Independent expression of Rep and RepA and their roles in regulating bean yellow dwarf virus replication

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Bean yellow dwarf virus (BeYDV) is a mastrevirus specific for dicotyledenous hosts. It contains four ORFs encoding a movement protein, a coat protein, and two Rep gene products, Rep and RepA, which are encoded by two overlapping ORFs. In this study, the roles of Rep and RepA in regulating replication of the BeYDV-based replicon were investigated by uncoupling them and placing Rep and RepA each under constitutive promoter control. Constitutive expression of both Rep and RepA supported replication and enhanced gene expression. When a reporter plasmid containing the Rep gene in the context of its native promoter was supplemented with additional Rep protein, replication was enhanced but the increase in gene expression was found to be more modest. Furthermore, expression of constitutively expressed RepA alone was found to reduce replication of this reporter construct as well as delay BeYDV replication in general. The effect of a RepA mutant with an altered retinoblastoma-related-protein binding motif on the efficiency of BeYDV replication was also examined. This mutant was found to severely diminish replication efficiency. Finally, the relationship of BeYDV coat protein to virus replication and reporter gene expression was investigated. Addition of coat protein increased accumulation of single-stranded DNA and had a detrimental effect on reporter gene expression.

INTRODUCTION

Bean yellow dwarf virus (BeYDV) is a member of a family of small, single-stranded (ss) DNA plant viruses known as the geminiviruses (Liu et al., 1997a). Since both BeYDV and tobacco yellow dwarf virus (TYDV), another closely related virus, possess a monopartite genome resembling that of mastreviruses and are transmitted by leafhoppers, they have been placed into a subclass of the mastreviruses whose host range is restricted to dicots (Morris et al., 1992; Liu et al., 1997a).

BeYDV contains four conserved open reading frames (ORFs); these are oriented bidirectionally between large and small intergenic regions known as LIR and SIR, respectively (Liu et al., 1998). The virion-sense strand, containing ORFs V1 and V2, encodes both the movement (MP) and coat protein (CP) genes (Liu et al., 1998). The Rep gene, which is encoded by two overlapping ORFs C1 and C2, and contains an 86 nt intron, resides on the complementary-sense strand and is expressed in the opposite orientation from the virion-sense genes. BeYDV complementary-sense gene expression is regulated by a splicing event in which RepA (C1) is expressed from the unspliced transcript, and Rep (C1 : C2) is expressed from the spliced transcript. The LIR is composed of an origin of replication as well as the cis-acting elements required for regulating both virion-sense and complementary-sense transcription (Kammann et al., 1991). The SIR contains elements necessary for transcriptional termination in addition to a host-derived primer, which is considered to play a role in complementary-sense strand DNA synthesis (Donson et al., 1984; Hayes et al., 1988a; Liu et al., 1998).

Virus replication is believed to take place via a rolling-circle mechanism in which a complementary-sense DNA strand is synthesized by host replication machinery from the primer located within the SIR to produce a double-stranded (ds) replication intermediate (Saunders et al., 1991; Stenger et al., 1991). Multiple ss circular copies of the virion-sense strand are produced by the introduction of a nick at a hairpin structure located within the LIR, followed by DNA synthesis and strand-displacement. Finally, the nascent DNA strand is ligated to produce a ss circular molecule. Both nicking and ligating events have been shown to be carried out by Rep, encoded by ORFs C1 and C2. RepA is thought to have an autoregulatory effect on Rep gene expression by binding to a Rep binding site located within the LIR and inhibiting complementary-sense gene expression (Palmer & Rybicki, 1998). In addition, RepA is believed to play a role in transactivating virion-sense expression. Several conserved motifs have been identified within the Rep
genes of other mastreviruses, including those required for oligomerization, DNA binding and cleavage (Palmer & Rybicki, 1998). An LXCXE consensus motif for RepA binding to the retinoblastoma-related (RBR) protein has also been identified; the interaction of RepA with RBR enables the virus to retain the environment of the cell in S-phase, thus generating conditions suitable for supporting viral DNA replication (Liu et al., 1999a).

