Functional analysis of proteins encoded by banana bunchy top virus DNA-4 to -6

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Green fluorescent protein (GFP)-tagging was used to determine the intracellular localization pattern of the proteins encoded by banana bunchy top virus (BBTV) DNA-3, -4 and -6. The protein encoded by BBTV DNA-4, which possesses a hydrophobic N terminus, was found to localize exclusively to the cell periphery while the proteins encoded by BBTV DNA-3 and -6 were found in both the nucleus and the cytoplasm. Co-expression of the DNA-4 protein and the proteins encoded by BBTV DNA-3 and -6 revealed that the DNA-4 protein was able to re-locate the DNA-6 protein, but not the DNA-3 protein, to the cell periphery. The 29 amino acid N-terminal hydrophobic region of the DNA-4 gene product appeared to be essential for specific localization of this protein since deletion of this region abolished its ability to localize to the cell periphery. These results indicate that BBTV may utilize a system analogous to that of the begomoviruses with the BBTV DNA-6 protein acting as a nuclear shuttle protein (NSP) while the DNA-4 protein transports the NSP–DNA complexes to the cell periphery for intercellular transport. The protein encoded by BBTV DNA-5 was found to contain an LXCXE motif and yeast two-hybrid analysis revealed that the DNA-5 protein has retinoblastoma (Rb)-binding activity. This activity was dependent on an intact LXCXE motif since specific mutations to either the C or E residue completely abolished Rb-binding activity. These results indicate that the gene product of BBTV DNA-5 is an Rb-binding-like protein and may play an important role in host-cell cycle manipulation.

Introduction

Banana bunchy top virus (BBTV) is an important pathogen of bananas (Musa spp.) in Australia, the South Pacific and parts of Asia and Africa. BBTV is a member of the proposed plant nanovirus group, which includes subterranean clover stunt virus (SCSV; Boevink et al., 1995), faba bean necrotic yellows virus (FBNYV; Katul et al., 1998), coconut foliar decay virus (CFDV; Rohde et al., 1990) and milk vetch dwarf virus (MDV; Sano et al., 1998). BBTV has a multi-component genome consisting of at least six ssDNA components (Burns et al., 1995). The functions of two DNA components have been identified; DNA-1 encodes a replication-associated protein (Rep) (Harding et al., 1993; Burns et al., 1995; Hafner et al., 1997a) while DNA-3 encodes the coat protein (CP) (Wanitchakorn et al., 1997). The functions of the proteins encoded by the remaining BBTV DNA components have not yet been determined. The nanoviruses, including BBTV, have a number of characteristics or predicted characteristics in common with geminiviruses including replicative strategy and movement in to and out of the nucleus as well as intercellular movement (Burns et al., 1995; Hafner et al., 1997b). It is likely, therefore, that the genes and the gene functions encoded by geminiviruses will have counterparts in BBTV.

The geminivirus replication cycle is dependent on host cellular proteins and is generally restricted to, or largely favoured in, S-phase cells (Acotto et al., 1993). It has been suggested that some plant viruses encode proteins which modify cell-cycle regulation in terminally differentiated cells of infected plants via a mechanism(s) similar to that found in animal cells (Nagar et al., 1995; Guiterrez, 1998). The replication protein (RepA) of wheat dwarf virus (WDV) has been shown to bind specifically to the members of the retinoblastoma (Rb) family of proteins, possibly through interactions with their pocket domain (Xie et al., 1995). The binding of RepA to Rb is believed to occur via an LXCXE motif, which is conserved in animal DNA virus oncoproteins as well as in a number of geminivirus replication proteins including those from maize streak virus (MSV; Mullineaux et al., 1984),

