Mutational analysis of bean yellow dwarf virus, a geminivirus of the genus Mastrevirus that is adapted to dicotyledonous plants

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Bean yellow dwarf virus (BeYDV) is an atypical member of the geminivirus genus Mastrevirus that infects dicotyledonous plants. BeYDV DNA contains six open reading frames (ORFs) with the capacity to encode proteins in excess of 10 kDa. Two virion-sense ORFs (V1 and V2) and two complementary-sense ORFs (C1 and C2) have homologues in all mastreviruses, while ORFs C3 and C4 are not conserved. To investigate their functions, each of the ORFs has been truncated by either frame-shifting or the introduction of a stop codon. We demonstrate that an ORF V1 mutant replicated efficiently in Nicotiana tabacum protoplasts but was unable to systemically infect Phaseolus vulgaris and Datura stramonium, consistent with a role for V1 protein in virus movement. However, the mutant was able to systemically infect Nicotiana benthamiana although the onset of symptoms was appreciably delayed in comparison with wild-type virus. Disruption of ORF V2, encoding the coat protein, prevented systemic infection of all three hosts but the mutant replicated in protoplasts. Both ORF C1 and ORF C2 were essential for replication in protoplasts. Modification of the complementary-sense splice donor and acceptor sequences also prevented replication. Removal of the intron prevented systemic infection, although the intronless mutant was able to produce functional replication-associated protein (Rep) and replicated efficiently in protoplasts. ORFs C3 and C4 were not required for systemic infection. Our results indicate that four ORFs are spatially and functionally conserved in mastreviruses that infect both monocotyledonous and dicotyledonous plants.

Introduction

Geminiviruses are small single-stranded (ss) DNA plant viruses that are subdivided on the basis of genome organization, host range and insect vector. All members of the genus Mastrevirus are transmitted by leafhoppers, and the majority infect monocotyledonous plants. There are currently only two known members of the genus that are adapted to dicotyledonous hosts, namely tobacco yellow dwarf virus (TYDV), originating from Australia (Morris et al., 1992), and bean yellow dwarf virus (BeYDV), originating from South Africa (Liu et al., 1997a). Their widely separated geographical locations and genetic diversity suggest that many more distinct mastreviruses that infect dicotyledonous plants await discovery.

Irrespective of host range, sequence analysis of several mastreviruses has revealed the presence of four conserved open reading frames (ORFs) distributed between the virion-sense (V) and complementary-sense (C) strands (Mullineaux et al., 1984; MacDowell et al., 1985; Donson et al., 1987; Andersen et al., 1988; Chatani et al., 1991; Briddon et al., 1992; Morris et al., 1992; Hughes et al., 1993; Liu et al., 1997a). Gene expression occurs bidirectionally from the large intergenic region (LIR) and genes on opposite DNA strands converge on the small intergenic region (SIR) (Morris-Krisinch et al., 1985; Dekker et al., 1991). The LIR contains cis-acting promoter elements that bind host factors and control virion-sense gene expression (Fenoll et al., 1988, 1990; Wright et al., 1997), as well as the origin of virion-sense DNA replication (Heyraud et al., 1993; Heyraud-Nitschke et al., 1995), which is believed to occur by a rolling circle mechanism (Saunders et al., 1991; Stenger et al., 1991). A putative primer for complementary-sense DNA synthesis is associated with the SIR (Donson et al., 1984; Hayes et al., 1988).
Table 1. BeYDV primers used for in vitro mutagenesis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
<th>Coordinates</th>
<th>Mutant†</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>V2472</td>
<td>GCTGGTGTACGAGCAGTCTCCTGGTGC</td>
<td>749–775</td>
<td>V2^1-111</td>
<td>Stop codon in ORF V2, SfiI site created</td>
</tr>
<tr>
<td>V2473</td>
<td>GTGAAATCAGCTGGGACCTTAATTGAG</td>
<td>1498–1527</td>
<td>C2^1-49</td>
<td>Stop codon in ORF C2, Hpal site created</td>
</tr>
<tr>
<td>V2474</td>
<td>TTTGATATGACTGACTTGTGTTAG</td>
<td>2097–2126</td>
<td>C3^1-82</td>
<td>Stop codon in ORF C3, XbaI site created</td>
</tr>
<tr>
<td>V2475</td>
<td>CAGAGCAACTAAGGAAAGAAAAATTAG</td>
<td>2251–2278</td>
<td>C1^1-31</td>
<td>Stop codon in ORF C3, AvrII site created</td>
</tr>
<tr>
<td>V2477</td>
<td>CTTTCCCTCTCAAGTTTGGATCCATCGC</td>
<td>1710–1739</td>
<td>3^BspHI</td>
<td>3′ Splice junction disrupted, two amino acid changes in ORF C1, BspHI site created</td>
</tr>
<tr>
<td>V2478</td>
<td>GGCATCGATGCTATGATAGTT</td>
<td>1793–1819</td>
<td>5^BspHI</td>
<td>5′ Splice junction disrupted, one amino acid change in ORF C1, BspHI site created</td>
</tr>
<tr>
<td>V3448</td>
<td>GATTCTGTGAGGGGTTACGTATGCTCA</td>
<td>1863–1892</td>
<td>C4^1-25</td>
<td>Stop codon in ORF C4, SnaBlI site created</td>
</tr>
<tr>
<td>V3856</td>
<td>GAGGCGAGGGCAATTGGGATATGGTAG</td>
<td>2457–2487</td>
<td>C1^1-316</td>
<td>Remove first in-frame ATG in ORF C1, AluI site created</td>
</tr>
</tbody>
</table>