The employment of geminivirus replicating vectors, or replicons, as putative gene amplification systems has been explored by a number of groups (Hayes et al., 1988b, 1989; Timmermans et al., 1994; Needham et al., 1998; Woolston et al., 1998). Timmermans et al. (1992) have shown that a geminivirus vector was capable of replication when the Rep gene was provided in trans from a second vector containing the complete virus genome. In the present study, we examined in more detail the expression of various Rep gene products and investigated their capacity to support replication in trans of a minimal Rep and RepA-deficient reporter cassette containing the cis-acting sequences required for BeYDV replication. We placed Rep and RepA under independent promoter control and analysed their respective effects on replication. In an attempt to maximize the expression levels of our minimal BeYDV construct, we investigated the role of the CP in regulating these processes. Finally, we analysed the effect of the constitutively expressed Rep gene products on BeYDV infection.

**METHODS**

**Cells, viruses and plants.** NT-1 tobacco cell suspensions were maintained in NT-1 liquid medium as shaker cultures (Paszty & Luiquin, 1987). They were prepared for biolistic DNA delivery by pipetting a 10-day-old culture onto NT-1 agar plates and pre-incubating the cells for 3–4 days prior to bombardment. BeYDV was generously provided by J. Stanley, John Innes Centre, UK. For bombardments, one micron gold particles (Bio-Rad) were used at 800 psi (~5.52 MPa) with the Bio-Rad Model PDS-1000/He Biolistic Particle Delivery System, to deliver 2 μg plasmid DNA prepared according to the Qiagen maxiprep kit protocol.

**Construction of plasmids.** Plasmids pSKBYD1.4 and pΔintron were kindly provided by J. Stanley (Liu et al., 1998). A schematic diagram of the constructs made is shown in Fig. 1. For the in trans studies, Rep, RepA and Δintron ORFs were PCR-amplified from pSKBYD1.4 and pΔintron, and subcloned into pIBT210 (Haq et al., 1995) to generate p35SRep, p35SRepA and p35SΔintron (Fig. 1b). A fourth construct, p35SΔRep, was generated by removal of the 727 bp BamHI fragment of p35SRep and religating the template. p35SCP was constructed by PCR-amplifying the CP ORF and inserting it into the plasmid pIBT210.

To create the Rep-deficient reporter constructs, the XbaI–SacI fragment of pSKBYD1.4 encompassing the Rep gene, the LIR and SIR

![Fig. 1. Schematic diagram of constructs used. (a) genomic organization of pSKBYD1.4; P, PstI; Xb, XbaI; S, SacI; B, BamHI; E, EcoRI; C, ClaI; Bg, BglII; C1, C2, V1 and V2 represent complementary and virion sense ORFs. Bar, 500 bp. (b) BeYDV-derived plasmids containing various forms of Rep, RepA and CP ORFs. Portions of Rep gene removed for 35SΔintron and 35SΔRep are indicated by a 'v'. Small rectangle represents tobacco etch virus leader sequences. (c) Reporter cassettes. Dashed line represents insertion of 35SGUS gene into BeYDV sequences. Double slashed lines indicate that only a portion of the ORF is shown.](http://example.com/fig1.png)
were subcloned into pBluescriptSKII+. This construct was then digested with HincII and Smal, and then religated to remove an EcoRI site located within the multiple cloning site of the vector. EcoRI fragments containing the GUS gene under both 35S promoter and terminator control were obtained from pBluesGFP-TYG-nos(SK) or pGUS2 (Dugdale et al., 1998) and inserted into the remaining EcoRI site located within ORF C1 to produce the construct pBGUS (Fig. 1c). To create pBYcisGUS (Fig. 1c), a site located within ORF C1 to produce the construct pBYGUS.

