A three-nucleotide mutation altering the *Maize streak virus* Rep pRBR-interaction motif reduces symptom severity in maize and partially reverts at high frequency without restoring pRBR–Rep binding

Dionne N. Shepherd,1 Darren P. Martin,1 David R. McGivern,2† Margaret I. Boulton,2 Jennifer A. Thomson1 and Edward P. Rybicki1

Geminivirus infectivity is thought to depend on interactions between the virus replication-associated proteins Rep or RepA and host retinoblastoma-related proteins (pRBR), which control cell-cycle progression. It was determined that the substitution of two amino acids in the *Maize streak virus* (MSV) RepA pRBR-interaction motif (LLCNE to LLCLK) abolished detectable RepA–pRBR interaction in yeast without abolishing infectivity in maize. Although the mutant virus was infectious in maize, it induced less severe symptoms than the wild-type virus. Sequence analysis of progeny viral DNA isolated from infected maize enabled detection of a high-frequency single-nucleotide reversion of C(601)A in the 3 nt mutated sequence of the Rep gene. Although it did not restore RepA–pRBR interaction in yeast, sequence-specific PCR showed that, in five out of eight plants, the C(601)A reversion appeared by day 10 post-inoculation. In all plants, the C(601)A revertant eventually completely replaced the original mutant population, indicating a high selection pressure for the single-nucleotide reversion. Apart from potentially revealing an alternative or possibly additional function for the stretch of DNA that encodes the apparently non-essential pRBR-interaction motif of MSV Rep, the consistent emergence and eventual dominance of the C(601)A revertant population might provide a useful tool for investigating aspects of MSV biology, such as replication, mutation and evolution rates, and complex population phenomena, such as competition between quasispecies and population turnover.

INTRODUCTION

*Maize streak virus* (MSV) is the type species of the genus *Mastrevirus* of the family *Geminiviridae*. These viruses have ~2-7 kb single-stranded circular DNA genomes that replicate in the nucleus via rolling-circle and recombination-dependent mechanisms (Jeske et al., 2001). Generic mastreviruses include MSV and related ‘African streak viruses’, a complex of viruses related to *Chloris striate mosaic virus*, *Miscanthus streak virus*, *Wheat dwarf virus* (WDV), *Tobacco yellow dwarf virus* and *Bean yellow dwarf virus* (BeYDV) (Fauquet & Stanley, 2003; Rybicki et al., 2000).

Replication of these viruses is believed to require both host factors and the virus-encoded replication-associated proteins Rep and RepA. Rep is expressed from spliced transcripts encompassing the two complementary sense/strand ORFs C1 and C2; RepA is expressed from the C1 ORF of unspliced transcripts (Wright et al., 1997). Although Rep and RepA share the same N terminus, each has a different C terminus and the proteins have distinct functions.

There is substantial evidence, mainly from work with WDV, that RepA is a multifunctional protein with unique features that are important at different stages during the replicative cycle (Gutiérrez, 1999). These potentially include down-regulation of replication (Collin et al., 1996) and interactions with cellular retinoblastoma-related protein (pRBR) (Horvath et al., 1998; Xie et al., 1995) and a group of host NAC domain-containing GRAB (geminivirus RepA-binding) proteins (Xie et al., 1999), which may influence
host developmental pathways for the benefit of viral processes (Gutierrez, 2000; Gutierrez et al., 2004). Properties common to Rep and RepA include sequence-specific DNA binding (Castellano et al., 1999; Missich et al., 2000), hetero- and homo-oligomerization (Horvath et al., 1998) and transactivation of virion (V)-sense gene promoters (Collin et al., 1996; Hofer et al., 1992; McGivern, 2002; Zhan et al., 1993).

Rep and RepA proteins contain a conserved amino acid sequence (LxCxE) that is common among oncoproteins of tumour-inducing viruses (Ludlow, 1993; Moran, 1994; Vossden, 1993). The LxCxE motif is also present in mammalian (Dowdy et al., 1993; Ewen et al., 1993) and plant (Dahl et al., 1995; Nakagami et al., 1999; Soni et al., 1995) D-type cyclins, in the nanovirus protein Clink (Aronson et al., 1999; Oruetxebarria et al., 2002), and mediates binding to both the mammalian retinoblastoma protein (pRB) and plant pRBR (reviewed by de Jager & Murray, 1999). However, in the mastreviruses MSV, BeYDV and WDV, only RepA, and not Rep, interacts with pRBR (Gutierrez et al., 2004; Horvath et al., 1998; Liu et al., 1999).

Mastrevirus replication is dependent on cellular replication factors that are generally absent or not functional in non-dividing cells. However, MSV replication has been detected in terminally differentiated cells (Lucy et al., 1996). The purpose of the LxCxE motif in the mastrevirus RepA may therefore be to induce a cellular S phase-like state that is permissive for viral DNA replication, by interaction with pRBR and subsequent activation of S phase-specific gene transcription.