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Digitaria streak virus (DSV; Donson et al., 1987), tobacco yellow dwarf virus (TobYDV; Morris et al., 1992), Panicum streak virus (PSV; Briddon et al., 1992) and bean yellow dwarf virus (BeYDV, Li et al., 1999). Furthermore, Xie et al. (1996) and Grafi et al. (1996) both reported the isolation of plant Rb-like proteins from maize. These proteins contained a conserved pocket domain and were able to interact with known Rb-binding proteins as well as plant D-type cyclin. These findings further confirm the possibility of Rb-mediated cell-cycle regulation in plant cells and also the ability of some plant viruses to manipulate their host-cell cycle to enhance replication. It is possible, therefore, that BBTV encodes a gene that is capable of influencing host-cell cycling. Interestingly, it has been shown that while point mutations within this motif severely diminish or completely eliminate binding of these proteins to Rb, these mutations may or may not affect virus replication. The RepA mutants of BeYDV were shown to retain their ability to replicate and systemically infect Nicotiana benthamiana and bean plants while replication of WDV mutants in wheat cell suspensions was significantly reduced or eliminated (Xie et al., 1995; Li et al., 1999).

The bipartite geminiviruses (begomoviruses) appear to encode two movement proteins, BV1 and BC1. These proteins are non-structural and are not required for replication or encapsidation but are essential for cell-to-cell and long-distance movement (Brough et al., 1988, Noueiry et al., 1994). Sanderfoot & Lazarowitz (1995) proposed that BV1 of squash leaf curl virus (SqLCV) binds the replicated SqLCV in the nucleus and shuttles the complex out to the cytoplasm where BC1 specifically binds the BV1–DNA complexes and directs them to the cell periphery, where they are transported into the adjoining cells. The requirement of both proteins for intercellular movement was also recently demonstrated for bean dwarf mosaic virus, where mutation of the BV1 and BC1 proteins restricted the cell-to-cell movement of viral DNA (Sudarshana et al., 1998).

The members of the mastrevirus group of monopartite geminiviruses encode a single movement protein (PV1) which has been shown to be associated with the plasmodesmata (Boulton et al., 1993; Dickinson et al., 1996). However, as the CP of MSV has been shown to possess strong ss- and dsDNA binding activities, it was proposed that the CP might fulfill a nuclear shuttle function, analogous to the BV1 protein of begomoviruses. Such CP–DNA complexes may then be recognized by the movement protein and redirected to the cell periphery for intercellular transport (Liu et al., 1997).

Burns et al. (1995) identified a short stretch of hydrophobic residues in the N terminus of the predicted gene product of BBTV DNA-4 and suggested that it may be a viral movement protein. Similar hydrophobic regions have been identified in the predicted gene products of FBNYV component 4 (C4) and SCSV component 1 (C1) and these are structurally similar to the movement protein of MSV (Boulton et al., 1993; Katul et al., 1997).

In this paper, we examined the localization of ‘green fluorescent protein (GFP)-tagged’ gene products of three BBTV DNA components in banana embryos and provide evidence that BBTV DNA-4 and DNA-6 encode proteins that are involved in virus movement. Further, we report that the gene product of DNA-5 is able to interact with the human Rb protein (p130) and may be an Rb-binding-like protein.

**Methods**

- **GFP fusion constructs.** Primers ORF3C.BAM (5' ACG CGG ATC CAT GGC TAG GTA TCC GC 3') and ORF3CR.PRI (5' AAC ATG ATA TGT AAT TCT GTT CTG G 3') and ORF4C.BAM (5' ACG CGG ATC CAT GCC ATT AAC AGA AGA 3') and BT4C2.PRI (5' GAA CAT AGG TCC AGC GT 3'), ORF6C.BAM (5' ACG CGG ATC CAT GGA GTT CGT GGA AT 3') and ORF6CR.PRI (5' TTT CTT GAT TCT TAA CGA AC 3') were used to amplify the entire gene, excluding the stop codon, of BBTV DNA-3, -4 and -6, respectively, using total DNA extracts from Australian BBTV-infected plants as template. PCR reactions were carried out using Pwo DNA polymerase (Boehringer Mannheim). The resulting PCR products were digested with BamHI, gel-purified using a Wizard purification kit (Promega) and cloned into BamHI/SnaI-digested pUBi-GFP (Dudgale et al., 1998). The resulting expression plasmids (pUBi-gene3/4/6-GFP) contained each BBTV gene sequence in-frame with the coding sequence of codon-altered GFP reporter gene (eGFP, Chiu et al., 1996), driven by a maize polyubiquitin (Ubi) promoter, followed by a nonaprole synthase (nos) terminator (Fig. 1). The deletion mutants of the BBTV DNA-4 gene were amplified using two specific sets of oligonucleotides. An N-terminal deletion mutant (ΔN) was amplified using Gene4ANT.PRI (5' CTT TGG ATC CAT GAG GAT TAT TAA GGA GC 3') and BT4C2.PRI while a C-terminal deletion mutant (ΔC) was amplified using BT4ATG.BAM (5' CTA GAG GAT CCA TGG CAT TAA CAA CA 3') and Gene4ACT.PRI (5' CGG TAC CTC AAA GAG CAA AAC 3'). The two gene deletions were cloned into BamHI/SnaI-digested pUBi-GFP and pGEM-Ubi-nos (J. McMahon, unpublished data) resulting in plasmids pGEM-Ubi-gene4ANT/CT-GFP and pGEM-Ubi-gene4ANT/CT-nos, respectively. The integrity of all BBTV coding sequences was determined by comparison with the published BBTV genome sequences (Burns et al., 1995).

