

Genetic Complexity of a Beet Curly Top Virus Population Used to Assess Sugar Beet Cultivar Response to Infection

Drake C. Stenger and Kristin M. Ostrow

Department of Biological Sciences and Plant Molecular Biology Center, Northern Illinois University, DeKalb 60115.

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ABSTRACT

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Four beet curly top virus (BCTV) isolates obtained from two sugar beet evaluation nurseries in Kimberly, ID, were characterized by restriction endonuclease profiles of double-stranded forms of BCTV DNA present in total DNA extracts. The results suggested that each isolate contained a mixture of at least two distinct BCTV genotypes, and analysis of full-length DNA clones verified that each nursery isolate contained at least two BCTV strains. Full-length DNA clones resembling the previously characterized Calif/Logan and CFH strains of BCTV were recovered from each of the nursery isolates, and a single clone resembling the

Worland strain of BCTV also was recovered from one of the nursery isolates. Detailed restriction endonuclease mapping of cloned BCTV genomes recovered from the nursery isolates revealed minor genotypic variability among cloned genomes of the same strain, and partial nucleotide sequencing of the origin of DNA replication confirmed strain identities assigned on the basis of restriction endonuclease maps. The complete nucleotide sequence (2,930 nt, GenBank accession U56975) of the Worland strain of BCTV was determined and verified as a third distinct strain with an overall nucleotide sequence identity of 79.0 to 80.2% relative to the Calif/Logan and CFH strains of BCTV.

Additional keyword: geminivirus.

Beet curly top virus (BCTV) is a monopartite, leafhopper-transmitted geminivirus with an unusually broad host range among dicots. BCTV exists in nature as distinct strains that have been characterized at the molecular level during the past 10 years. Stanley et al. (24) determined the complete nucleotide sequence of a cloned BCTV genome (pBCT028) recovered from the California isolate of BCTV. Subsequently, full-length clones (pLOGAN, pCFH, and pWORLAND) derived from three additional laboratory-maintained isolates of BCTV (Logan, CFH, and Worland) have been characterized for both genotypic and phenotypic properties (28). Infectivity assays and restriction endonuclease mapping indicated that the cloned Logan and California genomes may be considered as minor genotypic variants of the same strain (designated here as Calif/Logan). The cloned Logan genome is also very similar to the cloned California genome in nucleotide sequence (S. G. Hormuzdi and D. M. Bisaro, *unpublished data* [referenced in 14]).

In contrast, nucleotide sequence comparisons (25) indicated that the cloned viral insert of pCFH represents a second strain of BCTV that is distinct from the Calif/Logan strain. The CFH strain has since been demonstrated to be different from the Calif/Logan strain in virulence in the experimental host *Nicotiana benthamiana* (29), genetic interactions with *Arabidopsis thaliana* resistance genes (18), and determinants governing replication specificity (2,26). Although the origin of the isolate from which the CFH genome was cloned is unknown (28), a recent study (27) determined that minor genotypic variants of the CFH strain were present in BCTV-infected su-

gar beet (*Beta vulgaris*) samples collected from the Texas Panhandle during 1994.

The cloned viral insert of pWORLAND represents a third strain of BCTV that is noticeably less virulent on sugar beet relative to the Calif/Logan and CFH strains, although the Worland strain does retain the typical wide host range of BCTV and is capable of inducing severe disease symptoms on other hosts under experimental conditions (28). Restriction endonuclease mapping (28) and the inability to mobilize and amplify a Logan-derived defective-interfering (DI) DNA molecule present as a tandem repeat integrated into a chromosome of transgenic *N. benthamiana* (26) further suggest that the Worland strain has replication specificity determinants incompatible with that of the Calif/Logan strain (2).

