An intact RBR-binding motif is not required for infectivity of *Maize streak virus* in cereals, but is required for invasion of mesophyll cells

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The replication-associated protein (RepA) of *Maize streak virus* interacts in yeast with retinoblastoma-related protein (RBR), the negative regulator of cell-cycle progression. This may allow geminiviruses to subvert host cell-cycle control to provide an environment that is suitable for viral DNA replication. To determine the importance of this interaction for MSV infection, the RBR-binding motif, LxCxE, was mutated to LxCxK or IxCxE. Whilst RBR binding in yeast could not be detected for the LxCxK mutant, the IxCxE protein retained limited binding activity. Both mutants were able to replicate in maize cultures and to infect maize plants. However, whereas the wild-type virus invaded mesophyll cells of mature leaves, the LxCxK mutant was restricted to the vasculature, which is invaded prior to leaf maturity. Mature leaves contain high levels of RBR and it is suggested that the MSV RepA–RBR interaction is essential only in tissues with high levels of active RBR.

Geminiviruses have small, single-stranded DNA (ssDNA) genomes that replicate through double-stranded DNA (dsDNA) intermediates in the nuclei of infected cells. The viruses do not encode a DNA polymerase and must use the host machinery for viral DNA replication. However, DNA polymerases are not functional in mature leaf cells and, to infect these cells, geminiviruses must deregulate cell-cycle control to provide an environment that is permissive for viral DNA replication. This is probably accomplished by the interaction of geminivirus replication factors with the negative regulator of G1–S phase progression, the retinoblastoma-related protein (RBR; Gutierrez, 2002). This mechanism appears to be conserved throughout the geminiviruses, irrespective of whether they infect monocots [e.g. the mastreviruses *Maize streak virus* (MSV) and *Wheat dwarf virus* (WDV) (Horvath et al., 1998; Xie et al., 1995)] or dicots [e.g. *Bean yellow dwarf virus* (BeYDV; Liu et al., 1999) and the begomovirus *Tomato golden mosaic virus* (TGMV; Kong et al., 2000)]. For WDV and BeYDV, RBR binding occurs via the conserved LxCxE motif in the replication-associated protein RepA. The motif is not present in begomovirus proteins and RBR binding is mediated in part through a conserved α-helical region in the Rep protein (Arguello-Astorga et al., 2004; Hanley-Bowdoin et al., 2004; Kong et al., 2000).

Interactions with RBR have been shown in yeast, but not in plant cells, although RBR proteins have been cloned from both monocots and dicots (Dewitte & Murray, 2003). Genetic studies have not provided a definitive answer regarding the requirement for RBR binding. Geminivirus mutants with impaired RBR binding in yeast produce either wild-type (wt) (BeYDV; Liu et al., 1999) or attenuated symptoms (TGMV; Kong et al., 2000) or are unable to replicate in wheat suspension cells (WDV; Xie et al., 1995). To assess whether interaction of MSV RepA with RBR is necessary for MSV replication and infection, we made mutations in the RBR-binding motif that we predicted would reduce, or abolish, the interaction.

Plasmid MB64 was used for mutagenesis of RepA; it contains a BamHI/BglII fragment (co-ordinates 1203–2686) of the MSV Ns (renamed MSV–NG1) genome (GenBank accession no. X01633). Mutagenesis was performed by using a QuikChange site-directed mutagenesis kit (Stratagene) and the primers RbEKV (GGCTGGAGCCAATCATTGTATGTACTATTACAAAGTAAATCAGG) and RbEKC (CC-TGATTTACCTTGTGATATCAGCC) to replace the conserved glutamine (E) residue of the RBR-binding motif with lysine (K), thereby producing pMB64-LxCxK, which differs from the wt genome by only 1 nt. Primers RbLIV (CATCACCCTCATCACCTGATA-TCCTTTGTAATGTGCACTCAATC) and RbLIC (GATTGA-CTCATACAAAAAGAATACAGGTGAGGGTGAGT) were used to produce pMB64-IxCxE, in which isoleucine (I) replaces the conserved leucine (L) residue. All constructs were verified by sequencing.
The effect of these mutations on interaction with maize RBR was assessed by using the Matchmaker yeast two-hybrid system (Clontech) and the GAL4 binding domain (BD) construct pGBT9ZmRb1 (Horvath et al., 1998). RepA sequences were fused to the GAL4 activation domain (AD) of pGAD10 following PCR amplification from pMB64, pMB64-LxCxE and pMB64-LxCxK, thereby creating pGAD-LxCxE, pGAD-IxCxE and pGAD-LxCxK, which were used to transform yeast strain Y187 (Gietz & Woods, 1994). After mating with strain CG1945 containing either pGBT9ZmRb1 or control plasmids (pGBT9 or pLamC), transformants containing both AD and BD plasmids were selected as described in the Clontech manual.