Mutational analysis of the RepA LxCxE motif in WDV and BeYDV has shown the importance of the three conserved residues in mediating binding to pRBR. A mutation of E to K abolished the ability of WDV RepA to bind to pRB (Xie et al., 1995), whilst Liu et al. (1999) confirmed the importance of all three conserved residues in BeYDV RepA and showed that an E to Q mutation reduced binding efficiency by 95%. In the present study, the effects of a two amino acid substitution (LLCNE to LLCLK) in the MSV RepA pRBR-interaction motif on both virus replication in maize and infectivity in maize were determined.

**METHODS**

**Plasmid construction.** All Rep gene-based plasmids were derived from plasmid pKom602, containing the complete, infectious MSV-Kom isolate genome with reiterated long intergenic regions (1.1-mer; Schnippenkoetter et al., 2001). The C1/C2 ORFs of pKom602 were amplified by PCR using the primer set C1 (F) and C2 (R) (Table 1). The PCR product was inserted into the BamHI/HindIll

<table>
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<tr>
<td><strong>Primer name</strong></td>
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<tr>
<td>Cloning PCR</td>
</tr>
<tr>
<td>C1 (F)</td>
</tr>
<tr>
<td>C2 (R)</td>
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<td>AD/RepA (R)</td>
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<td>AD/RepA (R)</td>
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<td>Rb′ A (F)</td>
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*Lower-case letters denote nucleotide changes compared with the wt Rep gene sequence; underlined letters highlight engineered restriction-enzyme sites; letters in parentheses indicate degeneracies.
†Relative positions of the primers in the MSV-Kom genome (GenBank accession no. AF003952). Numbering starts from the penultimate nucleotide of the conserved TAATATTAC sequence of MSV-Kom. Primer co-ordinates are given in the 5’→3’ direction of the primer.
‡For mutations altering the Rep pRBR-interaction motif, the positions of the nucleotide changes relative to the Rep gene start codon are A(601)C, A(602)T and G(604)A (see also Table 3).
site of pBluescript SK+ (pSK; Stratagene) and all subsequent mutations and truncations were performed on this construct (pSKRep). All clones were confirmed by sequencing using an ALF Express automated sequencer (Pharmacia). Sequence analysis was done by using DNAMAN (version 4.0; Lynnon BioSoft). Standard cloning techniques were used (Sambrook et al., 1989).

The construct pMSV-Psfl – a replication-incompetent tandem dimer of MSV-Kom with an introduced Psfl site and premature stop codon in the C1 ORF N-terminal coding regions – was described previously (Palmer & Rybicki, 2001).

**PCR site-directed mutagenesis.** An MSV-Kom Rep gene containing mutations altering the pRRB-interaction domain (described below) was provided by Dr T. Mangwende (University of Cape Town, South Africa). A 3 nt mutation resulting in amino acid changes of LLCNE to LLCLK was introduced into pSKRep below) was provided by Dr T. Mangwende (University of Cape Town, South Africa). A 3 nt mutation resulting in amino acid changes of LLCNE to LLCLK was introduced into pSKRep below) was provided by Dr T. Mangwende (University of Cape Town, South Africa). A 3 nt mutation resulting in amino acid changes of LLCNE to LLCLK was introduced into pSKRep below) was provided by Dr T. Mangwende (University of Cape Town, South Africa). A 3 nt mutation resulting in amino acid changes of LLCNE to LLCLK was introduced into pSKRep below) was provided by Dr T. Mangwende (University of Cape Town, South Africa). A 3 nt mutation resulting in amino acid changes of LLCNE to LLCLK was introduced into pSKRep below) was provided by Dr T. Mangwende (University of Cape Town, South Africa). A 3 nt mutation resulting in amino acid changes of LLCNE to LLCLK was introduced into pSKRep below) was provided by Dr T. Mangwende (University of Cape Town, South Africa). A 3 nt mutation resulting in amino acid changes of LLCNE to LLCLK was introduced into pSKRep below) was provided by Dr T. Mangwende (University of Cape Town, South Africa).

**Construction of plant expression cassettes.** A 1:2 kb BamHI/ BglII fragment (containing the full-length Rep gene) from pSKRep, pSKRepBb and pSKRepBb–C(601)A (see below) was inserted into the BamHI site of pAHCh1 (Christensen & Quail, 1996) downstream from the maize ubiquitin promoter. The resulting plasmids were designated pRep, pRepBb and pRepBb–C(601)A, respectively.

