. The cloning and sequencing approaches were as described (25,34). All sequences were deposited in GenBank. BBTV components were amplified by PCR with primer pairs designed at specific regions of corresponding components. The nucleic acids were extracted from infected banana plants as described by Su et al. (55). The primer pairs were designed in an immediately adjacent outward-extending direction. The PCR conditions were as previously reported (55). The amplified products were cloned into a pGEM-T EASY vector (Promega Corp., Madison, WI) by incubation with DNA ligase overnight at 4°C, followed by transformation of *Escherichia coli* DH5α or Top 10. For each BBTV genomic component, at least 20 individual colonies were randomly selected for colony PCR with SP6 and T7 primers to check for size of inserted DNA. At least five clones were selected for sequencing from both directions by use of ABI PRISM DNA sequencer 310 or 377 (Applied Biosystems, Foster City, CA).

Real-time quantitative PCR. Nucleic acids were extracted from 0.5 g of banana leaf tissue as described by Su et al. (55) and dissolved in 150 μl of double-distilled (dd)H_2O. Nucleic acids (80 ng) and primer pairs were added into 2× SYBR Green PCR Master Mix (Applied Biosystems) for quantitative PCR in an ABI Prism 7000 Sequence Detection System following the manufacturer's instructions (Applied Biosystems). With the use of each cloned BBTV component, standard curves were generated of 10^2 to 10^{10} copy numbers, except for DNA-R, which involved 10^5 to 10^{10} copy numbers, and 10-fold increments of copies of each cloned BBTV component were used in real-time PCR for each interval of the standard curves. Banana 18S DNA was used as an internal control. Each real-time PCR reaction was analyzed in triplicate.

Southern blot hybridization. Nucleic acids were extracted from 0.5 g of banana leaves and dissolved in 20 μl of ddH_2O, then separated by electrophoresis in a 1.2% agarose gel. The gel was denatured by use of 0.5 M NaOH, then neutralized in 0.5 M Tris-HCl (pH 8.0). The gel was then transferred to Hybond NX membrane (Amersham) by use of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Digoxigenin-labeled probes were generated by PCR with cloned DNA-S and DNA-N used as templates, and primer pairs DNA-S-3F/DNA-S-3R and DNA-N-1F/ DNA-N-4R were used to generate part of the DNA-S and DNA-N ORF-specific probes, respectively. Immobilized RNA underwent hybridization, and signals were detected as previously described (36,68).

Sequences used for phylogenetic analysis. For BBTV integral components, sequences were derived from isolates of all integral-component sequences available in GenBank. For additional Rep DNAs, the Rep ORF sequences of nanoviruses and circoviruses were derived from all available sequences in GenBank. BBTV TW3 DNA-R, -U3, -S, -M, and -C are designated DRTW3, DU3TW3, DSTW3, DMTW3, and DCTW3, respectively. The ORF of additional Rep DNA-Y was not resolved by Yeh et al. (67). We recently cloned and sequenced several components of additional Rep DNA-Y and found deletions and insertions frequently in these clones; however, most additional Rep DNA-Ys contain 2-nt insertions (CC) between nucleotides 260 and 261 and a substitution G 633 C. With these modifications, we resolved an ORF of 286 amino acids.

Phylogenetic analysis. The sequence alignment was generated by use of CLUSTAL X (1.81) (58). Phylogenetic analyses involved Bayesian-inference (BI), maximum-parsimony (MP), and neighbor-joining (NJ) methods. BI was analyzed by use of MrBayes v3.1 (26) and MP and NJ by PAUP* 4.0 b 10 (56). NJ involved an HKY85 nucleotide substitution model, and node supports were analyzed by bootstrap with 1,000 replicates. MP involved heuristic searches with 500 random-addition replicates, bisection-reconnection branch swapping, and bootstrap with 1,000 replicates. BI analysis involved an HKY85 substitution model with gamma distribution ($\alpha = 0.5$) for rate distribution among sites. The BI analyses were performed with four chains of Markov chain Monte Carlo, sampling 1 tree per 500 generations for 1,500,000 generations; the first 500 trees were excluded to calculate the posterior probability of each node.

Recombination detection. The potential recombinant sequences and recombination breakpoints were analyzed by the Distance Plot method implemented in the RDP2 (35,41).

Transmission assay. *P. nigronervosa* aphids were placed in acquisition cages on healthy, BBTV severe-strain-infected, and TW3-infected banana leaves. The aphids were allowed 24 h of acquisition access feeding, and ≈50 aphids were transferred to each healthy Cavendish (cv. Pei Chiao, AAA) plant (≈15 cm tall) for a 48-h inoculation access period. The inoculated plants were sprayed with insecticide and grown in an insect-proof greenhouse. BBTV infections were determined by symptoms and confirmed by PCR with C1, CR-SL, and TS primer pairs.

RESULTS

Cloning and sequencing of BBTV TW3 integral components. We were able to clone components of DNA-R, -U3, -S, -M, and -C and the additional Reps DNA-Y and DNA-S2; however, we were unable to clone DNA-N. To reduce the possibility of nonappropriate primer design, we used PCR with two more primer pairs, DNA-N-1F and DNA-N-1R, and DNA-N-2F and DNA-N-2R, designed according to published DNA-N conserved regions, to amplify the DNA-N. These primer pairs were able to amplify DNA-N of all tested severe strains but not the expected DNA-N fragments from BBTV TW3. For each TW3 DNA component, at least five clones were selected for sequencing from both directions. Except for DNA-U3, clones derived from each TW3 DNA component share high sequence identity (at least 93% sequence identity), and consensus sequences were determined from these clones. Two types of DNA-U3 (designated as DU3aTW3 and DU3bTW3) share only 78% sequence identity, and both were included in our phylogenetic analysis.