*Mismatches are in lower case, introduced restriction sites are underlined.
†Superscript numbering indicates the coordinates of the amino acids encoded by the mutated ORFs.

Genetic analyses of mastreviruses have so far been confined to members that infect monocotyledonous plants. Maize streak virus (MSV) ORF V1 encodes a protein required for virus movement (Boulton et al., 1993) that is associated with plasmodesmata (Dickinson et al., 1996), and ORF V2 encodes the coat protein (Morris-Krsinich et al., 1985). Complementary-sense gene expression in digitaria streak virus (DSV), wheat dwarf virus (WDV), MSV and TYDV is accomplished by transcript splicing (Accotto et al., 1989; Schalk et al., 1989; Dekker et al., 1991; Morris et al., 1992; Wright et al., 1997), allowing expression of the replication-associated protein (Rep) from fused ORFs C1 and C2 (ORF C1C2), while ORF C1 is expressed from the unspliced transcript. The product of ORF C1 (C1 or RepA protein) activates virion-sense gene expression (Zhan et al., 1993; Collin et al., 1996), and Rep is required for nicking and joining of DNA strands at the origin of replication (Heyraud-Nitschke et al., 1995). Both proteins have been shown to bind to human retinoblastoma (Rb) tumour suppressor protein (Xie et al., 1995; Collin et al., 1996) and, more recently, to a maize Rb protein homologue (Grafi et al., 1996; Xie et al., 1996). This suggests that one or both of these proteins may induce the expression of S phase-specific host proteins required for viral DNA replication by removing the block to entry into S phase imposed by Rb protein. This is consistent with the observation that DSV is associated with nuclei in S phase (Accotto et al., 1993), although work by Lucy et al. (1996) showed no correlation between MSV infection and expression of the S phase-specific gene H2b.

Although the genomic organization of all mastreviruses is fundamentally similar, it remains to be established whether the genes are functionally conserved between viruses infecting monocotyledonous and dicotyledonous plants. To address this, we have investigated BeYDV gene functions by screening mutants for their ability to replicate in single cells (Nicotiana tabacum protoplasts), and to infect whole plants (Phaseolus vulgaris, the host from which the virus was originally isolated, Nicotiana benthamiana and Datura stramonium).

Methods

Construction of BeYDV mutants. The construction and sequence analysis of an infectious cloned copy of the BeYDV genome have been described (Liu et al., 1997a). Mutations were introduced into clone mpBYD081 (Liu et al., 1997a) using the Muta-Gene in vitro mutagenesis kit (Bio-Rad) and the primers summarized in Table 1. In this way, stop codons and novel restriction sites were introduced into specific ORFs in mutants V2^1-111, C2^1-49, C3^1-31, C1^1-82 and C4^1-25 (Fig. 1). BspHI sites were introduced at each complementary-sense splice junction in mutants 3^BspHI and 5^BspHI, and at both junctions in mutant 5′^BspHI. These mutations result in amino acid changes in ORF C1 from valine180 to glutamic acid in mutant 5′^BspHI, and from threonine225 and arginine278 to isoleucine and methionine, respectively, in mutant 3′^BspHI (amino acid numbering from the second in-frame ATG, as described in Results). The intron was excised from 3′^BspHI by BspHI digestion and religation, to produce mutant Antron (Fig. 1). The first in-frame ATG of ORF C1 was modified to AGT by in vitro mutagenesis to produce mutant C1^25-316 (Table 1).

