Mutagenesis of the BC1 and BV1 genes of African cassava mosaic virus identifies conserved amino acids that are essential for spread

Ann Haley,* Kim Richardson, Xiangcan Zhan and Bret Morris

The products of three open reading frames encoded by the bipartite geminiviruses have been implicated in viral spread: AC2, BV1 and BC1. Alignment of the DNA B encoded gene products, BV1 and BC1, from African cassava mosaic virus (ACMV) with six other bipartite geminiviruses showed several highly conserved regions. Specific amino acids were selected for mutagenic studies to ascertain the tolerance of the virus to change and to identify the regions within these two proteins required for normal functioning. Various mutant DNA B constructs, and a wild-type construct, were inoculated onto three host plant species with an equivalent DNA A construct. Three of the mutant constructs were infectious on Nicotiana benthamiana and N. clevelandii, but only two induced ACMV disease symptoms on N. tabacum cv. Samsun. Sequencing of the viral DNA extracted from the sap of systemically infected plants confirmed the maintenance of introduced base changes. The amino acid at position 95 on the BV1 gene product was identified as non-essential for normal functioning of the protein. The alteration of the amino acid at position 145 in BC1 demonstrated the ability of the virus to tolerate a conservative change. The lack of tolerance to other changes in amino acids has given an indication of the importance of maintaining protein structure for these proteins to function normally.

African cassava mosaic virus (ACMV) belongs to the subgroup (III) of geminiviruses that infect dicotyledonous plants, are transmitted by whiteflies and have genomes consisting of two single-stranded DNA circles called DNA A and DNA B (Hull et al., 1991). Four genes (AV1, AC1, AC2 and AC3) on DNA A provide viral functions required for replication (which occurs in the nucleus), viral gene regulation and encapsidation (Etessami et al., 1991; Haley et al., 1992; Morris et al., 1991; Townsend et al., 1985), whereas two genes (BV1 and BC1) on DNA B provide functions for virus movement (Etessami et al., 1988).

The movement of plant viruses is thought to occur via two processes: locally via cell-to-cell spread and systemically via the phloem. Most plant viruses are now known to encode a protein which is involved in the spread of the virus. The most characterized of these is p30, the 30 kDa protein of tobacco mosaic virus. The p30 protein functions by increasing the exclusion limit of plasmodesmata allowing the passage of RNA to which it has bound, thereby assisting in cell-to-cell movement (Wolf et al., 1989). Nucleic acid-binding experiments have since been carried out using the movement proteins from other groups of plant viruses indicating that this may be a general property of these proteins (Citovsky et al., 1990, 1992; Osman et al., 1992).

The mode of action of the ACMV DNA B genes is unclear, although it is known that their expression is under the control of the AC2 gene product (Haley et al., 1992) and it has been postulated that one of the proteins may be involved in localized or cell-to-cell spread and the other in long-distance vascular spread of the virus (Koonin et al., 1991; von Arnim et al., 1993; K. Richardson, A. Haley, X. Zhan & B. Morris, unpublished results). However, more recently, there has been growing evidence for a role of BV1 in nucleic acid binding and BC1 in increasing the plasmodesmatal exclusion limit. In this report, we examine the effect of introduced mutations in the ACMV BV1 and BC1 genes, at amino acid positions conserved in the corresponding genes of other geminiviruses, on their ability to spread in plants.

To identify conserved amino acid sequences an alignment was prepared using the GAP program (Devereux et al., 1984) for each of the ACMV BV1 and BC1 gene products and the corresponding gene products of seven other bipartite geminiviruses: tomato golden mosaic virus (TGMV) (Hamilton et al., 1984; von Arnim & Stanley, 1992a), bean golden mosaic virus (Howarth et al., 1985), mungbean yellow mosaic virus (Morinaga et
Short communication

(a) BC1

ACMV(1) MDtsv PvissdYlHsSrTeYkLtNdSpItLQFPSTlertrvRimgkCM
Consensus MdsQLvNPPnAFNYIES-RDYEQLSHDLTEIIILQFPSTAsQTAIRLSRNCM

ACMV(50) KvDHvVIEYRnQPnAqGSVIVtIrDtrlsDqndQAgfTfPIgCNVDL
Consensus KIDHCVIEYRQQPGINATGsvlEIHDKRMNDNESLQASWFTPIRCNIDL