To generate the HA-tagged Rep constructs, the coding regions of Rep, Ant and RepA were amplified by PCR, tagged at either the N or C terminus with a double haemagglutinin (HA) epitope and inserted into a plant expression vector containing the CaMV 35S promoter and nopaline synthase terminator (Kovtun et al., 2000). RepA and Rep RBR-binding mutants were constructed using the primer RBR1 (5'-GCAGATCTCTGTTGGCAGGGAGATCTGATTCTGTGAAGGG) by site-directed mutagenesis using the Bio-Rad mutagenesis kit.

**Immunoblot analysis.** Approximately 50 μg total protein extracted from NT-1 cells were ground in sample loading buffer (100 mM Tris-HCl, pH 6.8, 1% SDS, 20% glycerol). Each sample was boiled for 10 min and loaded onto a gradient gel. Protein was electro-transferred onto nitrocellulose membranes. Blots were incubated overnight with anti-HA antibody at a concentration of 0·1 μg ml⁻¹ and proteins were visualized using an enhanced chemiluminescence kit (Amersham).

**GUS assay.** NT-1 cells co-bombarded with pBYGUS as the reporter plasmid were analysed for GUS activity using the protocol of Jefferson (1987). Briefly, 1 g NT-1 cells was crushed using a micro-pestle and resuspended in GUS extraction buffer (50 mM NaPO₄, pH 7.6, 0·1% SDS, 10 mM Na₂EDTA and 10 mM 2-mercaptoethanol). Total protein concentrations were determined by Bradford assay (Bio-Rad) using BSA as a standard. GUS activities were determined by fluorometric assay using 1 mM 4-methylumbelliferyl β-D-glucuronide (MUG) as substrate and 4-methylumbelliferone (MU) as standard. Assays were monitored by fluorometry (DYNEX technologies Fluorolite 1000). Data are presented as μmol min⁻¹ (mg total protein)⁻¹.

**Southern blot analysis.** For Southern blot analysis, 2 μg of each plasmid DNA was co-bombarded into a thick slurry of NT-1 cells which had been slowly pipetted onto Petri dishes containing NT-1 media plus 8 g agar l⁻¹ (Sigma). Plates were then incubated at 28 °C for up to 8 days, depending on the experiment performed. DNA was extracted from cells using the procedure according to Wilke (1996). Ten μg total DNA of each sample was digested with HindIII and loaded onto a 1% agarose gel. DNA was transferred onto nitrocellulose by capillary action (Sambrook et al., 1989). A 1 kbp DNA fragment encoding GUS was 32P-labelled by random priming according to the conditions recommended by the manufacturer (Life Technologies) and used as a probe for hybridization in 25 mM Tris/HCl, pH 7·2, 1 mM EDTA and 7% SDS at 65 °C. Filters were washed in 40 mM Tris/HCl, pH 7·2, 1 mM EDTA and 5% SDS at 65 °C and signal detected by the STORM Optical Scanner (Molecular Dynamics) system. Signals were quantified using IMAGEQUANT software (Molecular Dynamics).

**RESULTS**

**Replication is supported by CaMV 35S promoter-driven Rep gene products**

To examine the expression profiles of Rep gene products and their respective roles in supporting a BeYDV-based replicon system, intron and intronless versions of the Rep ORF were each placed under CaMV 35S promoter control (Fig. 1b). A fourth construct containing a large deletion within the Rep ORF (Δ3Rep) was included as a negative control (Fig. 1b). We verified expression of C-terminally HA-tagged Rep gene products in NT-1 cells by immunoblot analysis using anti-HA antibodies (Fig. 2a). At 4 days post-bombardment, Rep was detected in cells bombarded with p35SRepHA and p35SΔintronHA (Fig. 2a, lanes 1 and 4), but not from samples bombarded with p35SΔBRepHA (Fig. 2a, lane 3). No RepA was detected from cells bombarded with construct p35SRepHA using HA-antibodies, because RepA is a spliced product of Rep and its ORF.

