Full-length infectious DNA clones were constructed for four distinct phenotypic variants of beet curly top virus (BCTV). Southern hybridization assays indicated that each cloned BCTV genome shared sequence homology with pBCT-028, a full-length infectious DNA clone of a California isolate of BCTV previously characterized by others. Restriction endonuclease maps of the cloned BCTV genomes were distinct from one another. Infectivity assays determined that plasmids containing tandem repeats of BCTV genomes were generally more infectious than excised linear DNA inserts. Progeny virus, derived from plants inoculated with cloned DNAs, differed in their ability to infect sugarbeet, Beta vulgaris L., and the severity of symptoms produced in B. vulgaris and other experimental hosts.

Introduction

Beet curly top virus (BCTV) is a leafhopper-transmitted, dicotyledonous plant-infecting, monopartite geminivirus of which many phenotypic variants have been described (Bennett, 1971; Magyarosy & Duffus, 1977; Duffus et al., 1982). Typical strains of BCTV have a wide host range and induce severe disease of sugarbeet, Beta vulgaris L. In contrast, some strains of BCTV cause only mild symptoms in B. vulgaris, whereas others have a restricted host range that does not include B. vulgaris.

Due to phloem-limited systemic spread (Esau & Hoeffert, 1973; Thornely & Mumford, 1979) and the lack of efficient mechanical transmission (Severin, 1924), BCTV has historically been a difficult virus to manipulate in the laboratory. These limitations have recently been circumvented by the application of molecular cloning techniques to the study of BCTV. Stanley et al. (1986) have constructed and completely sequenced a full-length BCTV DNA clone (pBCT-028), kindly provided by J. Stanley (Stanley et al., 1986). The horseradish strain of BCTV (HRCT) represents an atypical BCTV strain, having a limited host range (Duffus et al., 1982). The Logan (Duffus & Skoyen, 1977) and Worland (J. E. Duffus, unpublished) strains both have wide host ranges but can be distinguished by the symptoms they produce in sugarbeet. The Logan strain induces severe disease symptoms (obvious stunting with extensive leaf distortion) even in a tolerant cultivar such as Spreckles Sugar SS2, whereas the Worland strain does not elicit symptoms upon infection of SS2 and induces only mild symptoms (little or no stunting with limited leaf distortion) on the sensitive cultivar SL 742.

Cloning and characterization of BCTV DNA. The strains were propagated in shepherd's purse, Capsella bursa-pastoris (L.) Medic., inoculated using viruliferous beet leafhoppers, Circulifer tenellus (Baker). BCTV was purified from shepherd's purse (Larsen & Duffus, 1984) and circular ssDNA (3.0 kb) was recovered from the purified virions by disruption with 1% SDS, phenol–chloroform extraction and ethanol precipitation. Priming of second strand synthesis was mediated by a synthetic oligonucleotide complementary to nucleotides 96 to 114 of the BCTV sequence determined from pBCT-028 (Stanley et al., 1986). The primer site was selected so that the 3' end of the oligonucleotide was complementary to the conserved sequence element TAATATTAC that is present in a similar genomic location in all geminiviruses examined to date (Lazarowitz, 1987). The dsDNA products were then digested with EcoRI, BamHI or SalI and ligated to pUC8 that had previously been linearized with the appropriate enzyme. The BCTV DNA insert of pBCT-028 was excised from the original M13 construct of Stanley et al. (1986), recloned into pUC8 at the unique SalI site and designated p028SC2. Recombinant plasmids were used to transform Escherichia coli strain JM83, made competent by the CaCl2 method (Maniatis et al., 1982), and transformants harbouring recombinant plasmids were selected as described by Vieira.
& Messing (1982). Recombinant plasmids containing apparent full-length BCTV DNA inserts were selected and shown to be BCTV-specific by Southern hybridization using a nick-translated p028SC2 probe. Restriction endonuclease maps of cloned BCTV genomes were determined by single and double digestion of recombinant plasmids using the following enzymes: Apal, BamHI, BglII, BstXI, Csp45I, DraI, EcoRI, HindIII, KpnI, NcoI, PstI, PvuII, SalI, ScaI, SmaI, SstI, SpeI, SspI, SstI and XbaI.