ACMV(100) HYFSaSyFSldDnvPWgLlyKVeDSNVkngfTAgkaKLKLaSAKHzStD
Consensus HYFSsSFSKLDPMWKLrYrV-DtNVHRQTHFAKFGKGLKLaSTAKHSDV

ACMV(150) IkFqkPtIkILSKdYgdpcVDFWVsGkpKiRr1IqnepegtgydteYptgkyr
Consensus IFPRAPTVKILSKQF--KDVFSDHvDyGYKREWIR----AS--GL-G

ACMV(200) PItvqPGetWAkStIGyRytrsmrytrpnpididdskqtseAefPlRg
Consensus PIEgRPGWSAsrStIG-S-s---------------s----a-hPrY-

ACMV(250) LhQpeasLDPGdSiS qtQ sMSkddiesiieqTVnCiah
Consensus L-Ryl-DPGEASlVgAgRAQSNITMS--QLNelVR-TVQeCIn-N

ACMV(290) rgSh KdL
Consensus C--sqPKsL-

(b) BV1

ACMV(1) MYsirkqsrnlqrrksnisntnrypikrkyvaghtrpcvrrrllyepver
Consensus MY-----rRgs---QrY-R-Y-r---R-D-KRR-----k---dDP

ACMV(50) pfghnv1cEkQhGdvPnlqNTsytsFvtyysPrGpsgdgRsRdYIKLqsm
Consensus kM--QRIhENQgFGpFVM-hNtAISTF1-YP--GK-ePRNR-RSYIKLR

ACMV(100) svsgVihakanqDdpmevsqvngvFvFLim--DkPyl-PaGvqg
Consensus RFKGTmKIER--D-nMDG---PK-EGVFSLVlVDRKPHKL-pSGc--

ACMV(145) LpTFeELFGAsacyvNLrlLmnnqRhRyrvlHsvKRfvSssgDTkv
Consensus LHTDFELGARISIHsgGNL-v1P-LKDRFYIRHv-KRV-SVEKDT-M

ACMV(190) sqfrfnkrLsrrtyiWSFhDgDlnagynGnrNISKNAlLVsYafvSe
Consensus -D-EG-t-LSNr-NCW-tFKD-Dl-SC-GvY-NISKNAlLVYYCwMsd

ACMV(240) HamsckpFvqiEtSvYG
Consensus h-SKASTFVSFdLDY-G

Fig. 1. The predicted amino acid sequences (using single letter codes) of (a) the BC1 and (b) BV1 genes from ACMV (Kenyan strain) and the consensus of seven other bipartite geminiviruses are shown. Numbers indicate the position in the peptide sequence of the amino acid at the beginning of each line in the ACMV sequence. In the consensus sequence, upper case letters represent positions where an amino acid was conserved in five or more of the sequences and lower case letters represent positions where an amino acid was conserved in four sequences. Dashes show where no amino acid occurs more than three times. Upper case letters in the ACMV sequence show where the sequence is identical to the consensus. The asterisks are positioned above the sites selected for mutagenic studies.
Table 1. Summary of the mutations introduced into the DNA B ORFs BV1 and BC1.

<table>
<thead>
<tr>
<th>Construct*</th>
<th>Mutated ORF</th>
<th>Site of base change†</th>
<th>Codon alteration</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBV95M</td>
<td>BV1</td>
<td>870</td>
<td>AAG → ATG</td>
<td>Lys → Met</td>
</tr>
<tr>
<td>pBV95R</td>
<td>BV1</td>
<td>870</td>
<td>AAG → AGG</td>
<td>Lys → Arg</td>
</tr>
<tr>
<td>pBV229H</td>
<td>BV1</td>
<td>1269</td>
<td>AAT → CAT</td>
<td>Asn → His</td>
</tr>
<tr>
<td>pBV229Y</td>
<td>BV1</td>
<td>1269</td>
<td>AAT → TAT</td>
<td>Asn → Tyr</td>
</tr>
<tr>
<td>pBC52V</td>
<td>BC1</td>
<td>2108</td>
<td>GAC → GTC</td>
<td>Asp → Val</td>
</tr>
<tr>
<td>pBC52G</td>
<td>BC1</td>
<td>2108</td>
<td>GAC → GGC</td>
<td>Asp → Gly</td>
</tr>
<tr>
<td>pBC100Y</td>
<td>BC1</td>
<td>1965</td>
<td>CAT → TAT</td>
<td>His → Tyr</td>
</tr>
<tr>
<td>pBC100D</td>
<td>BC1</td>
<td>1965</td>
<td>CAT → GAT</td>
<td>His → Asp</td>
</tr>
<tr>
<td>pBC145I</td>
<td>BC1</td>
<td>1829</td>
<td>AAA → ATA</td>
<td>Lys → Ile</td>
</tr>
<tr>
<td>pBC145R</td>
<td>BC1</td>
<td>1829</td>
<td>AAA → AGA</td>
<td>Lys → Arg</td>
</tr>
<tr>
<td>pBC206Y</td>
<td>BC1</td>
<td>1646</td>
<td>GGA → GTA</td>
<td>Gly → Val</td>
</tr>
<tr>
<td>pBC206E</td>
<td>BC1</td>
<td>1646</td>
<td>GGA → GAA</td>
<td>Gly → Glu</td>
</tr>
</tbody>
</table>