**Construction of RepA genes.** An unspliced RepB gene of MSV-NG1 (formerly MSV-N; Faquet & Stanley, 2003) cloned in pMB1657, containing a 3’ splice site mutation of A37A/C974 to T234C34 (Wright et al., 1997), was used to construct MSV-Kom RepA genes. A fragment containing the splice-site mutation was excised from pMB1657 with XhoI and BglII and used to replace the XhoI/BglII fragment of pKom602, to produce pKomMB1657. The MSV-NG1 sequence remaining in pKomMB1657 is identical to that of MSV-Kom. The RepA gene was amplified from pKomMB1657 using the primer set C1 (F) and RepABglII (R) (see Table 1) and inserted into the BamHI site of pSK to give pSKRepA. RepA versions of Rb− mutants were made similarly, using pKomRb– and pKomRb–C(601)A (described below) as starting templates. The RepA plasmids, each containing the 3’ splice-site mutation, were called pSKRepAB and pSKRepAB–C(601)A, respectively.

**Construction of an intronless Rep gene.** pSKRep was used as a template to create an intronless Rep gene by inverse PCR, with the forward primer RepAI (F) and the reverse primer RepAI (R), which bind adjacent to the 3’ and 5’ ends of the intron, respectively. The whole template plasmid was amplified minus the intron, in such a way as to allow in-frame ligation. Primer sequences are given in Table 1. The PCR product was self-ligated to create the plasmid pSKRepAI.

**Plasmid construction for yeast two-hybrid analysis.** The RepA genes from pSKRepA, pSKRepArb and pSKRepArbc–C601A, and the intronless Rep gene from pSKRepAI were PCR-amplified by using primer sets ADSaIC1 (F) (forward primer for all Rep genes), ADBgIC2 (R) (reverse primer for RepA genes) and ADBgIC1C2 (R) (reverse primer for the RepAI gene; Table 1) and inserted into pGAD424 (Clontech) in-frame with the GAL4 activation domain (AD), to create the RepA– or RepAI–GAL4 AD fusion plasmids pADRepA, pADRepArb and pADRepArbc–C601A and pADRepAI, respectively. Plasmid GBT9ZmRb1 encodes the GAL4 binding domain (BD) fused to the maize RBR protein ZmRb1 and was provided by Dr G. Horvath (Horvath et al., 1998).

**Yeast two-hybrid analysis.** Interaction of MSV RepA and Rep proteins with ZmRb1 was assessed by using the Matchmaker yeast two-hybrid system (Clontech). Plasmids expressing GAL4 BD fusions (pGBT9; trp1 transformation marker) and GAL4 AD fusions (pGAD424; leu2 transformation marker) were used to transform Saccharomyces cerevisiae strains CG1945 and Y187, respectively, as described by Gietz & Woods (1994). The transformation mixture was plated onto yeast drop-out selection media lacking the appropriate amino acid to select for transformants. Yeast strains CG1945 (MATa; containing pGBT9-based plasmids) and Y187 (MATa; containing pGAD424-based plasmids) were mated according to a protocol modified from the Clontech Yeast Protocols handbook. The diploid yeasts were grown on synthetic drop-out medium lacking tryptophan (Trp) and leucine (Leu), to select for both plasmids, and also on drop-out medium lacking Trp, Leu and histidine (His) and containing 5 mM 3-amino-1,2,4-triazole (3-AT). Only cells containing interacting fusion proteins can grow on the latter medium.

**Transient-replication assays.** Replication of MSV replicons in Black Mexican sweet (BMS) suspension culture cells was assayed as described previously (Palmer et al., 1999). In each experiment (with a minimum of two replicates), up to nine plates of BMS were bombarded with gold microprojectiles by using a Bio-Rad PDS-1000/He particle gun. Particles carried the following plasmid combinations: pKom602 + pRep or pRepRb; pMSV-Psfl (replication-incompetent MSV) + pRep, pRepRb or pRepRb–C601A and pKom602, pKomRb or pKomRb–C601A without any Rep gene provided in trans. For the co-bombardments, up to nine plates of BMS were also co-bombarded with pKom602 + pAHCh1 or pMSV-Psfl + pAHCh1, with the latter as a non-Rep co-bombardment control. Four days after bombardment, total DNA was extracted from BMS cells by using the method of Dellaporta et al. (1983), including a step where DNA resuspended in 50 mM Tris/HCl, 10 mM EDTA (pH 8) had 600 µg RNaseA ml–1 added and the mixture was incubated for 1 h at 37 °C. After a second precipitation with 2-propanol, DNA was resuspended in water and quantified on a 0.8 % agarose gel by measuring band intensities densitometrically from digital images (GelTrak; Dennis Maeder, Center of Marine Biotechnology, MD, USA). Each sample was diluted to 50 ng µl–1 and again quantified on a 0.8 % agarose gel to ensure equal loading. Viral DNA replication was assayed by using a quantitative PCR (QPCR)-based technique (Fig. 1), using 100 ng total DNA as input. The amplification reaction used the primer set 215–234 and 1770–1792 (Willment et al., 2001; Table 1), which amplifies only replicationally released circular genomic DNA, but not linear viral DNA, from pKom602-derived input plasmids. To confirm this, DNA extracted immediately after bombardment (as a control for input DNA amplification) was subjected to the same PCR. Each PCR was ‘spiked’ with pMSV-Psfl as an internal control: this competes in amplification reactions with replicated (circular) viral DNA for primers and other PCR components. Twenty-five amplification cycles were used, as amplification was still exponential at cycle 25 in preliminary assays. Psfl digestion of amplified pMSV-Psfl yields products of 558 and 747 bp, allowing the competitor to be distinguished from amplified replicated viral DNA (Fig. 2a). The relative concentration of replicated viral DNA, expressed as pg viral DNA per 100 ng total DNA, was calculated by determining the ratio of the replicated virus band intensity to those of the two pMSV-Psfl competitor bands by using GelTrak. From individual replicate data obtained by using QPCR, a mean amount (in pg) of replicated virus in the presence and absence of each Rep gene derivative was calculated.