Infectious BeYDV genome was constructed by in vitro infection of tobacco protoplasts with BeYDV (strain Xilp, position 175) to excise a full-length copy of the genome, and religating the vector and insert after filling in recessed 3′ termini using DNA polymerase Klenow fragment (Amersham) to generate homology at the 5′ termini. The cloned BeYDV genome was digested by BstXI (Stratagene) to excise a full-length copy of the genome, and religated to produce infectious plasmid mpBYD1.4 (Fig. 1). This plasmid was linearized by NotI to introduce a NotI site at position 396 of the BeYDV genome, and linearized BeYDV DNA was used to produce infectious BeYDV genome by in vitro infection of tobacco protoplasts.
indicated. The deletion in the wild-type sequence or were introduced during mutagenesis are ORFs are shaded and the positions of restriction sites that either occur in bars demarcate the intron in the complementary-sense ORFs. Mutated Wild-type (wt) and mutant genomes are shown as linear maps. Vertical Genome organization of BeYDV and mutants produced Fig. 1.

**Results**

The phenotypes of mutants containing truncated ORFs were screened for systemic infectivity in *N. benthamiana*, *D. stramonium* and *P. vulgaris* (cv. Top Crop), and for replication in *N. tabacum* protoplasts. Wild-type BeYDV produced typical stunting and leaf curl symptoms in all three hosts approximately 10 days after agroinoculation (Liu et al., 1997 a).

**Assay for viral DNA replication.** The ability of BeYDV mutants to replicate was investigated using protoplasts derived from *N. tabacum* BY-2 suspension culture (Nagata et al., 1992). The preparation, inoculation and maintenance of protoplasts, and the extraction and analysis of replicating viral DNA has been described (Hong & Stanley, 1996). Wild-type and mutant viral DNAs (1.25 pmol aliquots) were introduced as partial repeat sequences in pBlueScript II SK(+) protoplasts were harvested after incubation for 72 h at 28 °C.

**Characterization of the complementary-sense splice junction.** Total nucleic acids were extracted from infected *D. stramonium* plants agroinoculated with cloned BeYDV DNA, and high molecular mass RNAs were isolated by salt precipitation and treated with DNase I (Pharmacia) as described by Covey & Hull (1981) replication of the viral genome has been described (Hong & Stanley, 1996). A cDNA to complementary-sense transcripts was synthesized by reverse transcription using primer V1540, corresponding to nucleotides 1435–1451 within ORF C2. DNA fragments were PCR-amplified from the cDNA using primers V3343 (nucleotides 1533–1562) and V3344 (complementary to nucleotides 2227–2256). Amplification conditions have been described by Liu et al. (1997 a).