- **Microprojectile bombardment of banana embryogenic cells.** Embryogenic suspension cultures of banana cv. ‘Bluggoe’ (Musa spp. – ABB) were maintained and harvested as described by Dudgale et al. (1998). Plasmids used for microprojectile bombardment were purified using a BresaGen Plasmid Maxi-Kit (Bresatec) according to the manufacturer’s instructions. Each embryogenic preparation was bombarded using a particle inflow gun. Preparation of 1 µm microcarrier gold particles (Bio-Rad) and coating of plasmid DNA were as described by Dudgale et al. (1998).

- **Visualization of GFP fluorescence.** GFP expression in the embryogenic cell preparations was determined 3 days after bombardment using a Leica MZ12 stereo microscope with GFP-Plus fluorescence module and green barrier filter (BG22, Chroma Technology). Strongly fluorescing embryos were removed using a sterile scalpel and placed into a 12-well microculture plate (Nunc) containing a small volume of sterile water. To minimize excessive vibration during confocal microscopy, a molten solution of 2% Phytagel (Sigma) was added to each well and allowed to set at room temperature. The cells collected were examined by confocal microscopy using a Leica TCS-4D CLSM with an argon/krypton laser and FITC filter set (488 nm excitation, 507 nm emission). The scanned images were directly recorded in TIFF format and compiled.
using the PowerPoint program (Microsoft). On average, three separate bombardments were carried out for each of the test combinations and approximately 20 representative cells were removed from the cell clumps for examination with the CLSM.

**Construction of plasmids used in two-hybrid analysis.** Primers BT5R.BAM (5'-CTG CAG GGA TCC TTA GAG TGT TAC 3') and BT5F.ECO (5'-GAG CTC GAA TAC ATG GAG TCC TGG GAA 3') and BT1R.BAM (5'-CTG CAG GGA TCC GCA AGA CAA CTT 3') and BT1F.ECO (5'-CTG CAG GGA TCC ATG GCC GGA TAT GTG 3') were used to amplify the full gene, excluding the stop codon, of BBTV DNA-5 and DNA-1, respectively, from total DNA extracts of Australian BBTV-infected plants. PCR reactions were carried out using Pwo DNA polymerase (Boehringer Mannheim). The resulting PCR products were double-digested with BamHI and EcoRI gel-purified using the Wizard purification kit (Promega) and cloned into BamHI/EcoRI-digested pGBT9 (Clontech). The resulting plasmids (pGBT9-gene5/gene1) contained each of the BBTV gene sequences in-frame with the coding sequence of the Gal4 DNA-binding domain (BD). Two specific mutants were generated from pGBT9-gene5; the primers BT5-LXCAG (5'-TCA AGA TCT TTA TGG TGA TAA GGT A 3') and BT5-LXEAK (5'-TCA AGA TCT TTA TGG TGA TAA GGT A 3') were used in conjunction with the Gal4BD sequencing primer (Clontech) to produce the C→G (amino acid 113) and E→K (amino acid 115) substitutions, respectively, in the LXCXE motif of DNA-5. The BglII-PsiI fragment of pGBT9-gene5 containing the wild-type gene5 sequence was replaced with the BglII/PsiI-digested fragments containing the point-specific mutations to produce pGBT9-LXCAG and pGBT9-LXEAK containing the (C113G) and (E115K) substitutions, respectively. The integrity of all BBTV coding sequences was determined by comparison with the published BBTV genome sequences (Burns et al., 1995).