BCTV is widespread throughout the western United States and continues to cause losses to the sugar beet industry despite continuous efforts to select cultivars with improved tolerance or partial resistance to BCTV since the 1920s (1). The sugar beet industry maintains two nursery facilities in Idaho dedicated to the assessment of cultivar response to BCTV infection. A resident population of BCTV is husbanded by the two nursery facilities, in which infected plants selected from field plots are collected at the end of each season, stored during the winter as bare taproots, and subsequently used as sources of inocula for the next field season. However, the BCTV population used by the industry for this purpose has not been characterized with respect to strain composition or genetic complexity. In this report, we characterize the BCTV genotypes present in isolates sampled from the BCTV population used by the industry to evaluate sugar beet cultivars. We also report the complete nucleotide sequence of the cloned viral insert of pWORLAND and verify that BCTV-Worland represents a third distinct strain of BCTV.

Corresponding author: Drake C. Stenger; E-mail address: dstenger@niu.edu

MATERIALS AND METHODS

Sources of cloned genomes and isolates of BCTV. Full-length, infectious DNA clones of the Calif/Logan, CFH, and Worland strains of BCTV have been described previously (24,28). BCTV genomes representing minor variants (Texas I to VI) of the CFH strain were cloned from isolates collected from a field population of BCTV in the Texas Panhandle (27). The four nursery isolates used in this study were provided by J. R. Stander (Betaseed, Kimberly, ID). Two of the nursery isolates (BS94-1 and BS94-3) were recovered from the Betaseed facility in Kimberly. The remaining two nursery isolates (BSDF94-1 and BSDF94-6) were obtained from the Beet Sugar Development Foundation (BSDF) facility, located approximately 4 miles from the Betaseed location. Each of the nursery isolates was collected as an individual sugar beet plant (no cultivar designation reported) from field plots at the end of the 1994 field season and stored for 6 months as bare taproots at 4°C. The stored taproots were placed in pots in the greenhouse and allowed to develop new leaves that exhibited vein swelling, leaf curling, and enation symptoms typical of BCTV infection. Five-gram leaf samples were collected for each isolate and processed for total DNA as described previously (27).

Characterization and cloning of BCTV nursery isolates. Total DNA (5 µg) extracted from each nursery isolate was incubated with *EcoRI*, *SalI*, or *BamHI*. Restriction endonuclease-digested or native DNA was assayed for the presence of double-stranded (ds) viral DNA forms in a Southern blot with a full-length riboprobe of virion-sense transcribed with T7 RNA polymerase with pCT9 as a template. pCT9 contains a genome-length *SalI* insert derived from pLOGAN (30), and riboprobes transcribed from pCT9 are capable of detecting DNA of all three BCTV strains (26).

Aliquots (50 µg) of total DNA extracted from each of the four nursery isolates were incubated with *EcoRI* or *SalI*. Linearized, genome-length BCTV dsDNA was size fractionated by agarose gel electrophoresis and recovered by the Gene Clean II system (BIO 101, La Jolla, CA). Gel-purified BCTV dsDNA was cloned into the plasmid vector pUC8, previously digested with the appropriate enzyme, by the cloning strategy described previously (27). Clones were selected for the presence of BCTV DNA inserts by colony hybridization (19) and verified by Southern hybridization of excised inserts with a riboprobe transcribed from pCT9. Each full-length

BCTV DNA clone identified was characterized by restriction endonuclease mapping with the following enzymes: *ApaI*, *BamHI*, *BstXI*, *Csp45I*, *EcoRI*, *KpnI*, *PvuII*, *SacI*, *SalI*, *ScaI*, *SnaBI*, *SpeI*, *XbaI*, and *XhoI*.

DNA sequencing. The complete nucleotide sequence for both strands of the cloned Worland genome was determined with pWORLAND (28) as a template in a primer-walking strategy with Sequenase 2.0 (Amersham/U.S. Biochemicals, Cleveland). Partial nucleotide sequences of the *ori* region of cloned genomes derived from the nursery isolates were obtained with Sequenase 2.0 and synthetic oligonucleotide primers based on the sequences of pLOGAN, pCFH, or pWORLAND. Nucleotide sequences were aligned by the CLUSTAL V program (13), with multiple alignment parameters kept at default values.