**Production of agroinfectious MSV constructs.** The Rb− mutation (altering the LLCLNE motif to LLCLK) was introduced into pKom602 by exchanging the wild-type (wt) NsiI/Xhol fragment of pKom602 with the mutated NsiI/Xhol fragment of pSKRepRb–, to create pKomRb–. Similarly, a virus genome isolated from maize that had been agroinfected with pKomRb–, containing a naturally occurring, single-nucleotide reversion of C601A relative to the Rep gene start codon, was converted into an agroinfectious 1.1-mer by replacing the NsiI/Xhol fragment of pKomRb– with the same
fragment from the revertant virus, resulting in the agroinfectious construct \( p\text{KomRb}^2 \text{C}(601)A \). The Rep gene was also PCR-amplified from \( p\text{KomRb}^2 \text{C}(601)A \) by using the primer set C1 (F) and C2 (R) (Table 1) and inserted into the BamHI/HindII sites of \( p\text{SK} \) to give \( p\text{SKRep} \text{Rb}^2 \text{C}(601)A \). DNAs of mutant and wt virus were inserted into the EcoRI and XbaI sites of the binary vector \( p\text{BI121} \) (Clontech) to obtain \( p\text{BIKom602}, p\text{BIKomRb}^2 \text{C}(601)A \), which were used to transform \( \text{Agrobacterium tumefaciens} \) C58C1 (Koncz & Schell, 1986) by the method of An et al. (1988).

**Agroinoculation and symptom analysis.** Maize seedlings (Zea mays L. cv. Jubilee) were inoculated with agroinfectious constructs as described previously (Martin et al., 1999). In each of three replicates, groups of 14 3-day-old maize seedlings were agroinoculated with \( p\text{BIKom602}, p\text{BIKomRb}^2 \) or \( p\text{BIKomRb}^2 \text{C}(601)A \), and 14 control plants were injected with sterile distilled water, for a total of 42 plants agroinoculated with each MSV construct. Chlorotic leaf areas (%) were measured for leaves 2, 3, 5 and 6 of each symptomatic plant by using a microcomputer-based image-analysis technique (Martin & Rybicki, 1998). Leaves 2 and 3 were assessed 15 days post-agroinoculation (p.i.); leaves 5 and 6 were assessed 29 and 35 days p.i., respectively. For each MSV construct, the mean percentage chlorotic area on leaves 2, 3, 5 and 6 was used as a representative measure of chlorosis.

**Cloning and sequencing of MSV DNA from infected plants.** Total DNA was extracted from infected maize plants; Rep genes were PCR-amplified from each sample by using the primer set C1 (F) and C2 (R) (Table 1), inserted into the pGEM-T Easy vector (Promega) and both strands were sequenced.

**Analysis of the C(601)A reversion in pKomRb".** For maize seedlings agroinoculated with \( p\text{BIKomRb}^2 \), a sample was taken from the leaf tip as it emerged from the whorl, then the leaf was sampled progressively downwards towards the base on or around every 10 days and total DNA was extracted. Sequence-specific forward primers were designed \([\text{Rb}^2 \text{A} (\text{F}) \text{and Rb}^2 \text{C} (\text{F}); \text{see Table 1}]\) to differentially amplify Rep genes containing the A(601) or C(601) nucleotide. The reverse primer was the C2 (R) primer (Table 1). The plasmids \( p\text{SKRepRb}^2 \) and \( p\text{SKRepRb}^2 \text{C}(601)A \) were included in each PCR as controls: the former was amplified by the \( \text{Rb}^2 \text{C} (\text{F}) \) primer, but not by the \( \text{RepRb}^2 \text{A} (\text{F}) \) primer; the latter was amplified by the \( \text{RepRb}^2 \text{A} (\text{F}) \) primer only.

**Analysis of the transition/transversion ratio in MSV.** Twenty-two full-length genomic MSV sequences, including maize-type and grass-type isolates from strains MSV-A, B, C, D and E (Martin et al., 2001), were aligned by using DNAMAN. The rate of each type of substitution relative to G to T was estimated by using PAUP* 4.0 (Swofford, 2001).