Detection of BBTV DNA-N by Southern blot hybridization. Because we were unable to amplify the DNA-N by PCR, we used Southern blot hybridization to determine whether the entire DNA-N coding sequence was missing or below detection levels in BBTV TW3. Similar amounts of DNA purified from healthy plants, plants infected with severe strains, and those with TW3 were separated on 1.2% agarose gels (Fig. 1A and C). We were able to detect DNA-S in both BBTV severe strains and in BBTV TW3 (Fig. 1B) but DNA-N only in the severe strains (Fig. 1D).

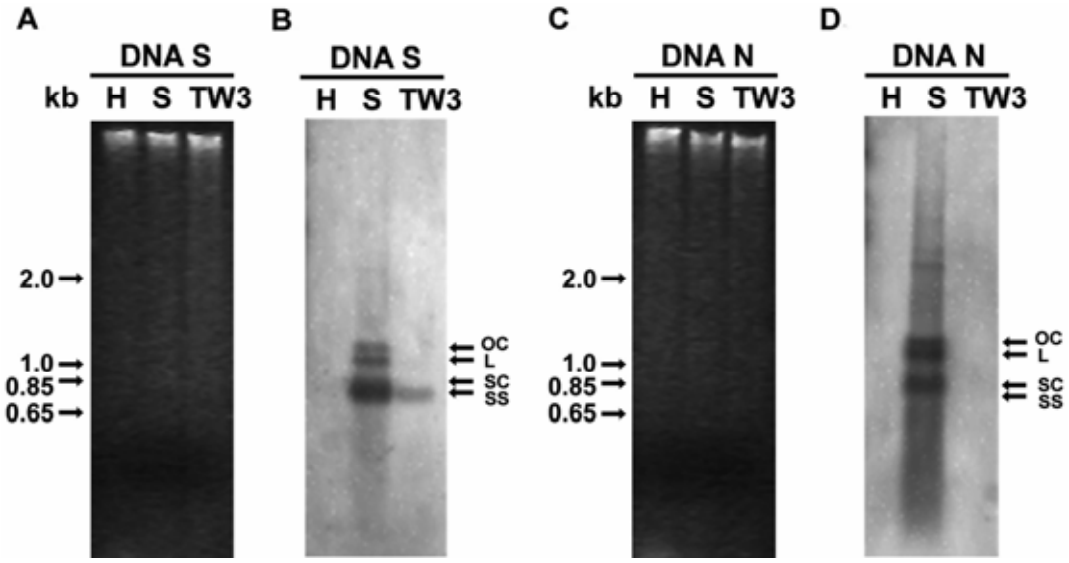

Fig. 1. Detection of DNA-S and DNA-N by Southern blot hybridization. **A and C,** DNA (22 µg) purified from healthy plants (H) and plants infected with severe strains (S) and TW3 were separated on 1.2% agarose gels. Southern blot hybridization performed with a digoxigenin-labeled probe corresponding to part of the open reading frame of *Banana bunchy top virus* (BBTV) DNA-S and DNA-N were used to hybridize **B,** (membrane directed from gel **A**) and **D,** (membrane directed from gel **C**). These probes were not cross-hybridized to other BBTV components (3). The molecular weight markers are indicated. Different forms of BBTV genomic DNA are indicated: open circle (OC), linear form (L), supercoiled (SC), and single-strand DNA (SS).

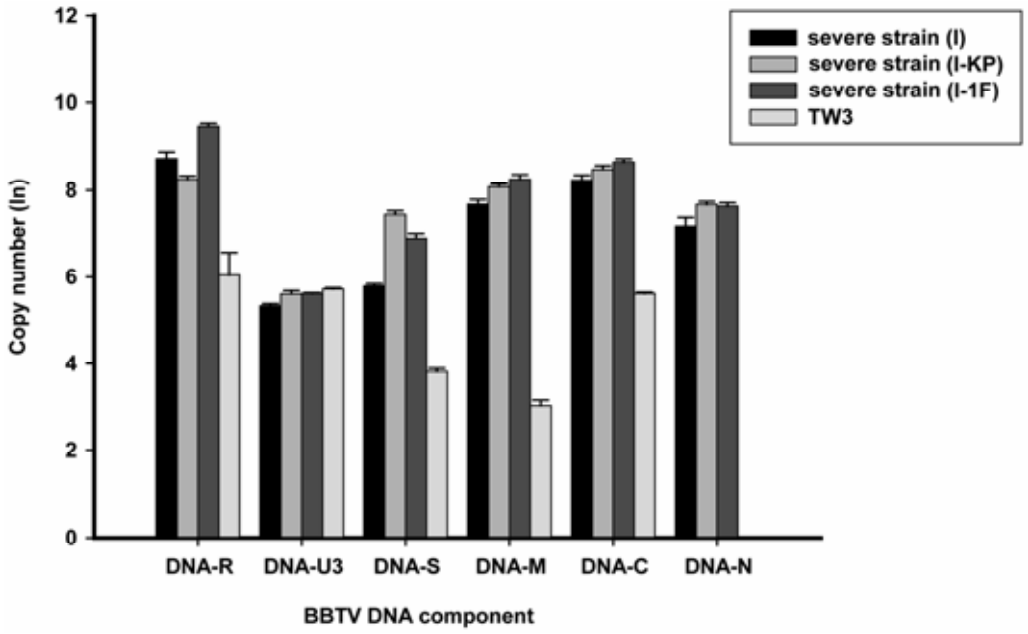

Fig. 2. Quantification of *Banana bunchy top virus* (BBTV) integral components by real-time polymerase chain reaction between severe strains I, I-KP, I-1F, and symptom-attenuating strain BBTV TW3. Banana 18S DNA was used as an internal control. The x-axis represents separate integral components and the y-axis represents copy number (in log scale).