* The names of the constructs indicate which open reading frame was mutated (BV or BC), the position of the altered amino acid in the gene product (e.g. residue 95) and the amino acid encoded after the introduction of the mutation, using the single letter abbreviations.
† The number refers to the position on ACMV DNA B.

al., 1987), abutilon mosaic virus (Frischmuth et al., 1990), squash leaf curl virus (SqiCV) (Lazarowitz & Lazdins, 1991), potato yellow mosaic virus (Coutts et al., 1991) and bean dwarf mosaic virus (BDMV) (Hidayat et al., 1993). The BC1 gene products showed a high level of homology in the first 190 amino acids with isolated blocks or single amino acids being conserved thereafter (Fig. 1a); whereas the BV1 gene products showed reduced homology, with fewer blocks of conserved amino acids being identified (Fig. 1b). From this information two conserved amino acids in BV1 (Lys-95 and Asp-228) and four conserved amino acids in BC1 (Asp-52, His-100, Lys-145 and Gly-206) were selected for mutagenesis. The two amino acid positions selected in BV1 were also conserved in the virus coat protein gene (AV1). These genes have previously been shown to be closely related by sequence homology (Kikuno et al., 1984).

For each of the amino acid positions selected, two mutant constructs were prepared. In one of these an amino acid with similar properties was substituted (e.g. Lys to Arg) while in the other a significantly different amino acid was substituted (e.g. Asp to Gly) where possible (Table 1). Because of significant preferences shown for codon usage in plants (Murray et al., 1989) consideration was given to codon usage in the design of the ACMV DNA B mutations so as to minimize any effect on the BC1 and BV1 gene expression. The codon bias for ACMV was examined (results not shown), demonstrating that only three of the 18 amino acids showed a preference for G or C in the third codon position. In ACMV the CG dinucleotide in second and third codon positions was infrequently used for serine or proline. Also the TA dinucleotide in second and third codon positions was infrequently used for leucine but was utilized for valine or isoleucine. Amino acid changes introduced into the BV1 and BC1 genes were made so as to reflect the ACMV codon bias.

Mutations were introduced into the DNA B construct pCL11 (Morris et al., 1988) using PCR by 30 cycles of 94 °C (30 s), 55 °C (30 s), 72 °C (2 min) using Taq DNA polymerase and oligonucleotides with a degenerate base. Two DNA fragments were generated for each mutant construct with pCL11 as a template and using pairs of specific oligonucleotide primers annealing to the 5' end of either the BV1 or BC1 gene and to the site of the mutation in one reaction and oligonucleotides annealing to the site of the mutation and to the 3' end of the same gene in the second reaction. The two DNA fragments for each construct were then joined in annealing and extension reactions (25 cycles) initially in the absence of primers, followed by further amplification (30 cycles) in the presence of the primers which annealed to the ends of the appropriate gene. Products containing a full-length but mutated BV1 or BC1 gene were subsequently cloned into the BamHI/EcoRI sites in pUC18 and mutations were confirmed by DNA sequencing. Mutations in the BV1 gene were cut out using XbaI and SphI and subcloned into the same sites in pCL11 to create the pbV95M, pBV95R, pBV229H and pBV229Y constructs. To generate constructs pBC52V, pBC52G, pBC100Y and pBC100D, pCL11 was digested with NcoI and SnaBI and the resulting fragment was replaced with one containing a mutation. The plasmids pBC145I, pBC145R, pBC206Y and pBC206E were created by cutting out a EcoRI–NcoI fragment and replacing it with the corresponding pieces from the pUC18 constructs. Each construct was sequenced to ensure that no additional errors had been introduced.