RESULTS

Characterization of the BCTV-Worland nucleotide sequence. The complete nucleotide sequence of the viral insert of pWORLAND was 2,930 nt and was deposited as GenBank accession U56975. The physical map of the BCTV-Worland genome (Fig. 1) depicts the results of a conceptual translation of the Worland DNA sequence in which seven open reading frames (ORFs) are present. The presence of four complementary-sense (C1, C2, C3, and C4) and three virion-sense (V1, V2, and V3) ORFs on the Worland genome (Fig. 1) is conserved among the two other BCTV strains for which sequence data are available and demonstrates that BCTV-Worland has a genome organization more similar to other strains of BCTV than to other geminiviruses.

A comparison of nucleotide sequence identities (Table 1) based on a multiple alignment of the sequences of four BCTV genomes representing three BCTV strains, the subgroup I geminivirus tobacco yellow dwarf virus (TYDV; [20]), and the distinct subgroup II geminivirus horseradish curly top virus (HrCTV; [16]) indicated that BCTV-Worland is a distinct strain of BCTV that shares an overall nucleotide identity of 79.0 to 80.2% with the Calif/Logan and CFH strains of BCTV. The percent nucleotide identity between the CFH and Calif/Logan strains was 82.3 to 82.8%, whereas two genomes of the same strain (California and Logan) were most similar to one another (96.3% nucleotide identity). All of the BCTV strains examined were more similar to one another than to HrCTV (56.5 to 60.0%) or TYDV (37.2 to 39.6%).

Genotypic characterization of the BCTV nursery isolates. Each of the nursery isolates of BCTV contained genome-length dsDNA replicative forms of viral DNA (Fig. 2). Also present were virus-specific dsDNA forms of high molecular weight that likely represent concatameric molecules, because these molecules did not produce larger than unit-length products resulting from digestion with *EcoRI* (Fig. 2). Smaller than genome-length dsDNA molecules also were associated with one nursery isolate (BSDF94-1). Similar molecules have been characterized as DI DNAs produced on first passage with cloned BCTV inocula (7,8,26,30) and also

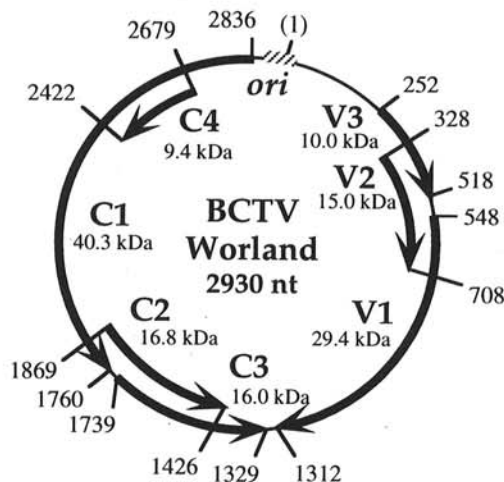


Fig. 1. Physical map of the beet curly top virus (BCTV)-Worland genome deduced from the complete nucleotide sequence deposited as GenBank accession U56975. Solid arrows denote the location and polarity of open reading frames (ORFs) conserved among all BCTV isolates characterized to date. Stippled arc denotes the location of the DNA origin of replication (*ori*). Nucleotide coordinates (virion-sense) of ORF start and stop sites are indicated; position of nucleotide 1 (parentheses) was defined as the A residue immediately 3' of the rep protein nick site within the *ori*.

TABLE 1. Percent nucleotide sequence identity among monopartite, leafhopper-transmitted geminiviruses infecting dicots^a

	TYDV ^b	BCTV ^c			
		California	Logan	CFH	Worland
California	38.3				
Logan	39.6	96.3			
CFH	39.4	82.8	82.3		
Worland	37.2	79.0	79.0	80.2	
HrCTV ^d	35.5	59.8	60.0	57.3	56.5

^a Based on a multiple alignment with a total of 3,288 characters examined in each two-way comparison. Gaps were treated as characters. If common gaps were ignored, percent identity values were reduced by less than 2%.