**Yeast two-hybrid analysis.** The yeast strains SFY526 (Harper et al., 1993) with the lacZ reporter gene, and HF7c (Feilotted et al., 1994), which contains both lacZ and HIS3 reporter genes, were used for two-hybrid analysis (Fields & Song, 1989). Yeast was transformed with Gal4 activation domain (AD) and BD constructs as previously described (Geitz & Schiestl, 1995) using approximately 1 μg of each plasmid per transformation. The transformation mixture was plated on the appropriate yeast synthetic drop-out (SD) medium to recover transformants (Bartel et al., 1993a). To screen for activation of the HIS3 reporter gene, the HF7c co-transformants were transferred to a drop-out selection medium lacking leucine, tryptophan and histidine and containing 10 mM 3-amino-1,2,4-triazole (Bartel et al., 1993b). SFY526 was also used to confirm positive protein–protein interactions. β-Galactosidase activity was assayed using a colony-lift filter method (Hannon et al., 1993). As a control for reporter genes, the plasmids pCL1, encoding the wild-type GAL4 protein (Fields & Song, 1989), and a known interacting pair of pTD1-encoding AD/SV40 large T-antigen (Li & Fields, 1993) and pVA3-encoding BD/murine p53 proteins (Iwabuchi et al., 1993) were used in each transformation. Two members of the Rb protein family were used in this study; the plasmid pGAD-Rbr2 encoded the large pocket domain of p105(Rb) fused to AD (Hannon et al., 1993), while pGAD-Rb1 encoded a fusion of full-length Rb1 from maize and AD (Xie et al., 1996).

**Results**

**Cellular localization of the protein products of BBTV DNA-3, -4 and -6**

Banana cv. ‘Bluggoe’ embryogenic cell suspensions were bombarded with gold particles coated with each plasmid construct. These cells were first examined with the GFP...
compound microscope, enabling expressing cells to be isolated from the embryogenic cell clumps. The localization of GFP was then recorded using a Leica CLSM. Cells bombarded with the native GFP control exhibited fluorescence in both the cell cytoplasm and nucleus (Fig. 2a). In contrast, cells bombarded with the gene4–GFP fusion construct showed bright highly localized fluorescence around the cell periphery (Fig. 2b). Fluorescence associated with GFP-tagged gene3 and gene6 proteins was also found throughout the cell but was most strongly localized to the nucleus (Fig. 2c, d).

To determine whether the protein encoded by BBTV DNA-4 was able to interact with other BBTV-encoded proteins, the GFP-fusion constructs of BBTV gene3 and gene6 were co-bombarded with an expression construct containing native BBTV gene4 driven by a Ubi promoter (pUbi-gene4). The embryos co-expressing both gene6–GFP and gene4 showed a marked increase in localization of GFP around the cell periphery compared with those expressing native gene6–GFP alone (Fig. 2e, d). The embryos transformed with gene3–GFP and gene4 showed a similar localization pattern to the embryos transformed with gene3–GFP alone (Fig. 2f, c).