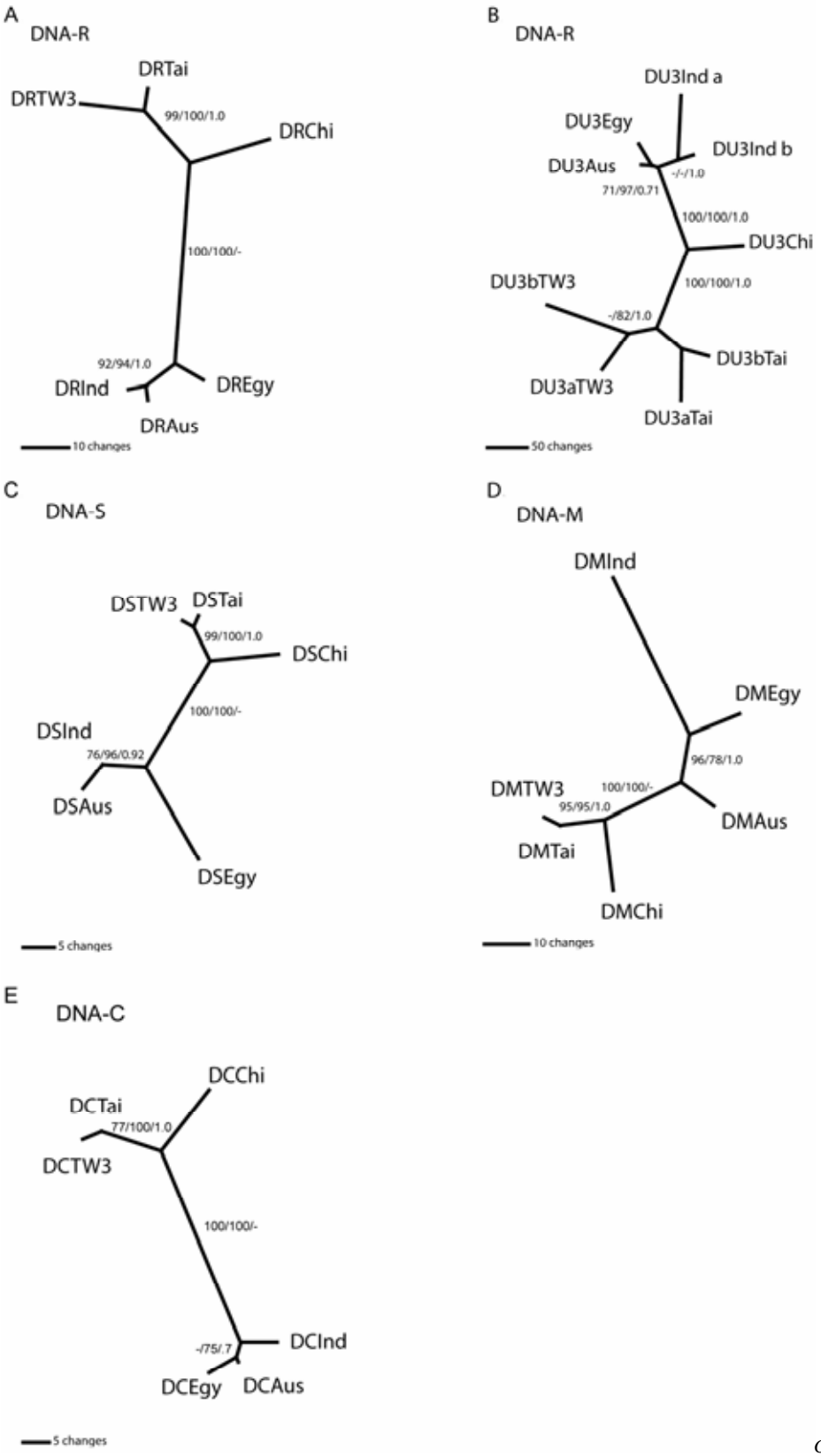

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Fig. 3. Phylogram of the maximum-parsimony tree based on open reading frame (ORF) nucleotide sequences (DNA-R, -S, -M, and -C) and the total nucleotide sequence (DNA-U3) from *Banana bunchy top virus* (BBTV) geographic isolates **A to E**. **F,** ORF sequences of replication-competent component (Rep)-encoded components of viruses within the *Nanoviridae* and circoviruses. Along the branches are the bootstrap supports of maximum-parsimony and neighbor-joining methods, followed by posterior probabilities from Bayesian inference; only values >50% are shown. For BBTV integral components, sequences were derived from isolates of all integral-component sequences available in GenBank. For additional Reps, the Rep ORF sequences of nanoviruses and circoviruses were derived from all available sequences in GenBank. BBTV TW3 DNA-R, -U3a,-U3b, -S, -M, and -C are designated DRTW3, DU3aTW3, DU3bTW3, DSTW3, DMTW3, and DCTW3, respectively. The ORF of additional Rep DNA-Y was not resolved by Yeh et al. (67). With some modifications (see Materials and methods), we resolved an ORF with 286 amino acids. The BBTV TW3-associated DNA-Y (DYTW3) and DNA-S2 (DS2TW3) are marked by open rectangles.