^b TYDV = tobacco yellow dwarf virus.

^c BCTV = beet curly top virus.

^d HrCTV = horseradish curly top virus.

have been observed in extracts derived from field-collected isolates of BCTV-infected sugar beets (27).

Restriction endonuclease digestion of BCTV dsDNA forms present in the four nursery isolate samples yielded complex patterns (Fig. 2). When incubated with *SalI* or *BamHI*, a portion of the unit-length viral dsDNA was converted to a full-length linear form, whereas some of the viral dsDNA retained the mobility of native open circular or supercoiled dsDNA. In contrast, *EcoRI* digestion resulted in three digestion products of 3.0 (full length), 2.3, and 0.8 kbp. In some samples, low amounts of native supercoiled and open circular DNA forms also were present after incubation with *EcoRI*. Collectively, these results suggested that each isolate contained a mixture of at least two genotypes. However, because these results also could be explained by incomplete digestion of a single genotype, the genetic complexity of each nursery isolate was examined further by analyzing the genotypic properties of cloned genomes recovered from each of the nursery isolates.

Full-length DNA clones recovered from each of the nursery isolates were identified and characterized by detailed restriction endonuclease mapping of cloned viral DNA inserts (Fig. 3). Based on an alignment of restriction endonuclease maps developed for each cloned genome, it was determined that each isolate contained minor genotypic variants of both the CFH and Calif/Logan strains (Table 2; Fig. 3). In addition, a single clone representing a minor genotypic variant of the Worland strain also was recovered from one of the nursery isolates (BS94-3) (Table 2; Fig. 3). These results verified that each isolate contained a mixture of BCTV genotypes with one or no *SalI* sites, one or no *BamHI* sites, and one or two *EcoRI* sites. Thus, the restriction endonuclease patterns obtained in Figure 2 can be explained best as representing a mixture of BCTV strains in each isolate, rather than incomplete digestion with each of the restriction endonucleases tested.

Strain identities as determined by restriction endonuclease mapping (Fig. 3) and reported in Table 2 were confirmed by partial nucleotide sequencing of the *ori* region for selected clones (Fig. 4). The nucleotide sequence of the *ori* previously has been demonstrated to be different among strains of BCTV (26), and a multiple alignment of the *ori* region sequences indicated that individual

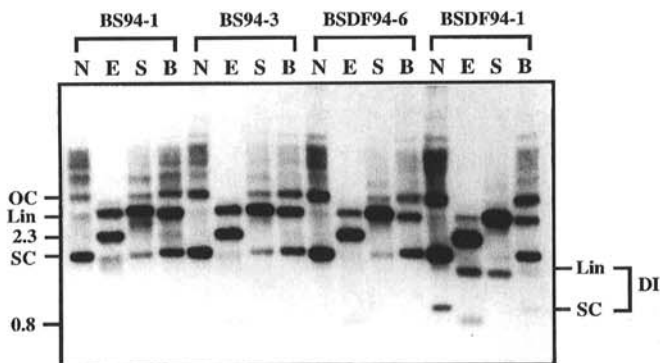


Fig. 2. Restriction endonuclease profiles of viral double-stranded (ds) DNA present in nursery isolates of beet curly top virus (BCTV). Presented is an autoradiograph of a Southern blot of total DNA (5 µg) extracted from four BCTV isolates (BS94-1, BS94-3, BSDF94-6, and BSDF94-1) obtained from two screening nurseries in Idaho. Total DNA samples were electrophoresed as native (N) viral DNA forms or after incubation with *EcoRI* (E), *SalI* (S), or *BamHI* (B). The mobilities of open circle (OC), linear (Lin), and supercoiled (SC) forms of full-length genomic BCTV dsDNA forms are indicated to the left. Virus-specific dsDNA forms with mobilities slower than genome-length open circle DNA likely represent concatameric molecules. The mobilities of linear and supercoiled DNA forms of smaller than genome-length DI-like (defective-interfering) molecules associated with sample BSDF94-1 are indicated to the right. Full-length linear DNA molecules resulting from digestion with restriction endonucleases have the same mobility as linear molecules occurring in low abundance in native samples. The mobilities of digestion products (2.3 and 0.8 kbp) derived from genomes containing two *EcoRI* sites are indicated to the left. A full-length virion-sense riboprobe transcribed from pCT9 was used as a probe.