Interaction between bait and prey proteins was evidenced by histidine prototrophy on medium supplemented with 6 mM 3-amino-1,2,4-triazole (3-AT). RepA and RepA-LxCxK, but not RepA-LxCxK, interacted with ZmRb1 (Table 1). The interaction of RepA and ZmRb1, assessed by expression of the lac reporter gene, resulted in β-galactosidase expression approximately 10-fold higher than the background activity associated with yeast transformed with pGBT9, pGAD10 or pLamC (Table 1). However, RepA-LxCxE showed a markedly reduced affinity for ZmRb1; approximately 10% of that obtained with the wt protein. This is consistent with the data reported for the equivalent mutation in BeYDV RepA, where binding was reduced by up to 85% (Liu et al., 1999). The activity obtained with RepA-LxCxK was not significantly different from the background (Table 1). The equivalent mutation in WDV RepA eliminated interaction with the human p130Rb and ZmRb1 in yeast (Xie et al., 1995, 1996). Similarly, MSV RepA proteins in which the latter two amino acids of the RBR-binding motif were substituted (Shepherd et al., 2005) do not interact with ZmRb1. Although the WDV mutant was unable to replicate in suspension-cultured wheat cells, the MSV mutants replicated efficiently.

To determine whether MSV carrying the LxCxK mutation could replicate in maize cells, a dimeric copy of the MSV-LxCxK genome was constructed. Plasmid MB64-LxCxK was digested with BamHI and Xhol to release a 1 kb fragment (MSV co-ordinates 1683–2686). An MSV-A[NG1] dimer in pUC19 (Boulton et al., 1993), was similarly digested and the fragment encompassing MSV co-ordinates 1–1682 was recovered. A three-way ligation was performed by using BamHI-linearized pAHC17 (Christensen & Quail, 1996) and the two MSV fragments. After confirming the sequence of the 1 kb region, the mutant MSV monomer was released from pAHC17 by digestion with BamHI and inserted into pUC19 using a 15-fold molar excess of insert to vector. This product was used to transform Escherichia coli DH5α. EcoRI plus XbaI digestion identified the dimer construct.

Maize (BMS) suspension cells were inoculated (Boulton et al., 1993) with dimeric copies of either MSV or MSV-LxCxK as described by Liu et al. (2002). Samples were taken 0, 2, 5 and 8 days post-inoculation (p.i.) and extracted DNA was subjected to Southern blot analysis as described by Boulton et al. (1993). In two experiments, the wt and mutant virus accumulated both ss- and dsDNA forms; the time-course of mutant viral DNA accumulation was similar to the wt in experiment 1, but was delayed in the second experiment (shown in Fig. 1a).

The lack of requirement for RBR binding for virus replication was not unexpected, as dividing cells possess the machinery for DNA synthesis and BMS cells contain low levels of active RBR (Huntley et al., 1998). Our data contrast with those obtained by Xie et al. (1995), who found that the equivalent WDV mutant could not replicate in cultured cells, but are consistent with the data of Shepherd et al. (2005) for MSV in BMS cultures. Either wheat cells are less competent for DNA replication, or the mutation in WDV disables a function that is necessary for viral DNA replication.