**Infectivity and phenotypic assays.** Biological activity of cloned BCTV genomes was evaluated by mechanical inoculation of excised linear BCTV DNA inserts or supercoiled plasmids containing head-to-tail tandem repeats of the BCTV inserts. Plasmids containing tandem repeats of BCTV DNA inserts were constructed as described by Stenger et al. (1990). Inocula (1 μl containing 2 to 3 mg/ml of linearized BCTV DNA inserts or 5 mg/ml of supercoiled plasmids containing tandem repeats) were applied by needle puncture to crowns of *B. vulgaris* or *C. bursa-pastoris* seedlings (Stanley et al., 1986). Mechanically inoculated plants were evaluated for BCTV symptoms and the presence of progeny virus was verified by ELISA and insect transmission assays utilizing *C. tenellus* (Larsen & Duffus, 1984). Additional host responses to infection were evaluated by transmission of progeny virus to experimental hosts by *C. tenellus*.

## Results

**Physical properties of cloned DNAs**

Potential full-length DNA clones were identified for each BCTV strain examined (Table 1). No variation in the number or relative position of SalI, EcoRI or BamHI sites was observed for 13 potential full-length clones derived from Worland DNA. Similarly, two clones of Logan DNA contained the same number and relative position of cleavage sites for these three enzymes. Of the clones obtained from HRCT, two distinct types were examined. Further analysis (see Discussion) indicated that the viral insert of pHRCT was derived from HRCT DNA, whereas pCFH (contaminant from HRCT) contained a viral DNA insert apparently derived from a typical wide host range BCTV strain present in the HRCT culture as a contaminant. Representative DNA clones for each strain were arbitrarily chosen for further analysis, as indicated in Table 1.

All of the representative BCTV clones examined in a Southern blot hybridized with nick-translated p028SC2 used as a probe (Fig. 1). Minor variations were observed in the apparent size of the cloned genome inserts (Table 1). Differences in the hybridization signals were also noted, particularly with the viral insert of pHRCT which hybridized only weakly with p028SC2 (Fig. 1). Specificity of the p028SC2 probe for BCTV sequences was demonstrated by the lack of hybridization with λ DNA fragments (Fig. 1) and by the absence of hybridization of BCTV inserts when pUC8 DNA was used as the probe (data not shown).

Restriction endonuclease maps determined for selected clones were distinct from one another (Fig. 2).

### Table 1. Representative DNA clones derived from BCTV strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Insert size (bp)*</th>
<th>Cloning site</th>
</tr>
</thead>
<tbody>
<tr>
<td>California</td>
<td>p028SC2</td>
<td>2993</td>
<td>SalI</td>
</tr>
<tr>
<td>Logan</td>
<td>pLogan</td>
<td>3040</td>
<td>SalI</td>
</tr>
<tr>
<td>Worland</td>
<td>pWorland</td>
<td>3000</td>
<td>BamHI</td>
</tr>
<tr>
<td>HRCT</td>
<td>pHRCT</td>
<td>3050</td>
<td>SalI</td>
</tr>
<tr>
<td>Unknown</td>
<td>pCFH</td>
<td>3000</td>
<td>EcoRI</td>
</tr>
</tbody>
</table>

* Estimated by agarose gel electrophoresis, except for the California strain for which the insert size is based upon the nucleotide sequence determined for pBCT-028 by Stanley et al. (1986).

However, there was sufficient similarity between the restriction map of pLogan and pBCT-028 (16 sites conserved) to permit their alignment. The pLogan insert contained four restriction sites not found in pBCT-028 and all of these sites mapped to the intergenic region. Restriction mapping also indicated that the slightly larger size of the pLogan insert relative to the insert of pBCT-028 is due to approximately 45 additional nucleotides located within the SstI-SspI fragment, encompassing part of open reading frame (ORF) R2 and part of
the intergenic region. Restriction endonuclease maps of pWorland and pCFH viral inserts share fewer common restriction sites with pBCT-028. The alignments of these clones with pBCT-028 were based upon the presence of five (pWorland) or six (pCFH) potentially conserved restriction sites. The alignments presented in Fig. 2 have since been confirmed by DNA sequencing of insert termini (data not shown). The restriction map of the viral insert of pHRCT (Fig. 3) does not have a sufficient number of conserved sites to permit alignment with pBCT-028.

**Biological properties of cloned DNAs**

Infectivity assays demonstrated that the cloned BCTV genomes were biologically active when mechanically inoculated into plants (Tables 2 and 3). Infectivity of excised linear DNA inserts was low (less than 4%) when *B. vulgaris* was mechanically inoculated with p028SC2, pLogan or pWorland, and moderate (up to 23%) when *C. bursa-pastoris* was mechanically inoculated with pCFH or pHRCT (Table 2). The use of uncut supercoiled plasmids containing tandem repeats of the BCTV inserts as inocula resulted in a three- to 10-fold increase (8 to 58%) in the number of plants infected for four or five BCTV genomes tested (Table 3).