**Analysis of viral DNA.** Total nucleic acids were extracted from systemically infected leaves as described by Covey & Hull (1981), and 5 μg aliquots were analysed by agarose gel electrophoresis in TBE buffer (40 mM Tris–acetate, 20 mM sodium acetate, 2 mM EDTA, pH 7.5), and blot hybridization using a α<sup>32</sup>P-labelled full-length BeYDV DNA probe prepared by random priming (Feinberg & Vogelstein, 1983). Extracts were screened for mutants by restriction analysis after PCR amplification of either a full-length fragment of the viral DNA using partially overlapping primers V2282 (nucleotides 1794–1819) and V2283 (complementary to nucleotides 1770–1803) or a subfragment using primers V3377 (nucleotides 1133–1167) and V3344 (complementary to nucleotides 2227–2256). Amplification conditions have been described by Liu et al. (1997 a).
Table 2. Infectivity of BeYDV mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Plants systemically infected/inoculated (no. of experiments)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>N. benthamiana</td>
</tr>
<tr>
<td>V1&lt;sup&gt;1-37&lt;/sup&gt;</td>
<td>4/17(2)*</td>
</tr>
<tr>
<td>V2&lt;sup&gt;1-114&lt;/sup&gt;</td>
<td>0/19(2)</td>
</tr>
<tr>
<td>C1&lt;sup&gt;1-27&lt;/sup&gt;</td>
<td>0/19(2)</td>
</tr>
<tr>
<td>C2&lt;sup&gt;1-49&lt;/sup&gt;</td>
<td>0/19(2)</td>
</tr>
<tr>
<td>C3&lt;sup&gt;1-82&lt;/sup&gt;</td>
<td>38/39(5)</td>
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<td>C3&lt;sup&gt;1-81&lt;/sup&gt;</td>
<td>17/18(3)</td>
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<tr>
<td>C4&lt;sup&gt;1-35&lt;/sup&gt;</td>
<td>24/31(4)</td>
</tr>
<tr>
<td>5&lt;sup&gt;°&lt;/sup&gt;BspHII</td>
<td>0/19(2)</td>
</tr>
<tr>
<td>3&lt;sup&gt;°&lt;/sup&gt;BspHII</td>
<td>0/19(2)</td>
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<tr>
<td>5&lt;sup&gt;°&lt;/sup&gt;AhdI</td>
<td>0/27(3)</td>
</tr>
<tr>
<td>Aintron</td>
<td>0/26(3)</td>
</tr>
<tr>
<td>C&lt;sup&gt;2&lt;/sup&gt;25–316</td>
<td>9/14(2)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>34/40(5)</td>
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<tr>
<td>V1&lt;sup&gt;1-37&lt;/sup&gt; + V2&lt;sup&gt;1-114&lt;/sup&gt;</td>
<td>7/15(2)</td>
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<tr>
<td>V1&lt;sup&gt;1-37&lt;/sup&gt; + C1&lt;sup&gt;1-27&lt;/sup&gt;</td>
<td>12/26(3)</td>
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<tr>
<td>V1&lt;sup&gt;1-37&lt;/sup&gt; + C2&lt;sup&gt;1-49&lt;/sup&gt;</td>
<td>5/15(2)</td>
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<tr>
<td>V1&lt;sup&gt;1-37&lt;/sup&gt; + Aintron</td>
<td>3/8(1)</td>
</tr>
<tr>
<td>V2&lt;sup&gt;1-114&lt;/sup&gt; + C1&lt;sup&gt;1-27&lt;/sup&gt;</td>
<td>7/18(2)</td>
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<tr>
<td>V2&lt;sup&gt;1-114&lt;/sup&gt; + C2&lt;sup&gt;1-49&lt;/sup&gt;</td>
<td>5/8(1)</td>
</tr>
<tr>
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<td>4/7(1)</td>
</tr>
<tr>
<td>C2&lt;sup&gt;1-49&lt;/sup&gt; + Aintron</td>
<td>5/15(2)</td>
</tr>
<tr>
<td>5&lt;sup&gt;°&lt;/sup&gt;BspHII + 3&lt;sup&gt;°&lt;/sup&gt;BspHII</td>
<td>0/9(1)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>29/34(4)</td>
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*The onset of symptom development was delayed by approximately 30 days in comparison with wild-type infection.

age that were infected with the wild-type virus, and the accumulation of both mutant and wild-type virus was similar in these tissues (Fig. 2). The mutant also replicated as efficiently as wild-type virus in N. benthamiana tissues using primers V2282 and V2283, and characterized by restriction analysis. The DNA fragment from tissues infected with the mutant had retained the introduced Smal site (Fig. 4, lanes 1–4), confirming that the frame-shift insertion had been maintained in the viral DNA.

The coat protein ORF (245 amino acids) was disrupted in mutant V2<sup>1-114</sup> by the introduction of a stop codon after 114 N-terminal amino acids. The coat protein mutant was unable to systemically infect N. benthamiana, D. stramonium and P. vulgaris (Table 2). However, it retained the ability to replicate in N. benthamiana protoplasts (Fig. 3, lane 2) although it produced reduced levels of ssDNA compared with the wild-type virus.

ORF C1 has the capacity to encode 316 amino acids when translated from the first in-frame ATG. This ATG (nucleotides 2468–2470) was modified to AGT in mutant C1<sup>25–316</sup>. The mutant retained the ability to infect plants and produce wild-type symptoms (Table 2). The retention of the AhdI site in the progeny of mutant C1<sup>25–316</sup> was verified by restriction analysis of a full-length copy of the viral DNA amplified by PCR (Fig. 4, lanes 13–16). The results are consistent with the second in-frame ATG, conserved in all other mastreviruses (Liu et al., 1997a), being functional in translation initiation. ORF C1 was disrupted in mutant C1<sup>1-27</sup> by the deletion of a dinucleotide at position 2386, to produce a truncated ORF encoding only 27 N-terminal amino acids (three from the second in-frame ATG) fused to eight amino acids in an adjacent reading frame. The mutant was unable either to systemically infect plants (Table 2) or to replicate in N. benthamiana protoplasts (Fig. 3, lane 7).