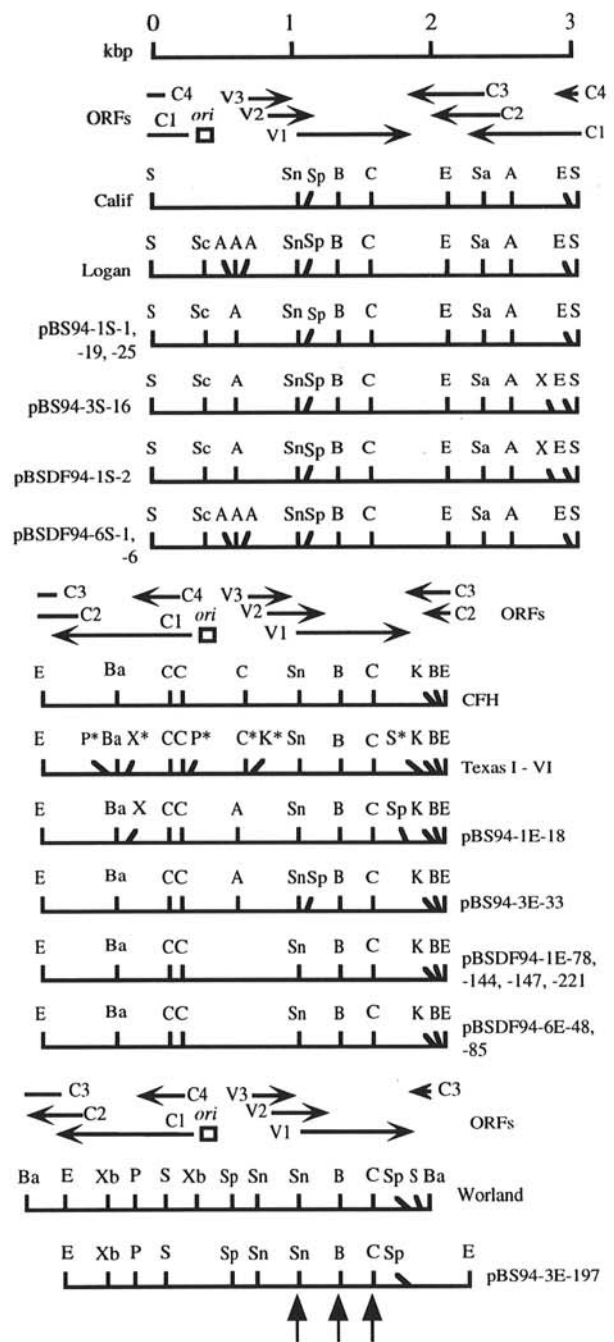


Fig. 3. Genotypic variability among cloned beet curly top virus (BCTV) genomes. Presented is an alignment of restriction endonuclease maps of cloned, full-length BCTV DNA inserts. The restriction endonuclease profiles of cloned DNA derived from laboratory-maintained isolates of BCTV (California, Logan, CFH, and Worland), previously characterized minor variants of the CFH strain derived from the Texas Panhandle (Texas I to VI), and genomes recovered from four Idaho nursery isolates (BS94-1, BS94-3, BSDF94-1, and BSDF94-6), as listed in Table 2, are compared. Terminal restriction endonuclease sites represent the cloning site used. Horizontal arrows denote location and polarity of open reading frames (ORFs) conserved among the four sequenced genomes of BCTV. The DNA origin of replication (*ori*) is denoted as a box. Vertical arrows indicate the location of three restriction endonuclease cleavage sites conserved among all cloned genomes displayed. Asterisks denote restriction endonuclease sites that exhibit polymorphism among the Texas I to VI genomes. Abbreviations used for restriction endonuclease sites are: A = *ApaI*; Ba = *BamHI*; B = *BstXI*; C = *Csp45I*; E = *EcoRI*; K = *KpnI*; P = *PvuII*; Sa = *SacI*; S = *SalI*; Sc = *Scal*; Sn = *SnaBI*; Sp = *SpeI*; Xb = *XbaI*; and X = *XhoI*. Although the Worland DNA sequence indicates that a second *SalI* site occurs within 20 nt of the *SalI* site depicted within the C1 ORF, only a single site is shown, and it was not determined whether pBS94-3E-197 contains both sites in this region.

TABLE 2. Strain composition of isolates of beet curly top virus obtained from Idaho screening nurseries and summary of cloning experiments

Isolate	Clone	Cloning site	Strain ^a
BS94-1	pBS94-1S-1	<i>SalI</i>	Calif/Logan
	pBS94-1S-19	<i>SalI</i>	Calif/Logan
	pBS94-1S-25	<i>SalI</i>	Calif/Logan
	pBS94-1E-18	<i>EcoRI</i>	CFH
BS94-3	pBS94-3S-16	<i>SalI</i>	Calif/Logan
	pBS94-3E-33	<i>EcoRI</i>	CFH
	pBS94-3E-197	<i>EcoRI</i>	Worland
BSDF94-1	pBSDF94-1S-2	<i>SalI</i>	Calif/Logan
	pBSDF94-1E-78	<i>EcoRI</i>	CFH
	pBSDF94-1E-144	<i>EcoRI</i>	CFH
	pBSDF94-1E-147	<i>EcoRI</i>	CFH
	pBSDF94-1E-221	<i>EcoRI</i>	CFH