### Table 2. Infectivity of linearized cloned BCTV DNA inserts

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Experiment†</th>
<th>Host‡</th>
<th>Infectivity§</th>
</tr>
</thead>
<tbody>
<tr>
<td>p028SC2</td>
<td>1</td>
<td>SS2</td>
<td>1/58 (1.7)</td>
</tr>
<tr>
<td>p028SC2</td>
<td>2</td>
<td>SS2</td>
<td>2/60 (3.3)</td>
</tr>
<tr>
<td>pLogan</td>
<td>1</td>
<td>SS2</td>
<td>0/50 (0.0)</td>
</tr>
<tr>
<td>pLogan</td>
<td>2</td>
<td>SS2</td>
<td>2/54 (3.7)</td>
</tr>
<tr>
<td>pWorland</td>
<td>1</td>
<td>SL 742</td>
<td>0/50 (0.0)</td>
</tr>
<tr>
<td>pWorland</td>
<td>2</td>
<td>SL 742</td>
<td>1/64 (1.6)</td>
</tr>
<tr>
<td>pCFH</td>
<td>1</td>
<td>SP</td>
<td>1/80 (1.3)</td>
</tr>
<tr>
<td>pCFH</td>
<td>2</td>
<td>SP</td>
<td>2/54 (3.7)</td>
</tr>
<tr>
<td>pHRCT</td>
<td>1</td>
<td>SP</td>
<td>1/80 (1.3)</td>
</tr>
<tr>
<td>pHRCT</td>
<td>2</td>
<td>SP</td>
<td>2/54 (3.7)</td>
</tr>
<tr>
<td>pUC8</td>
<td>1</td>
<td>SL 742</td>
<td>0/60 (0.0)</td>
</tr>
<tr>
<td>pUC8</td>
<td>2</td>
<td>SP</td>
<td>0/43 (0.0)</td>
</tr>
</tbody>
</table>

* After excision of insert DNA with the same restriction endonuclease used for cloning, pUC8 was linearized with SphI.
† Insert DNA concentration was 2 mg/ml (experiment 1) or 3 mg/ml (experiment 2). Linearized pUC8 concentration was 2 mg/ml for both experiments. Inocula (1 µl) were applied to the crown of test plants by needle puncture.
‡ SS2 and SL 742, cultivars of *B. vulgaris*; SP, shepherd's purse.
§ Number of plants infected/number of plants inoculated. Number in parentheses indicates percentage of plants infected.

### Table 3. Infectivity of plasmids containing tandem repeats of cloned BCTV DNA inserts

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Host‡</th>
<th>Infectivity§</th>
</tr>
</thead>
<tbody>
<tr>
<td>p028SC2-D</td>
<td>SS2</td>
<td>8/24 (33-3)</td>
</tr>
<tr>
<td>p028SC2-D</td>
<td>SS2</td>
<td>8/30 (26-7)</td>
</tr>
<tr>
<td>pLogan-D</td>
<td>SS2</td>
<td>11/33 (33-3)</td>
</tr>
<tr>
<td>pLogan-D</td>
<td>SS2</td>
<td>3/25 (12-0)</td>
</tr>
<tr>
<td>pLogan-D</td>
<td>SL 742</td>
<td>2/25 (8-0)</td>
</tr>
<tr>
<td>pLogan-D</td>
<td>SL 742</td>
<td>5/20 (25-0)</td>
</tr>
<tr>
<td>pWorland-D</td>
<td>SL 742</td>
<td>9/50 (18-0)</td>
</tr>
<tr>
<td>pWorland-D</td>
<td>SL 742</td>
<td>6/43 (14-0)</td>
</tr>
<tr>
<td>pCFH-D</td>
<td>SP</td>
<td>26/45 (57-8)</td>
</tr>
<tr>
<td>pCFH-D</td>
<td>SP</td>
<td>14/37 (37-8)</td>
</tr>
<tr>
<td>pHRCT-D</td>
<td>SP</td>
<td>3/30 (10-0)</td>
</tr>
<tr>
<td>pHRCT-D</td>
<td>SP</td>
<td>7/36 (19-4)</td>
</tr>
</tbody>
</table>