The protein encoded by BBTV DNA-4 has been predicted to have a hydrophobic β-sheet in the N terminus (Burns et al., 1995). To determine whether this region was responsible for the GFP localization pattern, two deletion mutants were constructed. The first mutant encoded DNA-4 protein without the 29 hydrophobic amino acid residues (residues 10–38) from the N terminus (gene4∆NT) while the second consisted of this 29 amino acid N-terminal region alone (gene4∆CT). GFP tagging of these mutants showed that deletion of the hydrophobic N terminus abolished the ability of the gene4 protein to localize to the cell periphery since the gene4∆NT–GFP fusion protein was subsequently found to localize in the cytoplasm (Fig. 2g). Further, there was very little fluorescence in the nucleus compared to the GFP fusions with gene3 and gene6. The C-terminal deletion mutant showed relatively
BBTV DNA-5 encodes an Rb-binding-like protein

The sequences of the predicted gene products of BBTV DNA-1 to -6, as well as the gene products of other nanoviruses and geminiviruses, were examined for the presence of the LXCXE motif normally associated with Rb-binding function. The predicted gene product of BBTV DNA-5 contained an LXCXE motif normally associated with Rb-binding function. The yeast two-hybrid system was used to determine the ability of the BBTV DNA-5 gene product to interact with known Rb-like proteins. The entire gene of DNA-5 was cloned into a yeast strain, HF7c, and the co-transformants assayed for protein–protein interactions via GAL4 activity. The HF7c co-transformants containing both pGAD-Rbr2 and pGBT9-gene6 were able to grow in the absence of His and Gal4-AD, respectively, with their interacting partners WDV RepA and human CDK2, in-frame fusions and human laminC, respectively, were obtained as in-frame fusions and human laminC, which directs low-level fusion protein expression in yeast cells. Two plasmids, pBD-ORF5 and pGAD424, encoding native AD, were used as controls for non-specific binding. The known interacting pair, AD–human laminC protein (pLAM5) and BD–murine p53 (pVA3), was used as a positive control for both reporter genes. Plasmid pCL1 encodes the wild-type full-length GAL4 gene. Positive growth on SD–His/Leu/Trp for HF7c transformants or β-galactosidase activity by filter assay for SFY526 transformants. – No growth on SD–His/Leu/Trp for HF7c transformants or no β-galactosidase activity by filter assay for SFY526 transformants. NA, Assay not applicable – transformants contain only one growth plasmid. HF7c cells transformed with pCL1 are β-galactosidase positive.

Table 1. Rb-binding ability of BBTV DNA-5 protein determined by yeast two-hybrid analysis

<table>
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<tr>
<th>Test/control vector</th>
<th>pGBT9-gene5</th>
<th>pGBT9LXCAG</th>
<th>pGBT9LXEAK</th>
<th>pGADRb1</th>
<th>pGADRbr2</th>
<th>pTD1</th>
<th>pCL1</th>
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<tr>
<td>Growth on SD–His/HIS</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Leu/Trp (HF7c)</td>
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<tr>
<td>β-Galactosidase activity (SFY526)</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>+</td>
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*SD–His/Leu/Trp contained 10 mM 3-AT.
colonies in HF7c showed weak β-galactosidase activity when compared to the SFY526 transformants, and no blue coloration was observed for the Rbr2/gene5 co-transformants after overnight incubation. This suggested that the interaction between the DNA-5 gene product and Rbr2 was relatively weak, or was transient in nature and therefore not detectable by the weakly expressing β-galactosidase marker in HF7c. In order to confirm this interaction, similar co-transformants were generated in the SFY526 host strain, which contains a highly expressed lacZ marker. Using this host strain, positive β-galactosidase activity was detected for Rbr2/gene5, Rbr2/CDK2 and Rb1/RepA co-transformants using colony-lift filter assays. As expected, transformants containing either pTD1/pVA3 or pCL1 were also found to express the β-galactosidase marker while transformants containing the BD–gene5 fusion and AD plasmids did not show detectable activation of this marker gene (Table 1).

To confirm the involvement of the LXCXE motif in Rb-binding, point-specific mutants were made with amino acid substitutions at the C (C113G) or E (E115K) residue. Both binding, point-specific mutants were made with amino acid substitutions at the C (C113G) or E (E115K) residue. Both substitutions were shown to severely reduce/abolish the ability of the BBTV DNA-5 gene product to bind Rbr2. The effect of these two substitutions was confirmed by the HIS marker in HF7c and also by lacZ screening in SFY526 (Table 1), indicating that binding of Rb by the DNA-5 gene product specifically involved the LXCXE motif.