The infectivity of each of the DNA B constructs was analysed by co-inoculation with the wild-type DNA A.
construct, pCL10 (Morris et al., 1988), onto host plants. Plasmid pCL10 contained a single copy of ACMV DNA A which is able to induce an ACMV infection when inoculated with a construct containing the wild-type DNA B molecule. The ACMV DNA A and DNA B constructs were linearized and mixtures of pCL10 and each of the DNA B constructs were inoculated onto Nicotiana benthamiana, N. clevelandii and N. tabacum cv. Samsun plants to examine whether systemic spread was affected in different host plants. Each leaf inoculated was marked to ensure they were identifiable for later analysis. Control plants were inoculated with distilled water.

The results, summarized in Table 2, indicated that two out of the four mutations introduced into the BV1 open reading frame (ORF) and seven out of the eight mutations introduced into the BC10RF prevented the virus constructs from being infectious. The infection rate for the wild-type constructs depended on the host plant species used to induce an ACMV infection and varied from 66 to 100%, which is in agreement with those of other workers who achieved infection rates varying from 50 to 100% for the wild-type clones but recorded lower efficiencies with mutant constructs (Stanley, 1983; Etesami et al., 1988).

The systemic symptoms which resulted from the inoculation of N. benthamiana plants with linearized constructs containing wild-type ACMV DNA A and B, typically, did not appear for approximately 3 to 4 weeks, during which time growth of the inoculated plants appeared to progress normally. The symptoms of an ACMV infection were evident as deformed new leaves, which failed to expand and became covered in a light- and dark-green mosaic. The symptoms were clearly visible after 6 weeks and were equally severe in both N. clevelandii and N. benthamiana. Symptoms resulting from an ACMV infection in N. tabacum cv. Samsun showed similar deformed leaves and mosaic symptoms but were slightly milder than those observed on the other two test species. Control plants did not develop symptoms.

The inoculation of the pBV95M and pBV95R constructs with the wild-type DNA A construct onto N. benthamiana, N. clevelandii and N. tabacum cv. Samsun resulted in a systemic infection of ACMV. The symptoms observed in the N. benthamiana plants inoculated with DNA A plus pBV95M were indistinguishable from the wild-type, whereas the symptoms from the inoculation of pBV95R were milder; the leaves were able to expand but still appeared deformed and showed a mosaic pattern. This difference in symptoms from the inoculation of the pBV95M and pBV95R constructs was not evident on N. clevelandii, but was clearly apparent on N. tabacum cv. Samsun. The infectious nature of the pBV95M and pBV95R constructs demonstrated that Lys-95 in the BV1 gene product can be mutated without affecting the functioning of the gene product in the spread of the virus but may have decreased the infectivity of the viral constructs. The alteration to an arginine, a conservative substitution, enables interactions with other residues in the protein to be maintained, whereas the alteration to methionine was considered to be radical suggesting that Lys-95 is not in an important structural position on the molecule.

ACMV disease symptoms were not evident on any of the plants inoculated with either of the constructs pBV229H or pBV229Y, demonstrating that the intro-

### Table 2. Analysis of the mutant DNA B constructs

<table>
<thead>
<tr>
<th>Construct*</th>
<th>N. benthamiana</th>
<th>N. clevelandii</th>
<th>N. tabacum cv. Samsun</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DNA</td>
<td>0/23</td>
<td>0/5</td>
<td>0/7</td>
</tr>
<tr>
<td>Wild-type DNA B</td>
<td>27/39</td>
<td>8/12</td>
<td>7/7</td>
</tr>
<tr>
<td>pBV95M</td>
<td>15/33</td>
<td>2/10</td>
<td>5/9</td>
</tr>
<tr>
<td>pBV95R</td>
<td>11/33</td>
<td>3/11</td>
<td>1/9</td>
</tr>
<tr>
<td>pBV229H</td>
<td>0/22</td>
<td>0/4</td>
<td>0/9</td>
</tr>
<tr>
<td>pBV229Y</td>
<td>0/24</td>
<td>0/2</td>
<td>0/7</td>
</tr>
<tr>
<td>pBC52V</td>
<td>0/30</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td>pBC52G</td>
<td>0/32</td>
<td>0/7</td>
<td>0/9</td>
</tr>
<tr>
<td>pBC100Y</td>
<td>0/29</td>
<td>0/6</td>
<td>0/7</td>
</tr>
<tr>
<td>pBC100D</td>
<td>0/31</td>
<td>0/10</td>
<td>0/7</td>
</tr>
<tr>
<td>pBC145I</td>
<td>0/26</td>
<td>0/5</td>
<td>0/7</td>
</tr>
<tr>
<td>pBC145R</td>
<td>9/28</td>
<td>1/5</td>
<td>0/9</td>
</tr>
<tr>
<td>pBC206V</td>
<td>0/27</td>
<td>0/6</td>
<td>0/7</td>
</tr>
<tr>
<td>pBC206E</td>
<td>0/26</td>
<td>0/5</td>
<td>0/7</td>
</tr>
</tbody>
</table>