**RESULTS**

A 3 nt mutation (Rb") altering the RepA pRBR-interaction motif abolishes the interaction between MSV RepA and maize pRBR in yeast

The ability of RepA containing the LLCNE to LLCLK substitutions to bind to maize pRBR (ZmRb1) was determined by using a yeast two-hybrid assay. Of the MSV RepA and Rep constructs tested, only MSV-Kom RepA and MSV-NG1 RepA (RepANs, a positive MSV control) interacted with ZmRb1 (Table 2). ZmRb1 interacted with...
neither MSV-Kom RepARb\(^{-}\text{N201L,E202K}\), consistent with earlier studies on WDV (Xie et al., 1995) and BeYDV (Liu et al., 1999), nor MSV-Kom Rep, as shown previously for MSV-NG1 (Horvath et al., 1998), BeYDV (Liu et al., 1999) and WDV (Gutierrez et al., 2004) Rep proteins.

**The Rb\(^{-}\) mutation has no effect on MSV replication in BMS**

The effect of the Rb\(^{-}\) mutation on replication of the MSV-Kom genome was tested by transient-replication assays. Fig. 2 shows QPCR assays of the trans replication of MSV-Kom and Rep-deficient MSV-Kom (MSV-PstI) by wt and Rb\(^{-}\) mutant Rep proteins. The replication of wt MSV-Kom and MSV-KomRb\(^{-}\) viruses was also compared (Fig. 2b). The Rb\(^{-}\) mutation had no effect on the trans-replicating ability of RepRb\(^{-}\text{N201L,E202K}\); both this and wt Rep, expressed in trans from the ubiquitin promoter, enhanced MSV-Kom genome replication ~30 fold when compared with the DNA levels achieved by MSV-Kom alone. Although, in this assay, wt Rep expressed by MSV-Kom was potentially available to bind to pRBR molecules, RepRb\(^{-}\text{N201L,E202K}\) and Rep also trans-replicated pMSV-PstI, which cannot express Rep or RepA, to similar levels.
Yeast cells containing plasmids encoding GAL4 BD- and AD-fusion proteins were grown on drop-out medium lacking Leu, Trp and His and supplemented with 5 mM 3-AT. Cells containing interacting fusion proteins can grow in the absence of His. Each of the Rep and RepA proteins was tested for interaction with ZmRb1. For negative controls, each Rep or RepA protein was tested for interaction with the GAL4 BD alone or with a GAL4 BD–human LamC protein fusion. Interaction of a BD–p53 fusion with an AD–SV40 T antigen was a positive control, using plasmids supplied with the Matchmaker yeast two-hybrid system (Clontech).

Growth is indicated by +; absence of growth is indicated by −.

<table>
<thead>
<tr>
<th>Bait protein (GAL4 BD fusion)</th>
<th>Prey protein (GAL4 AD fusion)</th>
<th>Growth of yeast (Trp+, Leu+, His+, +5 mM 3-AT)</th>
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<tr>
<td>None</td>
<td>RepA</td>
<td>−</td>
</tr>
<tr>
<td>ZmRb1</td>
<td>RepA</td>
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</tr>
<tr>
<td>LamC</td>
<td>RepA</td>
<td>−</td>
</tr>
<tr>
<td>None</td>
<td>RepARb−N201LE202K</td>
<td>−</td>
</tr>
<tr>
<td>ZmRb1</td>
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</tr>
<tr>
<td>p53</td>
<td>SV40 T antigen</td>
<td>++</td>
</tr>
<tr>
<td>ZmRb1</td>
<td>RepANs</td>
<td>+</td>
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(Fig. 2c). Similarly, there was no difference in the replication ability of the wt and Rb− mutant viruses (Fig. 2b), with the wt and mutant Reps being expressed solely from their native promoters rather than being highly expressed in trans. These data show that the Rb− mutation in RepRb−N201LE202K has no apparent effect on its ability to initiate rolling-circle replication of the MSV genome, indicating that, like BeYDV (Liu et al., 1999), but unlike WDV (Xie et al., 1995), RepA–pRBR interaction is not necessary for MSV replication in cultured cells.

**The Rb− mutation decreases symptom severity**

The relative severities of MSV-Kom and MSV-KomRb− infections were established by symptom assessment after agroinoculation of the MSV-sensitive maize cv. Jubilee. MSV-KomRb− systemically infected maize, although symptom severity on each of leaves 2–6 was clearly lower (Fig. 3) and plants were less stunted than those infected with wt virus. However, the timing of symptom development and the percentages of agroinoculated plants that became infected were the same in both mutant and wt infections.

The mutant virus was also transmissible to maize by Cicadulina mbila leafhoppers (data not shown).