BSDF94-6	pBSDF94-6S-1	<i>SalI</i>	Calif/Logan
	pBSDF94-6S-6	<i>SalI</i>	Calif/Logan
	pBSDF94-6E-48	<i>EcoRI</i>	CFH
	pBSDF94-6E-85	<i>EcoRI</i>	CFH

^a Strain designation determined by endonuclease restriction mapping as presented in Figure 3.

genomes recovered from the nursery isolates were highly similar in sequence to the strain they most resembled by restriction mapping. Few nucleotide substitutions occurred within minor variants of a single strain (Fig. 4), and most substitutions were in regions of the *ori* not implicated as *cis*-acting elements of strain-specific replication (2). The one exception to this was clone pBSDF94-1S-2, in which the nucleotides located between the direct repeats of the rep protein binding site (4-6) were different from those of the California and Logan genomes (Fig. 4).

DISCUSSION

The genotypic characterization of BCTV isolates sampled from the viral population used by the sugar beet industry to evaluate cultivar response to infection indicates that the evaluation program includes exposure of cultivars tested to all three currently recognized strains of BCTV. Because each of the isolates contained a mixture of the CFH and Calif/Logan strains, it appears that most, if not all, of the plants tested would be infected by both of these severe strains. Restriction endonuclease profiles examined for nine additional isolates derived from the Betaseed and BSDF nurseries (data not shown) were similar to those reported in Figure 2, further suggesting that cultivar screening at these facilities involves simultaneous exposure to more than one strain of BCTV. Although the Worland strain was recovered from only one of the four nursery isolates examined in cloning experiments, it is probable that genotypic variants of the Worland strain are present at both locations, because of the close proximity of the two nurseries and because the evaluations are conducted as open-air field plots with no restrictions placed on leafhopper vector dispersal. Furthermore, because the Worland strain does not cause severe disease symptoms on most sugar beet cultivars, the potential under representation of this strain may not have a major impact on the cultivar evaluation program as it is currently implemented.