PCR quantification of BBTV genomic components among strains. To detect DNA-N from BBTV TW3 by a more sensitive method and to analyze whether the accumulation of BBTV components differs among strains, we used real-time PCR to quantify the copy number of each BBTV integral component from three randomly selected BBTV severe strains (I, I-KP, and I-1F) and BBTV TW3 (Fig. 2). The copy number of each integral component was similar among severe strains. However, except for the copy number of DNA-U3, that of each detected integral component of BBTV TW3 was at least two orders lower than that of the severe strains. In addition, we were still not able to detect DNA-N in BBTV TW3 (Fig. 2).

Phylogenetic analysis of BBTV TW3 DNA components. We produced an unrooted phylogram of each DNA component on the basis of nucleotide sequences of ORFs encoded in BBTV integral components (DNA-R, -S, -M, and -C), additional Reps, and full-length sequences of DNA-U3 because published records of DNA-U3 show no consistent ORF (25) (Fig. 3). All integral components of TW3 formed a sister group with the corresponding integral components of the Taiwanese BBTV severe strain (Fig. 3A to E), and the additional Reps associated with BBTV TW3 (DYTW3 and DS2TW3) were related to the additional Reps Y1 and S2, respectively (Fig. 3F).

CR-SL phylogenies and alignments showed that TW3 DNA-U3b and BBTV additional Rep DNAs were grouped together (Fig. 4). The relationship has high posterior probability (1.0) and bootstrap support (100). Actually, the CR-SL of DU3bTW3 is identical to that of DYTW3 (Fig. 5A). In addition, the CR-M of DYTW3 is nearly identical to that of DU3bTW3 (Fig. 5B), which suggests that recombination had occurred between DU3bTW3 and the additional Rep DYTW3.

Recombination analysis with use of Distance Plot (35) implemented in RDP2 (41) revealed the breaking points located at DU3bTW3 (EU366170) (nucleotides 1 and 223; the genome of BBTV is circular and we arbitrarily designated the breaking point as the first nucleotides of BBTV for better representation) (Fig. 6A and B) and the additional Rep DYTW3 (FJ389724) (nucleotides 941 and 1,098) (Fig. 6A and B).

PCR quantification of nonrecombinant and recombinant forms of BBTV TW3 DNA-U3. To analyze the ratio of the nonrecombinant form (DU3aTW3) to recombinant form (DU3bTW3) of TW3 DNA-U3, primer pairs specific to DU3aTW3 (DNA-U3-3F/DNA-U3-3R) and DU3bTW3 (DNA-U3-4F/DNA-U3-3R) (Fig. 6B) were designed for quantitative real-time PCR. The mean ratio of DU3aTW3 to DU3bTW3 derived from three repeated experiments (nucleic acids purified from TW3 at three separate times) was 0.98 ± 0.08.

Transmission analysis. To compare the transmission efficiency of BBTV severe strain and TW3, we conducted an insect transmission assay by use of aphids. No infection was observed on