**A single nucleotide of the 3 nt Rb− mutation reverts at a high frequency**

Whilst BfrI cleavage of the PCR products from maize-leaf samples taken between 19 and 24 days after agroinoculation with MSV-KomRb− showed that they contained a mutant viral population, Rep genes amplified from leaf samples from the same plants and from leafhopper-inoculated plants around 60 days p.i. were no longer digestible with BfrI. The Rep genes from the samples that had lost the BfrI site, as well as from samples taken from leaves 5 and 12 of maize approximately 90 days after agroinoculation with MSV-KomRb−, were PCR-amplified and sequenced. All Rep genes sequenced from MSV-KomRb−-inoculated samples contained a single-nucleotide reversion of C(601) to A(601) (relative to the Rep gene start codon), which resulted in a conservative amino acid change of leucine to isoleucine (see Table 3). The A(602)T and G(604)A mutations that, together with A(601)C, comprised the original Rb− mutation were unaltered. Rep genes from MSV-Kom-infected plants contained no mutations. Plasmid DNA was extracted from the A. tumefaciens culture used for the agroinoculations and the RepRb− gene was PCR-amplified and subjected to BfrI restriction digest; products were digested completely (data not shown), indicating that no detectable reversion occurred in A. tumefaciens.

In order to study the nucleotide reversion more thoroughly, samples were taken from MSV-KomRb−-agroinoculated plants as each leaf emerged from the whorl and thereafter on or around every 10 days for 120 days, and total DNA was extracted. Sequence-specific PCR primers, designed to differentially amplify Rep genes containing the A(601)
or C(601) nucleotide, were used to determine when the nucleotide reversion first appeared in each sample (Fig. 4).

All symptomatic plants contained the A(601) revertant virus by 33 days p.i. (Fig. 4). In five plants, the revertant virus was detected as early as 10 days p.i. By day 90 p.i., the A(601) revertant population had outcompeted the C(601) population to the point where the latter population was no longer detectable. This was determined by non-digestibility of PCR products with BfrI, by non-amplification of the products with the C(601)-specific primer and by the fact that all Rep genes cloned and sequenced from infected plants around 90 days p.i. contained the C(601)A reversion. Whilst, in most cases, a 121 nt stretch of the Rep gene encompassing the mutated area was sequenced to detect a reversion, six full-length Rep genes amplified from two plants were also sequenced: five out of six contained no other mutations, indicating that, in most cases, the selection pressure was for the C(601)A reversion alone.

### Table 3. Wild-type, mutant and C(601)A revertant sequences in Rep genes and proteins

<table>
<thead>
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<th>Mutation</th>
<th>Rep gene</th>
<th>Nucleotide sequence*</th>
<th>Rep protein</th>
<th>Amino acid sequence (consensus = LxCxE)†</th>
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<td>Rep</td>
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<td>Rep</td>
<td>198LLCNE</td>
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<tr>
<td>Rb^ mutation</td>
<td>RepRb^</td>
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<tr>
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<td>RepRb^C(601)A</td>
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</tbody>
</table>

*The single-nucleotide reversion in RepRb^C(601)A is shown in comparison with the original mutated sequence in RepRb^ and the wt sequence in Rep (mutated nucleotides are in bold). Numbering is relative to the first nucleotide of the Rep gene start codon. Note that the BfrI site (underlined) that was introduced with the Rb^ mutation is lost in RepRb^C(601)A.

†The pRBR-interaction motif sequence in wt Rep and motif mutations in RepRb^N201L,E202K and RepRb^L201I,E202K are shown, with the amino acid changes highlighted in bold. Numbering is relative to the N-terminal residue of the Rep protein. Note that the C(601), T(602) mutations together altered a non-conserved asparagine residue to leucine and the C(601)A reversion resulted in a conservative change from leucine to isoleucine.

The Rep C(601)A reversion provides a selective advantage other than improved pRBR-binding affinity

Whilst it is possible that position 601 of the MSV-Kom Rep (or RepA) gene is a mutational hotspot, it is unlikely that the C(601)A reversion could be maintained without strong selective pressure. It has been noted that transitions (A«G and C«T) occur at higher frequencies than transversions (A«T, A«C, G«T and C«G) in virtually all DNA sequences examined from any genome (Yang & Yoder, 1999). MSV is no exception: our analysis of the relative transition/transversion ratios in MSV (the genome of which has equal base frequencies) revealed a mutational bias in favour of transitions (A«G=1.35; C«T=2.09; A«T=0.99; A«C=0.74; C«G=0.69; relative to Ge«T=1.0). As a C to A transversion is the second least likely substitution to occur in MSV, it must provide a strong selective advantage to be fixed in preference to the more frequently occurring substitutions.

Fig. 4. Detection of C(601) mutant and A(601) revertant viral populations in eight plants agroinfected with MSV-KomRb^-. Leaf samples were taken as each leaf emerged from the whorl and on or around every 10 days thereafter, and total DNA was extracted. PCR amplification with primers designed to specifically amplify Rep genes containing either the A or the C nucleotide at position 601 allowed determination of when the nucleotide reversion first appeared in each sample. Symptom development was observed by eye. Filled bars, first appearance of symptoms; hatched bars, first detection of C(601) virus; empty bars, first detection of A(601) virus.
The first option investigated for possible selection pressure was whether the C(601)A reversion increased the binding affinity of the mutant RepA to pRBR. This was determined by using a yeast two-hybrid assay. Whilst wt RepA interacted with ZmRb1, RepArbC(601)A,201K did not (Table 2). Therefore, it appears that the loss of pRBR-interaction ability in RepRbC(601)A was not detectably restored by the C(601)A reversion.