ORF C2 does not contain a suitably placed ATG codon for translation initiation but is expressed by post-transcriptional modification which serves to fuse ORFs C1 and C2. Comparison with nucleotide sequences of other mastreviruses identified possible splice junctions within BeYDV ORFs C1 and C2. Mutations were introduced within the splice donor (mutant 5<sup>°</sup>BspHII) and acceptor (mutant 3<sup>°</sup>BspHII) sites to modify the sequences from GT to GA (donor) and from AG to AT (acceptor) and create BspHII sites (Table 1, Fig. 5 B). The
Fig. 2. Southern blot analysis of viral DNA forms associated with mutant V11-17 infection of N. benthamiana. Total nucleic acids were extracted either from plants agroinoculated with wild-type virus (lane 9) or from symptomatic (lanes 1, 3–5) and asymptomatic plants (lanes 2, 6–8) agroinoculated with mutant V11-17. The blot was probed for BeYDV DNA. Tissue samples were taken from upper leaves 50 days after agroinoculation. The positions of single-stranded (SS) and supercoiled (SC) DNA are indicated.

Fig. 3. Southern blot analysis of mutant replication in N. tabacum protoplasts. Total nucleic acids were extracted from protoplasts inoculated with mutants V21-114 (lane 2), C21-69 (lane 3), 3′BspHI (lane 4), 5′BspHI (lane 5), 5′3′BspHI (lane 6), C11-27 (lane 7), V11-17 (lane 8), ∆intron (lane 9) and wild-type virus (lane 10). Viral DNA forms produced during wild-type virus infection of Arabidopsis thaliana are shown in lane 1. The blot was probed for BeYDV DNA. The positions of single-stranded (SS), supercoiled (SC), linear (LIN) and subgenomic (SG) DNA are indicated.

Mutations also change the amino acids encoded by ORF C1 from valine198 (numbering from the proposed functional initiation codon) to glutamic acid in mutant 5′BspHI and from threonine235 and arginine236 to isoleucine and methionine, respectively, in mutant 3′BspHI. Mutant 5′3′BspHI contains these modifications at both the donor and acceptor sites. Intron sequences were removed from mutant 5′3′BspHI by BspHI digestion, to produce mutant ∆intron. The presence of a functional intron was investigated by PCR-amplification of a fragment of the genome encompassing the putative splice junctions using cDNA synthesized from viral complementary-sense transcripts. Transcripts were treated with DNase I prior to cDNA synthesis to remove possible contaminating viral DNA. In addition to the anticipated 724 bp fragment produced from unspliced transcripts, a smaller, less abundant, fragment occurred with an electrophoretic mobility comparable to that of the fragment produced from the deletion mutant ∆intron (Fig. 5 A). Sequence analysis of clones derived from these fragments confirmed the presence of an intron encompassing nucleotides 1722–1807 (Fig. 5 B). Transcript splicing fuses ORFs C1 and C2, to allow the expression of a Rep protein homologous to its counterparts in other mastreviruses.

Mutants 5′BspHI, 3′BspHI and 5′3′BspHI were unable either to systemically infect plants (Table 2), or to replicate in N. tabacum protoplasts (Fig. 3, lanes 4–6). Mutant ∆intron, in which the intron sequence had been precisely removed, was also unable to systemically infect plants (Table 2). However, it replicated to high levels in N. tabacum protoplasts, in which it produced viral DNA forms slightly smaller than those associated with the wild-type virus as a result of the 86 bp intron deletion (Fig. 3, lane 9), confirming that functional Rep protein is being expressed.

ORF C2 (137 amino acids from the 3′ splice junction) was disrupted in mutant C21-69 by the introduction of a stop codon after 69 N-terminal amino acids. The mutant was unable either to systemically infect plants (Table 2) or to replicate in N. tabacum protoplasts (Fig. 3, lane 3).