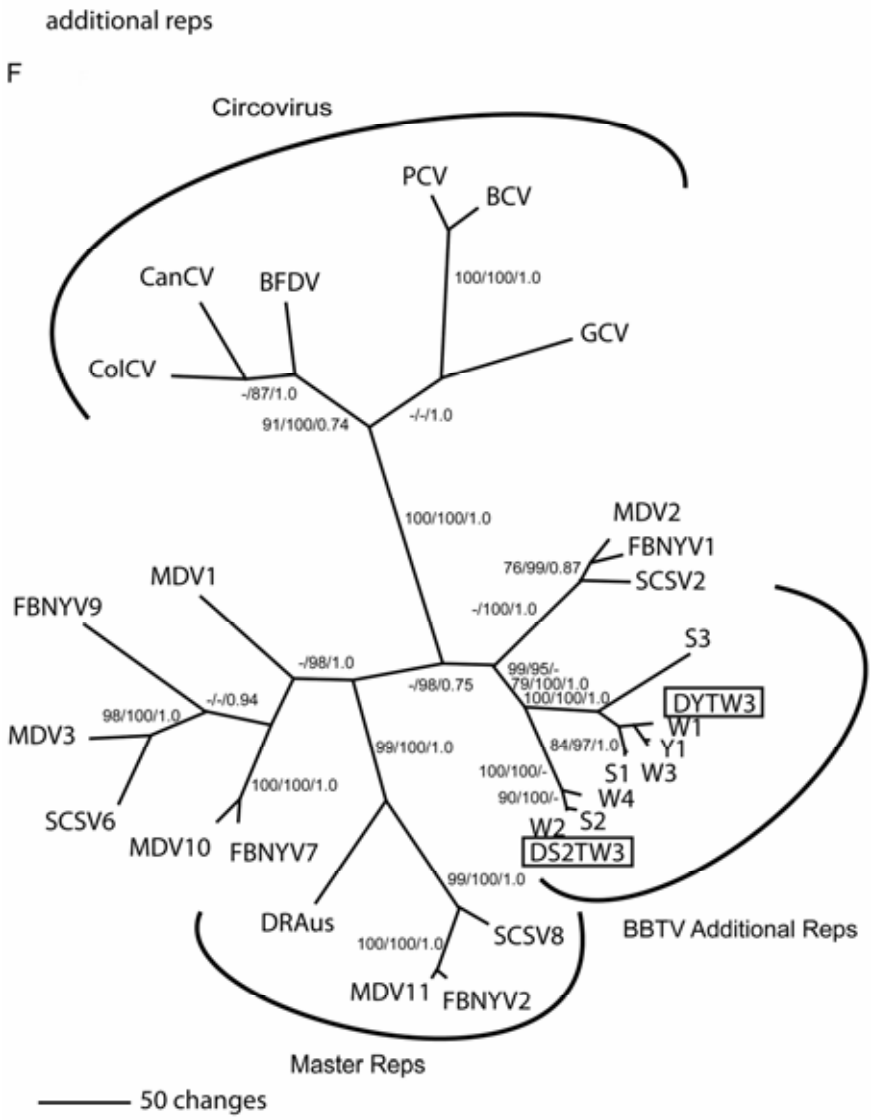

Fig. 3. (*Continued from previous page*)

plants inoculated with aphids first given acquisition access to the TW3-infected banana leaves (Table 1). By contrast, an average of 77% of banana plants were infected when plants were inoculated with aphids first given acquisition access to the BBTV severe-strain-infected banana leaves (Table 1).

DISCUSSION

The genome of nanoviruses is complicated and distinctive among viruses. Previously, we revealed recombination and reassortment in BBTV evolution (25). In the current study, we

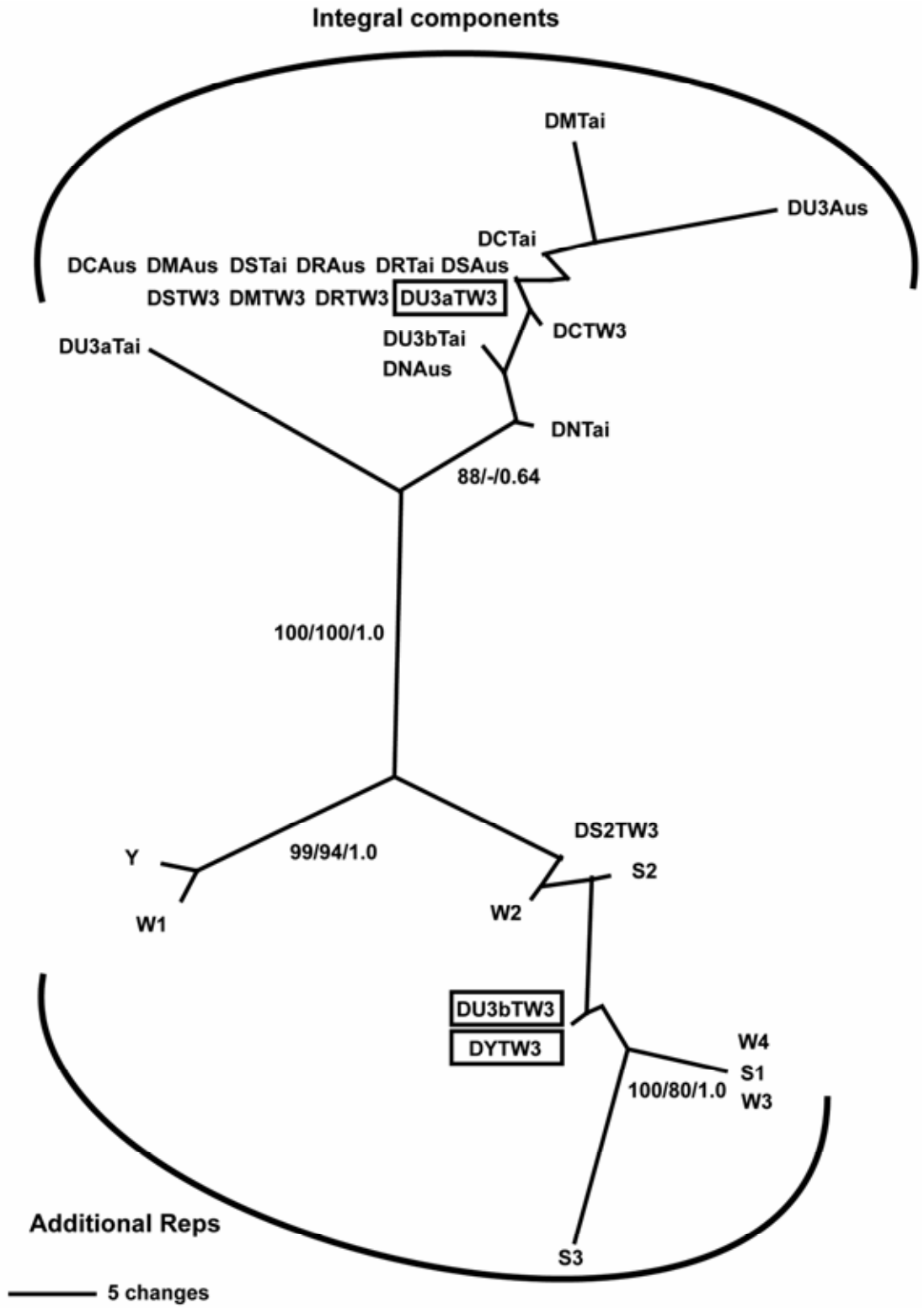

Fig. 4. Phylogram of the maximum-parsimony tree based on common region stem-loop nucleotide sequences. Along the branches are the bootstrap supports of maximum-parsimony and neighbor-joining methods, followed by posterior probabilities from Bayesian inference; only values >50% are shown. For *Banana bunchy top virus* (BBTV) integral components, sequences were derived from isolates with all integral-component sequences available in GenBank. For BBTV additional replication-competent component DNAs, all but the sequences of BBTV TW3 determined in this study were derived from GenBank. The BBTV TW3-associated DNA-Y (DYTW3) and integral-component DNA-U3 (DU3aTW3 and DU3bTW3) are marked by open rectangles.