* The constructs were inoculated in addition to the DNA A construct pCL10.
† The number of infected plants was determined by the number showing symptoms of infection after a period of 6 weeks.
duced changes of Asp to His or Tyr prevented the constructs from being infectious.

No deformed or curled leaves typical of an ACMV infection were apparent on any of the plants which were inoculated with the wild-type DNA A construct and each of the constructs pBC52V, pBC52G, pBC100Y, pBC100D, pBC145I, pBC206V or pBC206E which contained mutations in the BC1 ORF. At position 52 of the BC1 gene neither replacement of an aspartic acid with a valine or a glycine residue was considered conservative. The alteration of His-100 to an aspartic acid residue was intended as a non-conservative substitution reducing the size of the side chain, but the alteration to a tyrosine was a relatively conservative change. The lack of any symptoms indicated a lack of tolerance to change at these positions.

The alteration of the hydrophilic lysine residue, to an isoleucine, which is hydrophobic, in construct pBC145I was a non-conservative change. This construct was not infectious on any of the test plants indicating that the mutation caused malfunctioning of the protein and prevented normal viral spread. However, when *N. benthamiana* and *N. clevelandii* plants were inoculated with DNA A and the DNA B construct pBC145R, which contained an arginine at the same position, the plants exhibited severe deformation of the leaves and mosaic patterns, symptoms typical of those induced by ACMV, indicating that the mutation in this construct was tolerated by the virus. None of the *N. tabacum* cv. Samsun plants inoculated with the pBC145R construct became infected, although it remains to be determined whether the inoculation of large numbers of this host would reveal a low level of infectivity or whether the amino acid at this position determines differential symptoms in host plants.

Prediction of the secondary structure of the BV1 and BC1 proteins using the University of Wisconsin GCG 'peptidestructure' program indicated that the amino acids surrounding the mutated sites at positions 95 and 229 in the BV1 gene product and 52 and 100 in the BC1 gene product were likely to fold into β-sheets. However, the predicted structure for the region around Lys-145 in the BC1 gene product was an α-helix and may explain the tolerance to the arginine (which, similar to lysine, has a positively charged side chain), but not isoleucine, at this position.

Finally, mutagenesis of the second base of the glycine codon at position 206 in the BC1 protein (constructs pBC206V and pBC206E), introducing a valine or a glutamic acid residue, proved to be non-infectious. The glycine residue was preceded by a proline, a combination of amino acids that is predicted to be involved in a turn in the peptide structure. The conserved Pro–Gly–Glu peptide sequence occurs in a region of the protein which is not otherwise highly conserved suggesting that it is important for protein integrity. Consideration of this putative secondary structure may explain why the increased bulk of the valine, or the size and charge of the glutamic acid did not permit the normal functioning of the BC1 gene product.