ORF C3 (127 amino acids) was disrupted in mutants C31-31 and C31-82 by the introduction of stop codons after 31 and 82 N-terminal amino acids, respectively. ORF C4C1 (116 amino acids), produced by fusion of ORFs C4 and C1 as a result of splicing, was disrupted in mutant C41-25 by the introduction of a stop codon after 25 N-terminal amino acids. None of these mutations affected the amino acids encoded by the overlapping ORF C1. All mutants were systemically infectious in plants (Table 2), accumulating to wild-type viral DNA levels in N. benthamiana (data not shown) and producing symptoms in this host that were indistinguishable from the wild-type infection. Symptoms in P. vulgaris infected with mutant C31-82 were occasionally less severe than the wild-type infection. Restriction analysis of full-length viral DNAs, PCR-amplified from nucleic acids extracted from systemically infected N. benthamiana tissues, demonstrated the presence of XbaI, AvrII and SmaI sites in the progeny of mutants C31-32 (Fig. 4, lanes 9–12), C31-82 (data not shown) and C41-25 (lanes 5–8), respectively, confirming that the mutations had been maintained in the viral DNA.
Complementation of gene functions was investigated by co-agroinoculation of *N. benthamiana* with various combinations of mutants (Table 2).

With the exception of mutants $5'\text{BspHI}$ and $3'\text{BspHI}$, all combinations produced systemic infections, and the timing and severity of symptoms were similar to those associated with the wild-type infection. The lack of infectivity associated with mutants $5'\text{BspHI}$ and $3'\text{BspHI}$ may be due to the fact that recombination will be confined to the inoculum DNA as neither mutant can replicate. Also, the close proximity of the mutations within the genome will reduce the chance of recombination occurring between these points to generate wild-type virus. The progeny of mixed infections was analysed by PCR amplification and restriction analysis. Amplification of full-length fragments from a mixed infection of mutants $V1^{1-17}$ and $V2^{1-114}$ using primers $V2282$ and $V2283$, and digestion with $SmaI$ and/or $SpeI$ (Fig. 6 A, lanes 3–5) produced fragments that comigrated with those amplified from cloned DNA of mutants $V1^{1-17}$ (1614 and 959 bp; lane 6) and $V2^{1-114}$ (1540 and 1031 bp; lane 7). The presence of residual full-length fragment (lane 5) implies either partial digestion of the amplified DNA or recombination between the complementing mutants to produce wild-type viral DNA that lacks both restriction sites (lanes 1 and 2). Nonetheless, the results demonstrate that movement and coat protein defects in mutants $V1^{1-17}$ and $V2^{1-114}$, respectively, can be complemented by co-infected virus.

PCR amplification from extracts of plants co-infected with mutants $\Delta$intron and $C2^{1-69}$ using primers $V3344$ and $V3377$ produced two fragments (Fig. 6 B, lane 6) that comigrated with those amplified from wild-type viral DNA (1124 bp; lane 5) and cloned DNA of mutant $\Delta$intron (1038 bp; lane 7). Treatment with $ClaI$ and $HpaI$ (lane 1) failed to digest the 1038 bp fragment (the $ClaI$ site occurs within the intron), and produced fragments that comigrated with those amplified from cloned DNA of mutant $C2^{1-69}$ (458, 380 and 285 bp; lane 3). The presence of some residual 1124 bp fragment as well as a fragment comigrating with the 665 bp fragment amplified specifically from wild-type viral DNA (lane 4) suggests incomplete digestion of the amplified fragment, although recombination between complementing mutants to produce wild-type virus cannot be ruled out. The mutants were retained following graft transmission (lane 2), demonstrating that, if present, the recombinant virus did not rapidly become the predominant form within the virus population. The data demonstrate that defects in C1 and Rep proteins in mutants $\Delta$intron and $C2^{1-69}$, respectively, can be complemented by co-infecting virus. In another experiment, defects in both C1 and Rep proteins in mutant $C1^{1-47}$ were similarly complemented by mutant $V1^{1-17}$ (data not shown). The complementation data verify that mutants containing defects in C1, Rep and coat protein retain the ability to systemically infect plants when provided in trans with the appropriate gene product, implying that they are not defective in any cis-acting function.