Discussion

We have demonstrated that the protein encoded by BBTV DNA-4 is targeted to the cell periphery of banana embryogenic cells whereas the proteins encoded by DNA-6 and the coat protein (DNA-3) are localized in both the nucleus and cell cytoplasm. We have also shown that the DNA-4 gene product was able to redirect the DNA-6 gene product to the cell periphery but does not appear to interact with the coat protein. The 29 amino acid N-terminal hydrophobic region of DNA-4 gene product appeared to be essential for specific localization to the cell periphery.

These results provide the first evidence that BBTV DNA-4 encodes the virus movement protein. Further, it appears that the protein encoded by BBTV DNA-6 is a nuclear shuttle protein, as it is preferentially targeted to the nucleus when expressed alone but, in the presence of the movement protein, is targeted to the cell periphery. The characteristics of the proteins encoded by BBTV DNA-4 and DNA-6 are very similar to those of the BC1 (movement) and BV1 (nuclear shuttle) proteins, respectively, of squash leaf curl and other begomoviruses (Sanderfoot & Lazarowitz, 1995; Noueiry et al., 1994). This result was somewhat surprising, since it was expected that BBTV movement would be similar to that of the monocot-infecting mastreviruses in which the coat protein acts as a nuclear shuttle protein. However, FBNNV C4, MDV C8 and SCSV C1 each encode a 13 kDa protein similar to that of BBTV DNA-4, while FBNYV C8, MDV C6 and SCSV C4 encode proteins equivalent to that of BBTV DNA-6 (Katul et al., 1998; Sano et al., 1998). Since these viruses infect dicots, it would appear that, unlike the geminiviruses, the movement of nanoviruses does not differ between dicot and monocot hosts and more closely resemble the begomoviruses.

We have also demonstrated that the gene product of BBTV DNA-5 is able to bind a member of the Rb family of proteins and that site-specific mutations in either the C or E residues of the LXCXE motif severely reduced/abolished this interaction. It has previously been shown that the RepA protein of WDV, which also contains a LXCXE motif, also has the ability to bind to Rb (Xie et al., 1996). Interestingly, the Rep proteins (AC1) of begomoviruses do not have an LXCXE motif, but can still bind Rb in vitro (Ach et al., 1997). Further, expression of tomato golden mosaic virus is sufficient to induce the accumulation of the host DNA synthesis protein, proliferating cell nuclear antigen (PCNA), in terminally differentiated cells of tobacco plants which would suggest a role analogous to that of the animal virus oncoproteins (Nagar et al., 1995).

Thus, it would appear that the geminiviruses, and possibly the nanoviruses such as FBNNV, MDV and SCSV, which have DNA components equivalent to BBTV DNA-5, all encode proteins that can influence the accumulation of host DNA-replication proteins. It has been proposed that these Rb-binding proteins are responsible for altering the host cellular environment to facilitate viral DNA replication (Graft et al., 1996), a function which would require the protein to be expressed early in the virus replication process. This hypothesis is supported by the results of Dugdale (1998), who reported that the promoter region of BBTV DNA-5 directed one of the highest levels of transient GFP expression of any of the BBTV promoter regions in both banana embryogenic cells and regenerated plants where its expression was near constitutive. Further, Hafner et al. (1997 b) demonstrated that BBTV DNA-5 is far more efficiently self-primed than any of the other BBTV DNA components. These observations suggest that, upon infection, DNA-5 is the first component to be converted to the transcriptionally active dsDNA replicative form (RF) and that the DNA-5 gene product would be produced at high levels in virtually any cell type. The presence of this Rb-binding protein would be expected to result in the switching of these first infected cells to S-phase, an environment that enhances viral DNA replication. Interestingly, the mastreviruses, begomoviruses and the nanoviruses each appear to have a different strategy for expressing the protein required for cell-cycle manipulation. The mastreviruses incorporate this function into their RepA proteins, which have an LXCXE Rb-binding motif, while the begomoviruses also incorporate this function into Rep but these proteins do not have an LXCXE motif. Unlike the mastreviruses, the nanovirus Rep proteins do not possess a LXCXE motif. This motif is, however, contained in a protein encoded by another DNA component.
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References


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