To confirm the presence of ACMV DNA in systemically infected plants and to ascertain if the virus spread locally in plants which did not show systemic symptoms, sap was extracted from new leaves and from the inoculated leaves of individual plants approximately 6 weeks post-inoculation and treated as described by Maule et al. (1983). A probe corresponding to a fragment of ACMV DNA B hybridized to the DNA in the sap samples from new and inoculated leaves of *N. benthamiana* (Fig. 2) and *N. clevelandii* plants which had been inoculated with constructs containing ACMV DNA A and DNA B (wild-type), pBV95M, pBV95R and pBC145R. The probe also hybridized to the DNA in the samples extracted from *N. tabacum* cv. Samsun which had been inoculated with ACMV DNA A and DNA B (wild-type), pBV95M and pBV95R, but did not hybridize to DNA in the sap samples from plants inoculated with pBC145R, control plants, or any of the host plants inoculated with the nine constructs which failed to induce ACMV disease symptoms. These results confirmed that the symptoms observed were the result of ACMV infection and demonstrated that the constructs which were unable to spread systemically were also restricted from spreading locally within the originally inoculated leaf. No evidence was found that suggested that the milder symptoms observed on the *N. benthami-
Fig. 3. Amplification reactions using DNA extracted from systemically infected \textit{N. benthamiana} plants analysed on 1% agarose gels. (a) PCR was performed using primers to the BV1 gene with ACMV DNA A (pCL10) and DNA B (pCL11) (lane 1), DNA A plus pBV95M (lane 4) and DNA A plus pBV95R (lane 5). (b) PCR was performed using primers to the BC1 gene with DNA extracted from the sap of plants inoculated with distilled water (lane 3), ACMV DNA A and DNA B (lane 4) and DNA A plus pBC145R (lane 5). Markers are shown on gels (a) and (b) in lanes 3 and 1, respectively, and sizes are given in base pairs. Reactions in lane 2 of both gels were carried out using distilled water.

\textit{ian}a or \textit{N. tabacum} cv. Samsun plants that were inoculated with DNA A plus pBV95R were due to lower levels of the DNA B construct.

DNA was extracted from the sap of systemically infected \textit{N. benthamiana} plants, (essentially as described by Dellaporta \textit{et al.}, 1983) to analyse the maintenance of the introduced mutations during replication and systemic spread of the virus. Subsequent amplification reactions resulted in bands equivalent to the BV1 ORF being detectable in the sap extracted from plants which had been inoculated with constructs pBV95M, pBV95R and wild-type DNA B (Fig. 3). Similarly, bands corresponding to the BC1 ORF were produced from the sap extracted from plants inoculated with pBC145R and wild-type DNA B. No bands equivalent to products from the BV1 or BC1 ORF were observed from extracts from control plants. The amplification products from the systemically infected plants were subcloned and the DNA sequence determined. No mutations were found in the wild-type constructs, but the DNA derived from plants inoculated with the constructs pBV95M, pBV95R and pBC145R contained the introduced mutations demonstrating that these mutations did not affect the ability of the virus to replicate in a systemic host or to spread to uninoculated leaves.

Nine of the constructs containing DNA B mutations did not produce ACMV disease symptoms on \textit{N. benthamiana}, presumably because of their inability to spread owing to the altered movement gene. To analyse the ability of these constructs to replicate, leaf discs were agroinoculated with a DNA A construct and each of the nine mutant constructs (Morris \textit{et al.}, 1988). Southern blot analysis showed that all nine mutants replicated in a leaf disc system (A. Haley, unpublished results).

Inoculations were also carried out to determine whether the infectious mutants of ACMV were sap transmittable. Sap was extracted from \textit{N. benthamiana} plants which had become systemically infected after co-inoculation with constructs containing ACMV DNA A and one of the mutant DNA B constructs pBV95M, pBV95R, pBC145R or the wild-type DNA B construct and inoculated onto the leaves of healthy \textit{N. benthamiana} plants. The development of symptoms typical of an ACMV infection demonstrated that the virus which had accumulated in the leaves of the previously inoculated \textit{N. benthamiana} plants was transmittable via sap in all cases.

The alignments of the DNA B gene products highlight specific areas of conservation and give some indication of the important regions within these proteins. Use of the Protein interpretation program (Staden, 1990) to search the amino acid sequences encoded by the BC1 and BV1 genes did not reveal any conserved membrane or nucleotide binding sites that may have been expected considering the likely interaction of the transport proteins with the membrane at the site of plasmodesmata and possible involvement in the binding of the viral genome for transportation throughout the plant.

Complementation experiments have been carried out where the DNA B ORFs of TGMV have been inserted into ACMV DNA A in place of the coat protein (von
Arnim & Stanley, 1992b). These constructs were analysed for their ability to induce an infection in the common host *N. benthamiana* but neither the BV1 or BC1 gene products from TGMV was able to complement its ACMV homologue. Co-inoculation of both DNA B genes from TGMV (in ACMV DNA A vectors) was followed by replication and the induction of the symptoms of ACMV infection on inoculated leaves, but no systemic symptoms were observed. When a DNA A construct containing the TGMV BC1 ORF was inoculated with ACMV DNA B, the ACMV infection was suppressed. The presence of symptoms on inoculated leaves suggested that this suppression was acting at the level of long-distance spread.