The older literature on BCTV, as reviewed by Bennett (1), suggests that mixed infections with phenotypically distinct strains of BCTV are common and that the cross-protection phenomena seen with strains of plant RNA viruses do not occur with BCTV. However, these historical accounts of mixed infections do not address genotypic characterization of the BCTV strains involved, because at that time the etiology of curly top disease was undefined. Although genotypic heterogeneity among cloned genomes of BCTV recovered from a single isolate has been observed for the California isolate of BCTV (24), strain relationships for these variants were not reported. Also, minor genotypic variants of the same BCTV strain have been recovered from individual field-collected

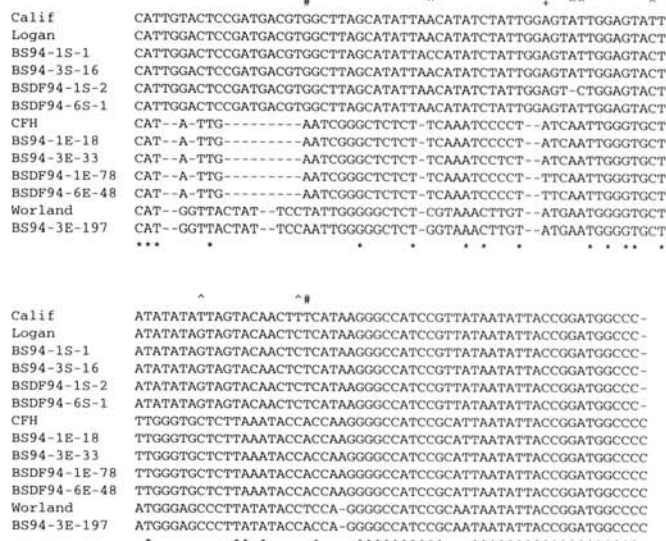


Fig. 4. Comparison of nucleotide sequences of the *ori* region of cloned beet curly top virus (BCTV) genomes. Presented is a multiple alignment of *ori* region virion-sense sequences of previously characterized cloned genomes of BCTV (California, Logan, CFH, and Worland) and cloned genomes recovered from the four nursery isolates of BCTV as described in Table 2. For each sequence aligned, the first three 5' nucleotides (CAT) represent the initiation anticodon of the rep protein complementary-sense open reading frame (ORF) C1. The 3' nucleotide represents the terminal nucleotide of the 3'-proximal inverted repeat of the *ori* stem-loop element. Asterisks below each block of sequences denote positions of nucleotides conserved among all sequences compared. Symbols placed above each block of sequences denote positions in which nucleotide substitutions occurred within a single strain (# = Worland; + = CFH; and ^ = Calif/Logan).

isolates of BCTV (27). However, our results, for the first time, clearly establish that different strains of BCTV may coexist in the same plant and that mixed infections with as many as three BCTV strains may occur. These results indicate that both breeding programs and field studies on BCTV must consider the potential consequences of mixed infections by more than one strain.

Sequence comparisons of the three strains of BCTV indicate that considerable divergence has occurred. The relatively low sequence identity values (79 to 82.8%) obtained when comparing the three strains of BCTV raises the question of whether each strain of BCTV should be considered as a separate virus species. However, because all three BCTV strains have a similar genome organization, are vectored by the same leafhopper species, and would be difficult to specifically identify on the basis of symptom expression in most hosts, the erection of new taxa to describe each BCTV strain as a separate virus species would contribute to confusion rather than facilitate clarity. Therefore, we do not propose to rename each strain of BCTV as separate virus species. Precedence for this course of action may be drawn from studies on genetic variability within the whitefly-transmitted geminiviruses bean golden mosaic (3,9-12), squash leaf curl (17,22), and tomato yellow leaf curl (15,21,23) viruses. However, we do present the message that three very different viral entities are each known as BCTV, and the classic observations of BCTV strain diversity and coinfection based on phenotypic differences now have been verified by precise measurements of genotypic variability.

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