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**Fig. 2.** Expression of Rep gene products and replication of BeYDV-derived reporter constructs in NT-1 cells. (a) Western blot analysis of HA-tagged Rep gene products using HA-antisera. Lane 1, p35SRepHA; lane 2, p35SRepAHA; lane 3, p35SΔintronHA; lane 4, p35SΔintronHA. Size markers are indicated on the left. (b) Replication of BeYDV-derived constructs. Southern blot analysis using 32P-derived GUS fragment as a probe. NT-1 cells were co-bombarded with pBGUS and samples collected at 4 days post-bombardment: lane 1, pSKBYD1.4; lane 2, p35SRep; lane 3, p35SΔintron; lane 4, p35SΔBRep; lane 5, p35SΔintron and p35SRepA. oc, open circular DNA; ds, double-stranded DNA; ss, single-stranded DNA; sc, supercoiled DNA.
terminates prior to the HA-tag (Fig. 2a, lane 1). RepA could be detected, however, from cells bombarded with the construct p35SRepAHA, and appeared to be in greater abundance than Rep (Fig. 2a, lane 2). In fact, when Western blots were performed on samples collected 6 and 8 days post-bombardment, RepA remained visible for a much longer period of time (data not shown).

Southern blot analysis was used to confirm that 35S promoter-driven Rep can support the replication of a reporter plasmid containing the BeYDV cis-acting sequences required for replication (Fig. 2b). NT-1 cells were bombarded with reporter construct pBYGUS and either p35SRep, p35SΔintron or p35SΔBRep. RepA is unable to support BeYDV replication and was not considered in this experiment (Palmer & Rybicki, 1998). pSKBYD1.4, containing the entire BeYDV genome, was included as a control (Fig. 2b, lane 1). At four days post-bombardment, various forms of viral DNA, including open circular, ds, ss and supercoiled DNAs, accumulated in cells cobombarded with pBYGUS and pSKBYD1.4 (Fig. 2b, lane 1). Samples cobombarded with pBYGUS and either 35SRep or 35SΔintron yielded predominantly a single band, representing the ds form of DNA (Fig. 2b, lanes 2 and 3). The absence of ssDNA from these samples is most likely due to the removal of the CP ORF from these constructs (Liu et al., 1997b). There were no significant differences between the replication efficiencies for spliced and unspliced forms of the Rep gene driven by the 35S promoter (Fig. 2b, compare lanes 2 with 3). No replication products could be detected for samples cobombarded with the non-functional p35SΔBRep (Fig. 2b, lane 4). However, replication levels were elevated when both Rep and RepA were each provided simultaneously under 35S promoter control (Fig. 2b, lane 5). Enhanced replication exhibited by the combined bombardment of both 35SRep and 35SRepA was fully reproducible, and was determined by the consistent observation of an increase in replication products over five repetitions of the experiment. Replacement of the 35SRep and RepA constructs with the tagged versions of the same constructs led to similar results (data not shown).

**Examination of Rep RBR-binding mutant**

Since RepA possesses an RBR-binding domain (LXCXE) which is believed to be involved in cell cycle control, we were interested in determining the effect of this domain on our expression system. To this end, we constructed N-terminally HA-tagged Rep and RepA as well as the mutants 35SΔRepARBR LXCXQ and 35SΔintronRBR LXCXQ (Fig. 3a). The LXCXQ mutation was selected for these experiments because constructs containing this mutation had been previously shown by Liu et al. (1999a), using the two-hybrid yeast system, to have a greatly reduced ability to bind to maize RBR. These constructs were cobombarded into NT-1 cells along with the pBYGUS expression cassette. We compared the expression profiles of each mutant to wild-type N-terminally HA-tagged Rep and Δintron by Western blot analysis. We found no difference in the level of expression of each of the mutant constructs (Fig. 3b).