* Inocula consisted of supercoiled plasmids at a concentration of 5 mg/ml (equivalent to 34 mg/ml of insert DNA) containing tandem repeats (D) of cloned viral inserts. Inocula (1 µl) were applied to the crown of test plants by needle puncture.
† SS2 and SL 742, cultivars of *B. vulgaris*; SP, shepherd's purse.
‡ Number of plants infected/number of plants inoculated. Number in parentheses indicates percentage of plants infected.
The symptoms exhibited by plants mechanically inoculated with cloned DNA, or with plants subsequently inoculated with progeny virus transmitted by *C. tenellus*, indicated that p028SC2, pLogan, pWorland and pHRCT displayed the same host range and symptom severity in *B. vulgaris* as the strains from which they were derived (Table 4). The exception was pCFH, which readily infected *B. vulgaris*, *Nicotiana tabacum* L. and *Phaseolus vulgaris* L., three species that are not hosts of HRCT (Duffus et al., 1982).

Host range studies conducted by leafhopper transmission tests of progeny virus, verified additional phenotypic differences among the cloned genomes (Table 4). Progeny virus of pHRCT was unique in producing a symptomless infection of spinach (*Spinacia oleracea* L.) and mild symptoms in *N. benthamiana* Domin., whereas progeny virus of p028SC2, pLogan, pWorland and pCFH caused severe symptoms in both hosts. Differences in symptom expression were also observed in *N. tabacum*, varying from symptomless infections (p028SC2 and pLogan) to mild stunting and leaf distortion (pWorland) and severe stunting, leaf deformation and vein clearing (pCFH).

**Discussion**

The genomes of four biologically distinct strains of BCTV were cloned and characterized. Although each of the cloned BCTV genomes shared detectable sequence homology with p028SC2 as judged by Southern hybridization, the variable strength of the hybridization signals and unique restriction maps indicated that considerable divergence exists in the nucleotide sequences of BCTV strains. Variation in nucleotide sequence also exists among strains of squash leaf curl virus that display differences in host range (Lazarowitz, 1987) and bean golden mosaic virus (BGMV) isolates from different geographical locations (Gilbertson et al., 1988). Variation in nucleotide sequence may also occur within a single strain; differences in restriction sites exist between BCTV DNA clones of the California isolate from which pBCT-028 was derived (Stanley et al., 1986), although the authors reported no differences in the biological properties of the variants. Thus, nucleotide sequence differences found in the BCTV clones described in this paper are not unusual when compared to other geminiviruses.

The low specific infectivity of linearized BCTV inserts mechanically inoculated into plants in this study was also noted by Stanley et al. (1986). The increased levels of infectivity resulting from the use of plasmids containing tandem repeats of BCTV inserts as inocula were reminiscent of results reported by Hayes et al. (1988) for tomato golden mosaic viurs and Morinaga et al. (1988) for BGMV. Although the infectivity of plasmids containing tandem repeats of BCTV inserts was generally three- to 10-fold higher than that obtained with excised linear inserts, exceptions have been noted. In particular, the infectivity of pHRCT was not increased by inoculating a plasmid containing tandem repeat sequences. It is possible that the level of infectivity depends upon the genomic location of the restriction site used to clone, and later excise, unit length viral inserts. More recently, agroinoculation procedures (Grimsley et al., 1986) have been applied to BCTV (Briddon et al., 1989), demonstrating that phloem-limited viral genomes can be efficiently delivered to host plants by this method as well.

Progeny virus of pCFH did not display the restricted host range properties typical of HRCT. As HCRT used in these experiments was initially unable to infect *B.
vulgaris, and because beet-infecting BCTV strains have been found to be incapable of infecting horseradish (Duffus et al., 1982), we believe that pCFH represents the cloned genome of a typical wide host range isolate of BCTV that was inadvertently introduced as a contaminant during the propagation of HCRT in shepherd's purse. The unique restriction map and distinct symptoms produced by pCFH on N. tabacum suggest that pCFH was not derived from the Logan, Worland or California strains, but instead represents the genome of a BCTV strain of unknown origin.

The results presented here demonstrate the biological activity and distinct phenotypic properties of cloned BCTV genomes. BCTV DNA clones have been identified that vary in the severity of symptoms produced on several experimental hosts, or that have a restricted host range. Examination of the similarities and differences in nucleotide sequences of these phenotypically distinct viral genomes should be informative. It is anticipated that analysis of recombinant viral genomes containing DNA fragments from phenotypically distinct BCTV strains may facilitate the identification of sequences controlling the expression of symptoms and host range.

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References


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