### Table 1. Yeast two-hybrid analysis of MSV RepA–maize RBR protein (ZmRb1) binding affinity

<table>
<thead>
<tr>
<th>Bait protein (GAL4 BD fusion)</th>
<th>Prey protein (GAL4 AD fusion)</th>
<th>Growth (His+, +3-AT)*</th>
<th>β-Galactosidase activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (pGBT9 only)</td>
<td>None (pGAD10 only)</td>
<td>–</td>
<td>0.26 ± 0.11</td>
</tr>
<tr>
<td>None (pGBT9)</td>
<td>RepA</td>
<td>–</td>
<td>0.26 ± 0.23</td>
</tr>
<tr>
<td>LamC</td>
<td>RepA</td>
<td>–</td>
<td>0.34 ± 0.16</td>
</tr>
<tr>
<td>ZmRb1</td>
<td>None</td>
<td>–</td>
<td>0.35 ± 0.16</td>
</tr>
<tr>
<td>ZmRb1</td>
<td>RepA</td>
<td>+ + +</td>
<td>3.80 ± 0.78</td>
</tr>
<tr>
<td>ZmRb1</td>
<td>RepA-LxCxE</td>
<td>+</td>
<td>0.72 ± 0.11</td>
</tr>
<tr>
<td>ZmRb1</td>
<td>RepA-LxCxK</td>
<td>–</td>
<td>0.39 ± 0.21</td>
</tr>
<tr>
<td>SV40 T</td>
<td>p53</td>
<td>+ + + + +</td>
<td>83.28 ± 15.65</td>
</tr>
</tbody>
</table>

*Growth of yeast was assessed visually on an incremental scale, from one to five colonies (+) to confluent growth (+ + + + +); –, no growth.
†Mean ± SD β-galactosidase activity [relative light units (μg protein)−1 min−1] was calculated from three individual yeast colonies for each construct.
A mutation (KEE146) in TGMV Rep that reduced RBR binding to 16% of wt levels in yeast resulted in limitation of the virus to the vascular cells (Kong et al., 2000). To test whether MSV mutants with impaired RepA–ZmRb1 binding can infect plants, constructs were prepared for agroinoculation of maize. An MSV monomer containing the LxCxE mutation was constructed as described for MSV-LxCxK. Dimeric copies of the genomes were inserted into pBIN19 as described for pUC19 constructs. Agrobacterium tumefaciens strain pGV3850 was transformed into pBIN19 as described for pUC19 constructs. 

**Fig. 1.** Southern hybridization analysis of the replication of wild-type MSV (E) and MSV-LxCxK mutant (K) in Black Mexican Sweet (BMS) maize suspension cultures (a) and infected maize plants (b). Samples were taken at 0, 2, 5 and 8 days post-bombardment (dpb) for cell samples and 28 days p.i. for plants. The results from two plants infected with each construct are shown in (b). The concentration of plant DNA loaded is approximately 1% of that loaded for the BMS samples. IP: Infected maize plant DNA; oc, open circular dsDNA; lin, linear dsDNA; ccc, covalently closed circular (supercoiled) dsDNA; ss, ssDNA.

MSV-A[NG1] is adapted to maize. To investigate whether the LxCxE motif is a host-range determinant, Cicadulina mbila was used to transmit the virus from MSV-LxCxK-infected maize to barley (cv. Igri) and wheat (cv. Chinese Spring). All nine of the barley and four of the five wheat plants became infected. Infectivity was equivalent to that seen with the wt virus (10/10 and 3/4 infected, respectively), although MSV-LxCxK caused less severe streaking and stunting (Fig. 2b). Clearly, an intact LxCxE domain is not required for insect transmission, encapsidation of viral DNA or infection of hosts to which MSV-A[NG1] is not adapted. Furthermore, infection is established irrespective of whether cloned DNA is delivered to the meristematic region of the plant (as ssDNA via agroinoculation) or virions are introduced into the phloem of mature leaves (via insect transmission). These data support the hypothesis that the initiation of viral dsDNA synthesis must occur in a cell that is competent for DNA replication.

As MSV infects most cells of the mature leaf, the narrower-streak phenotype could indicate a restriction of the MSV-LxCxK mutant to the vascular system, as shown for TGMV mutant KEE146 (Kong et al., 2000). Immunocytochemical staining of the MSV coat protein (Lucy et al., 1996) in sections from MSV-LxCxK- or MSV-infected maize showed that fewer vascular bundles contained virus in mutant-compared with wt-infected leaves (71 versus 88%, respectively); this was consistent with the lower titre of viral DNA in these plants (Fig. 1b). Furthermore, signal was detected in the mesophyll cells of leaves infected with wt MSV, but not in the MSV-LxCxK-infected leaves. However, staining in mutant-infected plants was generally weaker and, because maize has few mesophyll cells between the vascular bundles (Fig. 2c), estimation of mesophyll invasion was difficult.