**Fig. 3.** Effect of RBR-binding mutant on expression of Rep gene products and replication of BeYDV-derived construct. (a) Sequence comparison of wild-type and RBR-binding Rep and RepA mutant constructs. Portion of sequence depicted represents nt 1844–1858 within the Rep ORF. Nucleotide sequence is depicted at left, amino acid sequence on the right. Nucleotide changes are underlined. (b) Western blot analysis of Rep and RepA RBR-binding mutant expression using HA-antisera. NT-1 cells were bombarded with the following constructs and samples were collected at 3 days post-cobombardment. Lane 1, p35SRepA; lane 2, non-bombarded NT-1 cells; lane 3, p35SΔRepARBR LXCXQ; lane 4, p35SΔintronRBR LXCXQ; lane 5, non-bombarded NT-1 cells; lane 6, p35SΔintronRBR LXCXQ. Size markers are indicated on the left. (c) Effect of RBR-binding mutants on replication. Southern blot analysis of DNA purified from NT-1 cells 3 days post-bombardment and bombarded with pBYGUS and various Rep and RepA wild-type and RBR-binding mutant constructs using a 32p-labelled DNA fragment encompassing the GUS ORF as a probe. Lane 1, pBYGUS; p35SRepA and p35SΔintron; lane 2, pBYGUS, p35SΔRepARBR LXCXQ and p35SΔintron; lane 3, pBYGUS, p35SRepA and p35SΔintronRBR LXCXQ.
of expression of Rep gene products between wild-type and RBR-binding mutant constructs (Fig. 3b, compare lane 1 with 3 and 4 with 6). In both cases, the level of RepA expression exceeded that of Rep. To determine whether the reduced ability of RepA to bind to RBR plays a role in replication, we co-bombarded NT-1 cells with the pBYGUS expression cassette and either p35SRepARBR^{LXCXQ} and p35SΔintron, or p35SRepA and p35SΔintronRBR^{LXCXQ}. Despite the consistency among levels of gene products throughout these constructs, the levels of corresponding replication products from tissue co-bombarded with p35SΔintron and p35SRepARBR^{LXCXQ} were significantly reduced (Fig. 3c, compare lane 1 with 2). On the other hand, cells co-bombarded with constructs p35SRepA and p35SΔintronRBR^{LXCXQ} resulted in an accumulation of replication products comparable to that of wild-type p35SRepA and p35SΔintron (Fig. 3c, compare lane 1 with 3), suggesting that the drop in replication efficiency is more likely to be due to the decreased ability of RepA to bind to RBR rather than a general mutation which reduced replication efficiency in general.

Factors which influence gene expression for the BeYDV replicon system

We examined in more detail the ability of BeYDV gene products to influence the efficiency of BeYDV replication using the reporter plasmid pBYcisGUS, which contains the Rep gene under the control of its native promoter (Fig. 4). To determine whether the addition of BeYDV gene products could increase gene expression to greater levels, we supplemented pBYcisGUS with p35SRep, p35SΔintron or p35SRepA (Fig. 4, lanes 1–4). We found that the addition of more Rep protein to the system via a constitutive promoter increased replication levels significantly; however, increases in GUS activity were more modest (Fig. 4a and b, compare lanes 1 and 2 with 3). Since RepA may play a role in altering the cellular environment to one that is more permissive for BeYDV replication, we supplemented the pBYcisGUS system with constitutively expressed RepA. To our surprise, we found under these conditions that both replication and reporter gene expression were reduced (Fig. 4a and b, compare lanes 3 and 4). Diminished replication efficiency and GUS activity were also observed when the RepA^{LXCXQ} RBR-binding mutant was substituted in place of RepA (data not shown). Furthermore, analysis of BeYDV replication products from a time-course performed in NT-1 cells co-bombarded with pSKBYD1.4 and the p35SRepA construct indicated a delay in replication when compared to cells bombarded with pSKBYD1.4 alone (Fig. 5, compare lanes 1–4 to 9–12). When the p35SRepA construct was replaced with the 35SΔintron construct in this experiment, the delay in replication was eliminated (Fig. 5, compare lanes 1–4 to 5–8).

Earlier reports have implicated a role for the CP of geminiviruses in regulating the switch between ds and ssDNA accumulation (Azzam et al., 1994; Liu et al., 1999b; Padidam et al., 1999). Reduced dsDNA levels could result in subsequently lower reporter gene expression since a dsDNA template is required for transcription. To determine the effects of CP expression on the BeYDV replicon system, NT-1 cells were co-bombarded with p35SΔintron, pBYcisGUS and p35SCP. We found that the increased production of ssDNA was accompanied by a significant reduction of GUS activity (Fig. 4a and b, lane 5).