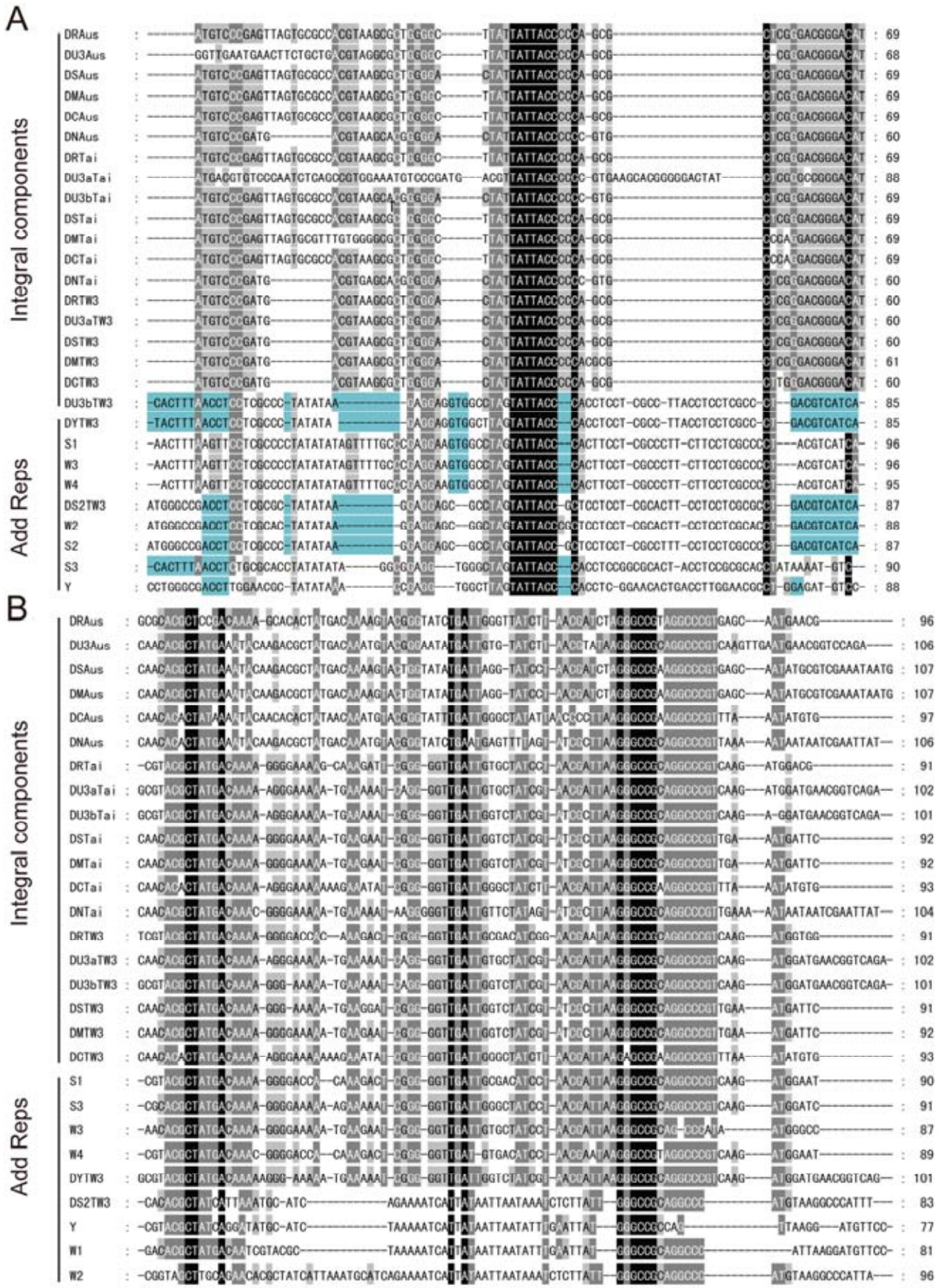

Fig. 5. Sequence alignment of **A,** stem-loop common region and **B,** major common region of *Banana bunchy top virus* (BBTV) integral components of the Australian (Aus); Taiwanese severe strains (Tai); BBTV TW3; previously published additional replication-competent component (Rep) DNAs-S1, -S2, -S3, -W1, -W2, -W3, and DNA-W4; and additional Rep DNAs-Y (DYTW3) and DNA-S2 (DS2TW3) associated with TW3. Identical and conserved sequences within all aligned sequences are in black and gray shadow; some identical sequences found between particular isolates are in blue shadow.

identified some unusual genomic features, including a missing integral component, and recombination between an integral genomic DNA and an additional Rep DNA in BBTV.

Several sensitive methods were applied to detect BBTV components; however; unexpectedly, we were not able to detect BBTV TW3 DNA-N. Although the copy number of each genomic component in BBTV TW3 is much lower than that of severe strains (Fig. 2), we were able to detect DNA-S in BBTV TW3 by Southern blot hybridization (Fig. 1) even though the copy number of DNA-S is lower than that of DNA-N in all detected severe isolates (Fig. 2). DNA-N either did not associate with BBTV TW3 or the copy number of DNA-N in BBTV TW3 is below the detection ability of current approaches.