To further assess tissue tropism in the MSV-LxCxK-infected leaves, sections were subjected to transmission electron microscopy (TEM) (Wells, 1985) and immunogold staining. Four areas were sampled for each of the inocula. TEM confirmed the decreased invasion of the vasculature by the mutant compared with the wt virus, and suggested that the infected cells contained fewer virions [evidenced as smaller nuclear crystalline arrays; compare Fig. 2(d) and (e)]. The lack of infection of cells outside the vasculature in mutant-infected leaves was also confirmed: out of 43 infected bundles that were examined, only one infected mesophyll cell and one infected epidermal cell were seen (Fig. 2f–i), whereas virus was seen in over 80% of mesophyll cells adjoining vascular bundles infected with MSV (Fig. 2c).

The ability of MSV-LxCxK to replicate in the vasculature of maize plants, despite its inability to bind RBR in yeast, suggests that the vascular cells are DNA replication-competent. MSV replication occurs in the vascular tissue of leaf primordia and immature leaves (Lucy et al., 1996).
Fig. 2. Infection of plants by wild-type MSV and MSV-LxCxK. (a) Symptoms obtained in maize leaves infected with wild-type (WT) MSV and mutants MSV-IxCxE and MSV-LxCxK; H, uninfected leaf. (b) Symptoms obtained in barley plants inoculated with the non-viruliferous vector C. mbila (H) or vector carrying wild-type MSV (WT) or mutant MSV-LxCxK. Note the decreased stunting obtained in plants infected by the mutant virus. (c) Diagrammatic representation of sections showing cells infected by WT MSV (left panel) or MSV-LxCxK (right panel). Tissue tropism for WT MSV is typical of that seen previously (Lucy et al., 1996; Pinner et al., 1993; this study); that for MSV-LxCxK is typical of that seen by immunochromic staining and electron microscopy (this study). Shaded areas represent infected cells. (d–i) Electron micrographs of leaf samples infected with wild-type MSV (d) or mutant MSV-LxCxK (e–i); the arrows depict MSV aggregates within the nuclei of infected cells. Comparatively more cells were infected in plants inoculated with MSV. The MSV-LxCxK-infected mesophyll (MES) cell is shown in (f); MSV-specific immunogold labelling [panel (g)] confirmed the presence of virus in the aggregate arrowed in (f). The MSV-LxCxK-infected epidermal cell (EP) is shown in panel (h); immunogold labelling [panel (i)] confirmed the presence of virus in the aggregate arrowed in (h). All other MSV-LxCxK-infected cells were within the vasculature. M, Metaxylem; PX, protoxylem; P, phloem; BS, bundle sheath.
and DNA polymerases are likely to be present in these cells. In contrast, invasion of the mesophyll occurs only as the mature leaves unfold from the whorl. Mature mesophyll cells do not incorporate [3H]thymidine (M. I. Boulton, unpublished data) and, although RBR is present at low levels in proliferating basal tissue, mature maize tissue contains high levels of active pocket domain forms (Huntley et al., 1998). Thus, it is likely that MSV RepA–RBR binding is needed to sequester active (hypophosphorylated) RBR and, thereby, to overcome the block to G1–S phase progression for efficient virus replication, only in mature leaf cells. Taken together, our data, and those of Kong et al. (2000) using TGMV KEE146, suggest that geminiviruses that are naturally phloem-limited may not require RBR binding and that disruption of RBR binding affects symptom production only in viruses that invade the mesophyll. This conclusion is supported by the reduced symptom severity that is seen in maize infected by other MSV RBR-binding domain mutants (Shepherd et al., 2005). Thus, the wt symptoms produced by the BeYDV RBR-binding mutants (Liu et al., 1999) may reflect vascular limitation of BeYDV; this is currently under investigation.

The decreased immunochemical signal and size of crystaline arrays in cells infected by MSV-LxCxK suggest that replication efficiency of this mutant is impaired slightly; delay in accumulation of mutant viral DNA in BMS, in one of two experiments, supports this premise. Decreased replication could have pleiotropic effects on virus (or viral DNA) transport to adjacent cells. For example, if infection must occur during a developmental ‘window of opportunity’, it may not be established in cells receiving suboptimal amounts of viral DNA and uninfected vascular bundles could result. Nevertheless, replication is not impaired sufficiently to delay timing of symptom appearance or reduce infectivity.

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References