The effect of the C(601)A reversion on virus replication was analysed by bombardment of BMS with pKomRbC(601)A and co-bombardment with pMSV-Pstl + pRepRbC(601)A. As can be seen in Fig. 2(b), replication levels of MSV-Kom, MSV-KomRb− and MSV-KomRbC(601)A were indistinguishable from one another. Similarly, although RepRbL201,E202K apparently replicated MSV-Pstl to slightly higher levels than did either Rep or RepRbC(601)A, these differences were not significant (P>0.05).

Finally, MSV-Kom, MSV-KomRb− and MSV-KomRbC(601)A infectivities were compared by agroinoculation of maize and analysis of symptom severities. The symptoms induced by MSV-KomRb− and MSV-KomRbC(601)A were not significantly different at any point in the infection (Student’s t-test; Fig. 3). As A(601) in the Rep gene of MSV-KomRb−C(601)A did not obviously increase its virulence relative to that of MSV-KomRb−, the selective pressure for the reversion did not appear to be increased pathogenicity. However, as the C(601)A reversion in MSV-KomRb− occurs so rapidly (the earliest that the infected leaf samples were analysed by PCR was on day 10 p.i., therefore the reversion probably appears earlier), it is impossible to separate the symptom-severity profiles of the mutant and C(601)A revertant viruses. It is possible that, by the time symptoms were analysed, the disadvantage of the C(601) mutation had been overcome and the mutant had effectively become the C(601)A revertant. This is further corroborated by the fact that, in some plants (see Fig. 4), the reversion was detected before symptoms were even detectable. It can, therefore, only be concluded that the C(601)A reversion did not increase symptom severity to wt levels, which is not surprising, as MSV-KomRbC(601)A and MSV-KomRb− shared an inability to interact with maize pRBR.

**DISCUSSION**

Recent studies have supported the view that geminivirus replication in terminally differentiated cells requires an interaction between viral Rep or RepA proteins and host RBR proteins, for the induction of host replication-associated genes (Hanley-Bowdoin et al., 2004). It has been established that, with the begomovirus Tomato golden mosaic virus (TGMV), Rep–pRBR interaction and associated proliferating-cell nuclear-antigen accumulation is important for infectivity in Nicotiana benthamiana (Egelkrout et al., 2001, 2002; Kong et al., 2000). In mastreviruses, indications that RepA–pRBR interaction is required in the viral life cycle came from observations that: (i) MSV and BeYDV mutants that are unable to express RepA are unable to infect plants (Liu et al., 1998; Wright, 1995); (ii) RepA binds pRBR in yeast (Gutierrez et al., 2004; Horvath et al., 1998; Liu et al., 1999); and (iii) RepA can stimulate cell division in tobacco cell cultures (Gordon-Kamm et al., 2002). Recently, it was shown that MSV V-sense promoter activation by RepA apparently depends on an intact pRBR–interaction domain, although the heterologous WDV and not MSV RepA was used in the expression studies (Muñoz-Martin et al., 2003). As has been demonstrated previously for BeYDV pRBR interaction-domain mutants (Liu et al., 1999), we have shown that MSV mutants that cannot interact detectably with pRBR retain their ability to establish a systemic infection in maize. However, unlike the attenuation of MSV–KomRb−-induced symptoms in maize, the BeYDV mutants were able to produce an apparently wt infection in N. benthamiana and bean. Until now, the only geminivirus for which a mutation in Rep abolishing pRBR binding has been shown to affect symptom development was TGMV (Hanley-Bowdoin et al., 2004), which led the authors to conclude that the role of pRBR binding in a variety of geminivirus–host combinations needs to be examined. This report therefore adds MSV and maize to the few combinations that have thus far been studied.

The difference in apparent fitness of BeYDV and MSV mutants relative to wt viruses may be attributable to differential requirements for pRBR interaction in viruses infecting dicots (BeYDV) and monocots (MSV). It has been suggested that BeYDV, when adapting to replicate in dicots, may have gained a begomovirus-type interaction between Rep and pRBR that does not require an LxCxE motif (Gutierrez, 2000). However, our results indicate that a monocot-infecting mastrevirus can also replicate and establish a systemic infection (albeit attenuated) in the absence of RepA–pRBR interaction. Alternatively, the difference would be explained if BeYDV, unlike MSV, is phloem-limited, as suggested by Hanley-Bowdoin et al. (2004) and McGivern et al. (2005).