**Discussion**

On the basis of the arrangement of ORFs predicted from nucleotide sequence data, both TYDV and BeYDV (Morris et al., 1992; Liu et al., 1997a) appear to be organized in a similar manner to members of the genus that are confined to monocotyledonous hosts. Our systematic mutagenesis of BeYDV ORFs with the capacity to encode proteins in excess of 10 kDa supports this view. The phenotype of coat protein mutant $V2^{1-114}$ resembled those reported for its counterparts in MSV (Boulton et al., 1989; Lazarowitz et al., 1989) and WDV (Woolston et al., 1989). The BeYDV coat protein is essential for virus systemic infection, which may reflect a requirement either for encapsidation for intercellular movement or for intracellular trafficking of viral DNA. In this respect, it is notable that the BeYDV coat protein has a basic domain within its N terminus which may be important for DNA binding and/or nuclear localization, as recently proposed for MSV coat protein (Liu et
**Fig. 5.** Characterization of the BeYDV complementary-sense intron. (A) Viral DNA fragments encompassing the putative intron were PCR-amplified from either cDNA synthesized from complementary-sense transcript extracted from infected *D. stramonium* (lane 1) or clones pSK-BYD1.4 (lane 2) and Δintron (lane 3) using primers V3343 and V3344. (B) Sequence analysis of representative clones from the major (lane 1) and minor (lane 2) bands synthesized by PCR amplification of complementary-sense transcript cDNA. Sequences across the 5′ and 3′ splice junctions, and their modifications in mutants 5′BspHI (T → A) and 3′BspHI (C → T and G → T) to produce BspHI sites, are shown.

**Fig. 6.** Restriction analysis of mutant DNA extracted from systemically infected *N. benthamiana* tissues. (A) Viral DNA fragments were amplified by PCR using primers V2282 and V2283 either from nucleic acids extracted from plants agroinoculated with wild-type virus (lanes 1 and 2) and mutants V11–17 and V21–114 (lanes 3–5), or from partial repeats of mutants V11–17 (lane 6) and V21–114 (lane 7) cloned into pBluescript II SK(+) as described in Methods. DNA was digested with either *SnaBI* (lanes 1, 3 and 6), *SpeI* (lanes 2, 4 and 7) or both *SnaBI* and *SpeI* (lane 5) before gel fractionation. (B) Viral DNA fragments were amplified by PCR using primers V3344 and V3377 either from nucleic acids extracted from plants infected with mutants C21–69 and Δintron by agroinoculation (lanes 1 and 6) and grafting (lane 2), and wild-type virus (lanes 4 and 5), or from partial repeats of mutants C21–69 (lane 3) and Δintron (lane 7) cloned into pBluescript II SK(+) as described in Methods. DNA in lanes 1–4 was digested with *ClaI* and *HpaI* before gel fractionation. Fragments were detected by ethidium bromide staining. The sizes (bp) of markers (Gibco BRL) in lane M are indicated.

Mutant V11–17 was unable to systemically infect *P. vulgaris* and *D. stramonium* but replicated efficiently in *N. tabacum* protoplasts, consistent with the proposed function of the gene product in virus movement based on the inability of a replication-competent MSV mutant to systemically infect *Zea mays* (Boulton *et al.*, 1993). However, mutant V11–17 caused a systemic infection in *N. benthamiana*, albeit at a slower rate than the wild-type virus. This is not inconsistent with a role in virus movement because *N. benthamiana* is widely
regarded as being particularly permissive for many viruses, and some geminivirus mutants are already known to behave atypically in this host (Stanley et al., 1992; Ingham & Lazarowitz, 1993). For this reason, mutants were screened in *D. stramonium* as well as *P. vulgaris*, the host species from which the virus was originally isolated. It should be noted that the mutant was introduced by agroinoculation in our experiments, and it remains to be seen if it can systemically infect plants following insect transmission. This awaits the identification of the BeYDV vector. The ability of BeYDV movement protein mutants to infect *N. benthamiana* should facilitate the analysis of movement protein functional domains in vivo. Constitutive expression of BeYDV V1 protein in transgenic *N. tabacum* has suggested that it is an important symptom determinant (C. Pitaksutheepong, M. I. Boulton & J. W. Davies, unpublished data), as has been proposed for other geminivirus movement proteins (von Arnim & Stanley, 1992; Pascal et al., 1993; Duan et al., 1997). The fact that plants infected with mutant V1 expressed qualitatively similar symptoms to those associated with the wild-type infection implies either that the 17 N-terminal amino acids of the V1 protein are important in this respect or that additional viral factors contribute to the symptom phenotype.