In addition to the involvement of the DNA B-encoded proteins in the movement of the virus, it has been demonstrated that the severity of symptoms of TGMV and ACMV infections was related to the level of DNA B (Revington *et al.*, 1989; Stanley *et al.*, 1990), although this contradicted earlier work where the induction of vein necrosis was shown to segregate with DNA A (Stanley *et al.*, 1985). Experiments using pseudorecombinants of two strains of TGMV also supported the involvement of DNA B in symptom production and indicated that the phenotypes observed correlated with a fragment of DNA containing the BL1 gene (equivalent to BC1 in ACMV) and most of the common region (von Arnim & Stanley, 1992a). Symptom development has also been mapped to BL1 in SqLCV (Pascal *et al.*, 1993).

The essential differences between the genome of tomato yellow leaf curl virus (TYLCV) S strain and ACMV DNA A, which enable the former to infect dicotyledonous hosts (Kheyr-Pour *et al.*, 1992), are still unclear. However, reports have indicated that agro-inoculated ACMV DNA A is able to replicate to low levels and spread systemically in the absence of DNA B, but fails to produce any symptoms (Klinkenberg & Stanley, 1990), again showing the relevance of DNA B for symptom induction. Beet curly top virus (BCTV) genome, which again resembles ACMV DNA A in layout, is able to induce severe symptoms on *N. benthamiana*. Both TYLCV and BCTV are, however, limited to the phloem of infected plants (Mumford & Thornley, 1977; Cherif & Russo, 1983). This suggests that the DNA B-encoded gene products are responsible for cell-to-cell spread, but may not necessarily be required for longer distance movement of the viral genome via the phloem.

Microinjection studies using BDMV have now shown that, in addition to its role in symptom production, BL1 (BC1) is involved in increasing the size of the exclusion limit of plasmodesmata and is able to move to neighbouring cells (Noueiry *et al.*, 1994). This correlates with its cellular location as it cofractionated with the cell wall (von Arnim *et al.*, 1993; Pascal *et al.*, 1993). The use of fluorescently labelled nucleic acid also demonstrated the ability of BC1 to assist in the rapid movement of double-stranded but not single-stranded DNA from the injected cell. This is in contrast to SqLCV BL1 which bound weakly to ssDNA but was unable to form a complex with dsDNA (Pascal *et al.*, 1994). Whether these differences are dependent upon the source of the protein or are species specific remains to be determined.

Although BDMV BR1 (BV1) does not appear to be involved in interactions with plasmodesmata, it has now been shown to mediate the movement of ss- and dsDNA out of the nucleus (Noueiry *et al.*, 1994), again correlating with the localization of the BR1 protein with the microsomal membrane fraction (Pascal *et al.*, 1993). However, Pascal *et al.* (1993) demonstrated that although SqLCV BR1 binds strongly to ssDNA, and also binds to RNA, it did not bind with dsDNA. The overall picture emerging indicates that both DNA B-encoded proteins are required for local spread of the virus.

The results presented here have identified specific regions within the BV1 and BC1 gene products that have been conserved in the different bipartite geminiviruses. Selected amino acids within these proteins have been altered by mutagenesis of the coding sequence. This has identified five amino acids that are essential for normal functioning of the DNA B-encoded products and for infection by the virus. Lys-95 within the BV1 gene product has also been identified as not being important either structurally or in the movement of the virus in the host plants used in this study, but does not rule out a possible role in viral spread in other hosts or in quantitative effects such as the level of replication. The introduction of glutamic acid in the same position in BDMV rendered the virus non-infectious (Noueiry *et al.*, 1994). This may possibly be explained by the change in charge from the original lysine.

These results presented here correlate with those of other workers as none of the mutations that prevented systemic spread, permitted local spread of the virus, therefore providing additional evidence that both DNA B genes are involved in cell-to-cell and long-distance spread of the virus. However, the ability of TYLCV, BCTV and to some extent ACMV DNA A to spread, leads us to hypothesize that DNA A is also involved in long-distance spread. It is likely that this has so far remained undetected as the DNA A genes are essential for replication and as yet the two functions have remained inseparable.

**References**