DISCUSSION

Geminiviruses are well known for their small genome and its organization, nuclear-based replication and collective broad host range. Geminiviruses have been widely considered for use as potential vectors for foreign gene expression due to their ability to replicate to high copy numbers in an extrachromosomal fashion (Davies & Stanley, 1989; Kammann et al., 1991; Matzeit et al., 1991; Kanevski et al., 1992; Shen & Hohn, 1994, 1995; Timmermans et al., 1994). Rep (C1:C2) is the sole virally-encoded protein required for replication, and this dependence on host products makes geminiviruses an
difficult to utilize (Scholthof have host range and size restrictions rendering them more been employed in the past are restricted to the cytosol and numbers in plants. Many plant RNA virus vectors that have attractive vector for replicating foreign genes to high copy state that promotes DNA synthesis rather than transcription. Such a phenomenon would provide a similar explanation of the results presented in this study. It is not clear whether changes in gene expression brought about by independent expression of Rep and RepA would be masked by changes in the replication efficiency. A more refined approach using non-replicating constructs to further examine the roles of Rep and RepA in regulating gene expression from complementary and virion-sense promoters is currently under way.

With the exception of BeYDV and TYDV, all geminiviruses that infect dicots possess a single continuous Rep gene; however, in monocots, splicing of Rep ORFs C1 and C2 is a general feature (Schalk et al., 1989). Gene expression has been demonstrated to be enhanced by the presence of an intron, most likely by affecting mRNA stability (McCullough et al., 1991). Some monocot genes containing introns are prevented from being spliced in dicots. Digitaria streak virus (DSV), for example, exhibits inefficient splicing in wheat and maize; however its splicing is non-functional in tobacco (Mullineaux et al., 1990). It has been considered that a component of the splicing machinery that predominates in monocots is expressed in dicots in limiting amounts or in a temporal or tissue-specific manner (Mullineaux et al., 1990). Previously, examination of replication using a virus containing an intronless version of the Rep gene of BeYDV resulted in several-fold higher levels of replication products than the spliced version (Liu et al., 1998). While such results were not observed in the present study, this discrepancy may be due to a difference in cell preparation and DNA delivery technology (virus-infected protoplasts were used in previous studies, as opposed to biolistic delivery into intact cells used in the current study). In addition, we did not observe significant differences in expression of C-terminally tagged versions of Rep derived from 35SRep or 35SΔintron constructs, which could also explain the similarities in replication efficiency and GUS activity (Fig. 2a). The results of our Western blot analysis of N-terminally tagged Rep gene products expressed in NT-1 cells implies that RepA accumulated to greater levels than Rep. Using a yeast expression system, Liu et al. (1999a), recorded that expression of BeYDV Rep and RepA yielded bands on a Western blot of similar intensity; perhaps the splicing efficiency varies between these two systems. Since intron splicing may be host-specific and is either inactive or works less efficiently in other plant hosts, it may be wise to use the spliced form for more widespread application of this vector system.

It is interesting to note that whereas providing Rep and RepA from construct 35SRep or Rep alone from construct p35Δintron conferred comparable increases in replication efficiency, suggesting a more minor role for RepA in this assay, we found that supplementing Rep with RepA both individually under 35S promoter control enhanced replication. Perhaps this discrepancy in our results can be accounted for by the artificial alteration in the ratio of Rep

![Fig. 5. Effect of Rep and RepA gene products on virus replication. Comparison of BeYDV-replication profiles in virus-infected NT-1 cells supplemented with 35SΔintron or 35SRepA. A Southern blot was performed using 32P-labelled BeYDV DNA as a probe. Lanes 1–4, pSKBYD1.4; lanes 5–8; pSKBYD1.4 + 35SΔintron; lanes 9–12; pSKBYD1.4 + 35SRepA. Lanes 1, 5 and 9, 2 days post-bombardment; lanes 2, 6 and 10, 4 days post-bombardment; lanes 3, 7 and 11, 6 days post-bombardment; lanes 4, 8 and 12, 8 days post-bombardment. Positions of ds and ss forms of viral DNA are indicated.

attractive vector for replicating foreign genes to high copy numbers in plants. Many plant RNA virus vectors that have been employed in the past are restricted to the cytosol and have host range and size restrictions rendering them more difficult to utilize (Scholthof et al., 1996). In this study, we created recombinant BeYDV replicons and examined the role of Rep gene products in trans by placing them under 35S promoter control.