Demonstration of a spliced complementary-sense transcript supports the contention that BeYDV, like other mastreviruses (Accotto et al., 1989; Schalk et al., 1989; Mullineaux et al., 1990; Dekker et al., 1991; Morris et al., 1992; Wright et al., 1997), expresses at least two complementary-sense proteins, C1 and Rep. In comparison with its counterparts in other members of the genus, BeYDV ORF C1 has an additional 24 amino acids at its N terminus. However, removal of the first in-frame ATG had no effect on infectivity, suggesting that it is the second, conserved ATG that is functional. The relatively low level of BeYDV spliced transcript is reminiscent of its abundance in plants infected with WDV, DSV and MSV (Mullineaux et al., 1990; Dekker et al., 1991; Wright et al., 1997), but contrasts with the high level of splicing observed for TYDV (Morris et al., 1992). This difference between BeYDV and TYDV may be attributable either to the different hosts used for transcript isolation (BeYDV RNA was isolated from *D. stramonium*, TYDV from bean and tobacco) or to the stage of infection of the sampled tissues which may not necessarily reflect steady-state levels of transcript processing. In common with complementary-sense introns from other mastreviruses, the BeYDV intron is not particularly AU-rich (52%), being less so than the adjacent ORF C1 (58%) and C2 (54%) sequences. As AU-rich intron sequences are a feature of dicotyledonous species, this suggests that BeYDV and TYDV evolved from geminiviruses that infect monocotyledonous plants rather than vice versa.

Modifications adjacent to the splice junctions prevented replication of mutants 5′Bsp HI, 3′Bsp HI and 5′3′Bsp HI. As these modifications occur within the intron they do not alter the Rep protein sequence and, hence, have no detrimental effect on replication of mutant Δintron in which the intron has been removed. The ability of mutant Δintron to replicate demonstrates that intact C1 protein is not prerequisite for replication, implying that modifications to valine197, threonine225 and arginine226 in the C1 protein sequence of mutants 5′Bsp HI, 3′Bsp HI and 5′3′Bsp HI are not responsible for their inability to replicate. It is more likely that modifications to the splice donor (GT → GA) and acceptor (AG → AT) motifs prevent or alter transcript processing, as has been demonstrated for several *Arabidopsis thaliana* gene mutants (reviewed by Brown, 1996), thus preventing expression of functional Rep protein. Rather than having a direct role in viral DNA replication, C1 protein may participate in the control of viral and host gene expression, as suggested for WDV (Xie et al., 1995; Collin et al., 1996). Its proposed role in controlling cell cycle progression to S phase by binding to Rb protein is presumably redundant in dividing protoplasts, explaining why mutant Δintron is able to replicate under these conditions. Interestingly, the level of replication of the intronless mutant was noticeably higher than that of the wild-type virus. A similar observation was made by Collin et al. (1996) for WDV, who postulated a regulatory role for C1 protein to explain this effect. However, increased levels of the intronless mutant may be due simply to more efficient replication of a slightly smaller molecule. Alternatively, channelling of complementary-sense gene expression solely into production of Rep protein in the mutant may contribute to higher replication efficiency, particularly in view of the relatively low level of splicing detected in BeYDV-infected plants.

Considering the ORFs unique to BeYDV and TYDV, only mutant C3 had a slightly altered phenotype, and this was confined to infection of *P. vulgaris*. While we cannot rule out functional expression from this ORF, it is also possible that perturbation of a cis-acting element may be responsible for the phenotype. It is unlikely that a change in ORF C1 codon preference is a determining factor because, of the two modifications in mutant C3 (GTC → GTT and CTT → CTA), GTT frequently occurs in the BeYDV functional ORFs and, although CTA is not used by the wild-type virus, an identical modification has been made in mutant C31 which exhibited the wild-type phenotype. We can conclude that ORF C3 plays no essential role in BeYDV proliferation. Finally, the wild-type phenotype of mutant C4 suggests that ORF C4C1 is also non-functional.

In conclusion, our genetic analysis indicates that BeYDV encodes two virion-sense gene products required for virus movement (V1 and coat protein), and two complementary-sense gene products (C1 and Rep, expressed by post-transcriptional processing), of which only Rep is essential for viral DNA replication. The results support the view, based on sequence comparisons (Morris et al., 1992; Liu et al., 1997), that mastreviruses are similarly organized and have functionally equivalent genes irrespective of their origins from monocotyledonous or dicotyledonous plant species.
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