The proposed function of BBTV DNA-N encoded protein (64) is similar to that of nuclear shuttle protein (NSP) encoded in DNA B of bipartite begomoviruses, suggested to be involved in virus movement (32,45,47). Recently, the NSP of *Tomato leaf curl New Delhi virus* was found to play roles as a pathogenicity determinant and a target of host defense responses (27). Similarly, an NSP encoded by *Tomato golden mosaic virus* was found to bind a leucine-rich repeat receptor-like kinase, and the binding was

TABLE 1. *Banana bunchy top virus–Pentalonia nigronervosa* transmission analysis[a]

Experiment	Healthy	Severe strain	TW3
1	0/10	9/10	0/12
2	0/10	8/10	0/10
3	0/10	6/10	0/20

[a] Indicates sources of virus acquisition; number of infected plants/total number inoculated.

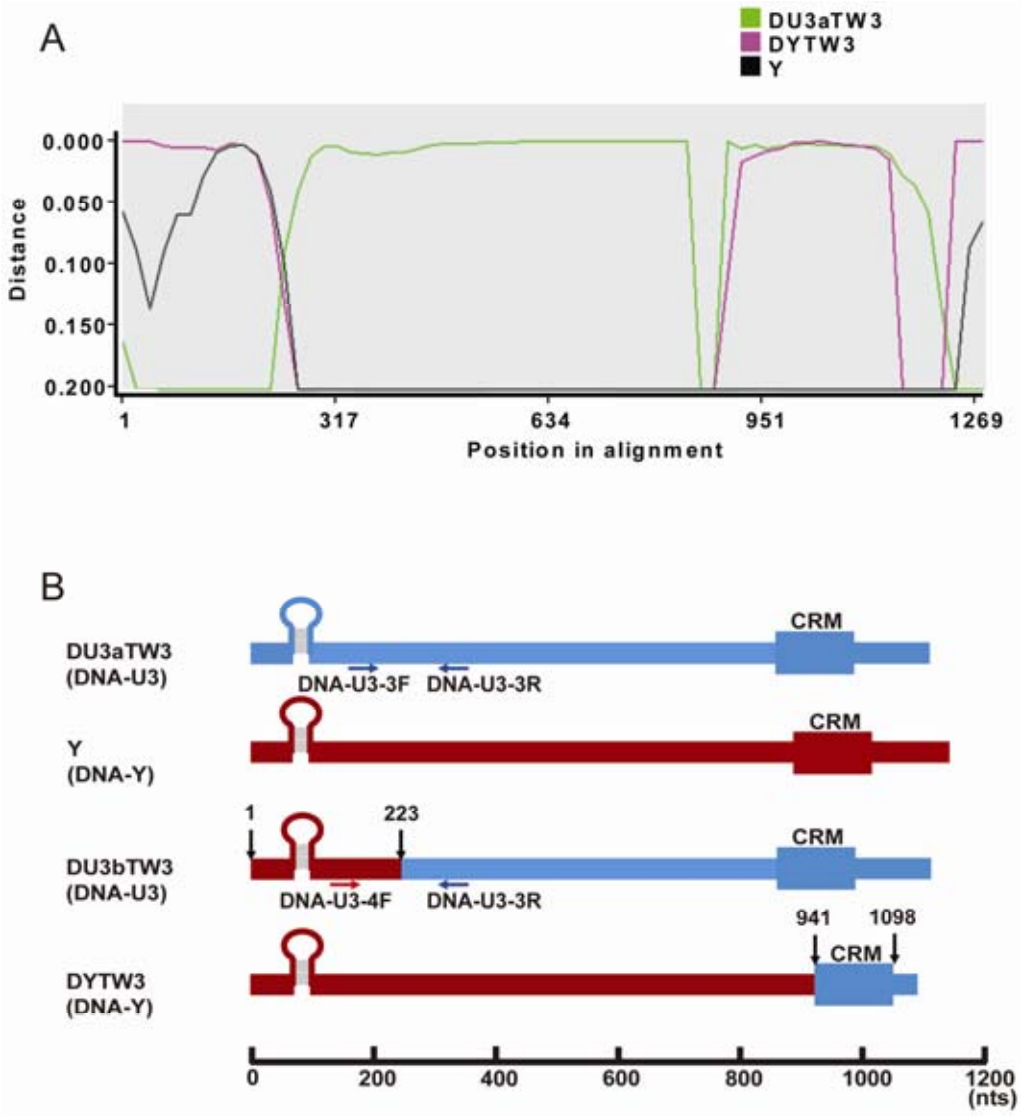

Fig. 6. Recombination analysis of *Banana bunchy top virus* (BBTV) TW3 DNA-U3 and additional replication-competent component (Rep) DYTW3. **A,** Recombination analysis involved use of Distance Plot (Simplot) implemented in RDP2 (41). The BBTV TW3 DNA-U3, DU3bTW3, was selected as the potential recombinant sequence, and another BBTV TW3 DNA-U3 (DU3aTW3; green; FJ773283; DU3aTW3 is more similar to reported DNA-U3 of Taiwanese common severe strain), BBTV TW3 additional Rep DNA-Y (DYTW3) (pink; FJ389724), and the common additional Rep DNA-Y (black; U02312) were selected as parental sequences. Window size and step size were set at 100 and 20, respectively. The x-axis and y-axis represent the sequence location and genetic distance, respectively. The putative recombination sites are located at the cross-site. **B,** Schematic representation of potential recombination between BBTV TW3 DNA-U3 and additional Rep DNA-Y. The genomes of TW3 DNA-U3a (DU3aTW3; FJ773283) and additional Rep DNA-Y (Y; U02312) are indicated by blue and red lines, respectively. The mosaic blue and red lines in DU3bTW3 and DYTW3 indicate their putative origins. The junction between blue and red lines in DU3bTW3 and DYTW3 indicate the putative recombination breaking points. Primer pairs specific to DU3aTW3 (DNA-U3-3F/DNA-U3-3R) and DU3bTW3 (DNA-U3-4F/DNA-U3-3R) used for real-time polymerase chain reaction quantification are indicated. Scale bar, in nucleotides, is at the bottom.

suggested to prevent the induction of a resistance response (14, 40). In addition, an NSP of *Cabbage leaf curl virus* was found to interact with nuclear acetyltransferase of *Arabidopsis thaliana*, and the interaction is involved in *Cabbage leaf curl virus* infection and pathogenesis (10,11,42). However, whether the NSPs encoded by nanoviruses play similar functions as in geminiviruses has yet to be determined.

Interesting examples for the lack of requirement of NSP to complete the viral infection cycle exist within the genus *Begomovirus* in the family *Geminiviridae*. Most begomoviruses have a bipartite genome (DNA-A and DNA-B). DNA-A encodes replication-association proteins and DNA-B encodes proteins for movement (including NSP). However, *Tomato yellow leaf curl virus*, *Tomato leaf curl Sardinia virus*, and *Tomato leaf curl virus* typically contain only one DNA (such as DNA-A of the bipartite geminiviruses), and introducing the cloned DNA into tobacco or tomato by agroinoculation induced systemic symptoms on inoculated plants (13,30,44). Furthermore, the progeny virus can be transmitted between plants by whitefly vectors to produce typical symptoms (30,44). Therefore, some *Begomovirus* isolates can overcome the need for DNA-B to complete the life cycle of viruses. Recently, whole-plant infection by cloned nanovirus DNA was established in FBNYV (59). Unexpectedly, even without including DNA-N, the inoculated plants still showed symptoms similar to that of plants inoculated with all eight cloned DNA components, including DNA-N (59). However, aphids cannot transmit viruses from plants inoculated with all eight cloned DNA components (59).