Assuming that MSV infectivity depends on V-sense promoter activation by Rep/RepA, it appears that MSV RepA–pRBR interaction is not required for activation of the MSV V-sense promoter. Although Muñoz-Martin et al. (2003) found that WDV RepA-mediated activation of the MSV V-sense promoter depended on an intact pRBR–interaction domain, they did propose that MSV RepA could potentially activate the MSV V-sense promoter by a pRBR-independent route, as the present study suggests. However, whilst the lack of yeast growth in yeast two-hybrid studies has been interpreted as a lack of interaction between Rb− mutant RepAs and pRBR, the possibility exists that an alternative binding domain in the mutant RepAs allows the binding of pRBR with a very low affinity in plants, which was undetectable in our assay. TGMV Rep (AL1) does not have an LxCxE motif and interacts with pRBR through another sequence, designated helix 4. A
similar structure is also predicted in MSV and BeYDV RepA (Arguello-Astorga et al., 2004; Kong et al., 2000).

It is possible that the attenuation of infection symptoms induced by MSV-KomRb relative to wt virus was due to an abolition or dramatic reduction of pRBR binding by the mutant RepA. The pattern of chlorotic streaks on maize leaves is correlated directly with the pattern of virus accumulation (Lucy et al., 1996). As MSV-KomRb-infected plants developed slightly narrower streaks than those infected by wt virus, the mutation may have lowered virus replication efficiency in terminally differentiated cells or altered the tissue specificity of the virus. For example, it might only replicate in vascular tissue, using pre-existing host replication factors that are present in some phloem-associated cells, as suggested for TGMV by Hanley-Bowdoin et al. (2000). This suggestion is supported by the data of McGivern et al. (2005), using an MSV RepA mutant that is also unable to bind pRBR. These factors limiting virus replication would not be expected to have an effect in the replication assays used in this study [and in those of Xie et al. (1995) and Liu et al. (1999)], which employed the use of actively dividing cell cultures that were already competent for host DNA replication.

Taking into account the high-frequency occurrence of a reversion of C(601)A in the mutated RepA gene, which had no detectable effect on pRBR-binding efficiency, the 3 nt Rb− mutation may have had effects separate from pRBR binding. Considering the small size of the MSV genome, it would not be surprising for any particular stretch of sequence to have more than one function in the viral life cycle. The fact that the Rb− mutation and the C(601)A reversion had no effect on virus replication in BMS may indicate that it did not affect the functions of Rep, but rather those of RepA. A single nucleotide change could affect splicing of the complementary-sense transcripts, thereby altering the ratio of Rep to RepA. Exonic sequences upstream of the Rep intron could affect splicing efficiency, either by influencing RNA secondary structure or by comprising splicing-enhancer sequences (Manley & Tacke, 1996). In turn, RepA expression is determined by how efficiently the transcript is spliced. Increased splicing efficiency of the Rep transcript could result in lower expression of coat protein (CP), due to both lower RepA-mediated activation and increased Rep-mediated repression (Muñoz-Martín et al., 2003) of the CP promoter. Thus, decreasing splicing efficiency to wt levels could be the selective pressure for the C(601)A reversion. Alternatively, the MSV genome secondary structure may have inherent biological properties, perhaps influencing virus location or the mechanisms by which the genome is replicated, transported or encapsidated. For example, single-stranded DNA (ssDNA)–capsid interactions may be dependent on ssDNA secondary structure. Preliminary experimental results indicate that the predicted structures of wt and C(601)A revertant Rep transcripts (which may be similar to Rep DNA structure) are more stable and thermodynamically favourable over the C(601) Rb− mutant structure, providing a possible reason for the C(601)A reversion (data not shown).

Interestingly, a single nucleotide mutation in MSV-NG1, equivalent to one [G(604)A] of the three mutations in MSV-KomRb−, which alters the LLCNE motif to LLCKN, does not revert (McGivern et al., 2005). The fact that reversion to a wt motif does not occur, despite the favoured status of A to G transitions in MSV, reinforces the proposal that the selective pressure for the C(601)A reversion in MSV-KomRb− may be operating at the nucleic acid (structure) level, rather than at the level of Rep and RepA expression or function.

In conclusion, we have demonstrated that an intact MSV Rep pRBR-interaction motif is not required for virus replication in culture cells or infectivity in maize, although it is possibly required for wt symptom development. As Liu et al. (1999) reported similar results for BeYDV, it appears that WDV is exceptional amongst mastreviruses in that it requires a consensus pRBR-interaction motif, even in wheat suspension cells, where host replication factors should be abundant. The high-frequency single-nucleotide reversion in the DNA encoding the MSV pRBR-interaction motif may shed some light on the reason that the apparently non-essential LxCxE motif has been retained in mastreviruses, whilst it has been lost in begomoviruses. The consistent emergence and eventual dominance and easy detection of the C(601)A revertant viral population can be used as a system to investigate aspects of MSV biology, such as transcript or ssDNA secondary-structure constraints, movement models, replication, mutation and evolution rates, and complex population phenomena, such as competition between quasispecies and population turnover.

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