Replication was supported and reporter gene expression enhanced when RepA and Rep, comprising both C1 and C1 : C2 gene products, and Δintron, comprising the C1 : C2 gene product, were either expressed from the context of the native Rep promoter or placed under 35S promoter control. Attempts to increase reporter gene expression by simultaneously providing Rep in cis as well as in trans resulted in a decrease in the relative level of gene expression, although the replication efficiency increased more significantly. It was interesting to note in this case that the levels of GUS activity did not increase proportionally with the increase in DNA accumulation. Gooding et al. (1999) demonstrated that wheat dwarf virus virion-sense gene expression may be tissue-dependent, as it became compromised in suspension cell cultures compared with embryogenic tissues. The authors suggested that the lack of corresponding increase in gene expression was due to an association of the viral DNA with a subset of minichromosomes that renders the virus in a replicationally committed
to RepA, brought about by the coexpression of these two gene products under independent constitutive promoter control. The fact that a decrease in replication was observed when 35SRepA and pBYcisGUS (containing Rep in cis and under native reporter control) were cobombarded into NT-1 cells would strengthen this argument.

It was previously demonstrated that RepA of BeYDV contains an RBR-binding domain and binds to a plant homologue of the cell cycle regulator retinoblastoma protein (Liu et al., 1999a). RepA uses this property to reprogram the host and create a replication-competent environment (Gutierrez, 2000a, b). By altering this LXCXE motif, we showed that while the level of expression of the mutant Rep gene products remained similar to that of wild-type Rep gene products, replication efficiency was severely diminished, most likely due to the reduced ability of RepA to bind to RBR. Liu et al. (1999a) conducted a series of experiments with Rep mutants in which altering the strength of RBR binding, as determined by the two-hybrid yeast system, did not appear to significantly affect virus replication in systemically infected Phaseolus vulgaris tissue. In the present report, we have demonstrated that the introduction of a mutation to the LXCXE consensus motif of RepA resulted in a marked reduction in replication efficiency of our BeYDV replicon system. Since our experiments were performed on NT-1 cells, which were plated out for synchronous growth, it is possible that the efficiency of RBR binding to RepA is more pronounced under these conditions than when an entire plant is infected.

In a number of geminiviruses, the absence of CP has been shown to result in the abolition of virus infectivity as well as a diminishment of ssDNA levels (Azzam et al., 1994; Qin et al., 1998; Woolston et al., 1998; Liu et al., 1998). When a non-specific ssDNA binding protein was used as a substitute for tomato leaf curl virus CP, ssDNA accumulated to wild-type levels (Padidam et al., 1996, 1999). Liu et al. (1997b, 1999b, 2001) have indicated that the CP of maize streak virus, another mastrevirus, binds to ssDNA to facilitate both nuclear trafficking of viral DNA as well as cell-to-cell movement. It is possible that early on in infection, the CP is available to interact with but not abundant enough to fully encapsidate viral DNA until later on in the infection process. Consequently, the CP may regulate ssDNA accumulation by sequestering it away from the replication pool (Qin et al., 1998). In this report, expression of the CP gene resulted in a marked increase in ssDNA accumulation and a decrease in reporter gene expression. Since we have eliminated the native CP gene from our expression system, we have optimized reporter gene expression.

In this report, we have examined the effects of constitutively expressed Rep and RepA on BeYDV replication. Further work concerning their effects on reporter gene expression using replication-deficient constructs is currently under investigation.

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