In contrast to the previously published data indicating that the original collected BBTV TW3 were transmissible by aphids (55), we were unable to transmit BBTV from TW3-infected plants to healthy banana plants by aphids (Table 1). Prolonged vegetative propagation in the absence of the vector may lead to viruses losing their insect transmission ability (33,62). Because the BBTV TW3 isolate has been maintained for more than 10 years by vegetative propagation, the loss of insect transmission ability may be due to prolonged vegetative propagation. However, we have recently collected another symptomless BBTV isolate from the field; we were also unable to transmit BBTV from the symptomless BBTV-infected plants to healthy banana plants by aphids (*unpublished data*), possibly because BBTV-infected banana plants with severe symptoms usually die (including all suckers) within 1 to 2 years, when the stored nutrients are completely consumed. Therefore, if the BBTV is not transmitted to new hosts, the virus will eventually disappear in nature. BBTV mild-strain-infected plants, however, showed mild symptoms on banana leaves, which allowed for suitable photosynthesis to take place in the plants and allowed the plants to survive in the field and to be propagated vegetatively for a long time. Interestingly, we could not detect DNA-N from the newly collected isolate as well (*unpublished data*), which suggests that a loss of the DNA-N also occurred in nature. Unfortunately, the DNA of the original BBTV TW3-infected plants was not stored, and we cannot analyze whether the original BBTV TW3 contained any DNA-N. Whether the DNA-N plays roles in BBTV insect transmission has yet to be determined.

Previously, we showed a concerted evolution in CR-M but not CR-SL among the Pacific BBTVs (25). Although the CR-SL regions of BBTV are very similar, the CR-SLs of DNA-U3 and DNA-N are easily differentiated from other DNA components (Fig. 5A). Of note, all the CR-SLs of TW3 DNA components are identical to that of the DNA-N from other isolates (Fig. 5A). Therefore, DNA-N probably existed, and recombination occurred between the integral components DNA-R, -U3, -S, -M, -C, and -N. However, at some point, DNA-N was either lost from BBTV or still existed but at extremely low levels.

We also observed some small stretches of identical sequences between DU3bTW3/DYTW3 and additional Rep S1/W3/W4, DU3bTW3/DYTW3 and DS2TW3S2/W2, and DU3bTW3/DYTW3 and S3 (Fig. 5A). Recombination may have occurred between these groups during the evolution of BBTV.

Unexpectedly, our analysis also revealed recombination between TW3 DNA-U3 and additional Rep DNA-Y (Fig. 6A and B). The biological significance of the nanovirus additional Reps associated with viruses within the family *Geminiviridae* has not been well resolved (4,7,38). In nanoviruses, the additional Reps were demonstrated to reduce the rate of infection in plants with use of cloned DNAs of FBNYV as an inoculum (59). Although, currently, the biological significance of BBTV additional Reps in terms of symptoms and longevity of BBTV-infected plants have yet to be analyzed (4), our data suggest that the additional Rep DNAs can serve as sources of additional genetic diversity for the integral BBTV components.

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