Replication modes of Maize streak virus mutants lacking RepA or the RepA–pRBR interaction motif

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ABSTRACT

The plant-infecting mastreviruses (family Geminiviridae) express two distinct replication-initiator proteins, Rep and RepA. Although RepA is essential for systemic infectivity, little is known about its precise function. We therefore investigated its role in replication using 2D-gel electrophoresis to discriminate the replicative forms of Maize streak virus (MSV) mutants that either fail to express RepA (RepA−), or express RepA that is unable to bind the plant retinoblastoma related protein, pRBR. Whereas amounts of viral DNA were reduced in two pRBR-binding deficient RepA mutants, their repertoires of replicative forms changed only slightly. While a complete lack of RepA expression was also associated with reduced viral DNA titres, the only traces of replicative intermediates of RepA− viruses were those indicative of recombination-dependent replication. We conclude that in MSV, RepA, but not RepA−pRBR binding, is necessary for single-stranded DNA production and efficient rolling circle replication.

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Introduction

Maize streak virus (MSV), the type member of the genus Mastrevirus in the family Geminiviridae, causes the most severe viral disease of maize in Africa (Shepherd et al., 2009). It has a 2.7 kb monopartite single-stranded (ss) DNA genome (Fig. S1, reviewed by Gutierrez et al., 2004) composed of a long intergenic region (LIR), a short intergenic region (SIR) and four open reading frames (ORFs). These express a movement protein (MP; from the V2 ORF) and a coat protein (CP; from the V1 ORF) from the virion-sense strand, and two replication-initiator proteins, Rep (spliced from the C1 and C2 ORFs) and RepA (C1 ORF alone), from the complementary strand. Rep and RepA share the same N-terminus and differ in their C-termini, which allows their different and multiple functions in the viral life cycle. Rep cleaves virion-strand DNA at the origin of replication in a hairpin structure situated in the LIR to initiate rolling circle replication (RCR; see Laufs et al., 1995), resulting in new circular ssDNA progeny which is converted to double-stranded DNA (dsDNA) by complementary-strand replication (CSR) and covalently closed circular DNA (cccDNA) upon packaging around host nucleosomes (Saunders et al., 1991; Stenger et al., 1991; reviewed by Hanley-Bowdoin et al. (1999), Jeske (2009)). The precise role of RepA is less well defined, although it is at least involved in the induction of a cellular environment that is amenable to viral replication (see Gutierrez, 2000, for a review).

In addition to RCR, recombination-dependent replication (RDR) has been found for plant begomoviruses (Jeske et al., 2001; Jovel et al., 2007), curtoviruses (Preiss and Jeske, 2003) and mastreviruses (Erdmann et al., 2010) as well as animal circoviruses (Cheung, 2012). RDR uses cccDNA as a template and does not rely on an origin of replication. Instead, replication is initiated by base-pairing interactions between the template cccDNA strand and the homologous nucleotides on the single-stranded 3' overhanging end of a linearised geminiviral genome or genome fragment. Replication then proceeds by extension of the free 3' end of this “invading” linearised DNA molecule (Jeske, 2007).

Since geminiviruses do not encode a polymerase, viral replication relies on host replication enzymes that are mainly inactive in differentiated cells, but which can be activated by the binding of
Rep/RepA to cell cycle-regulating factors like the plant retinoblastoma-related protein (pRBR) (Nagar et al., 1995; Xie et al., 1995, 1996; Ach et al., 1997; Kong et al., 2000; Egelkrout et al., 2001). However, Rep–pRBR interaction may not be sufficient to reactivate host replication in older leaves, as shown for the begomovirus African cassava mosaic virus (ACMV; Bruce et al., 2011).

Even though the mastrevirus Rep and RepA both contain the conserved pRBR-binding motif (LXCXE) (Fig. S1B), only RepA binds pRBR (Horvath et al., 1998; Liu et al., 1999b; Gutierrez et al., 2004).

An intact pRBR-binding motif of RepA in MSV is not required for replication in the phloem, but it promotes the invasion of terminally differentiated tissues in the mesophyll thereby increasing symptom severity (McGivern et al., 2005).

In addition to its role in replication, RepA activates V-sense gene transcription (Hefferon et al., 2006; Munoz-Martin et al., 2003), possibly by binding directly to the viral DNA, or by interfering with cellular transcription control, like the pRBR/E2F pathway (Munoz-Martin et al., 2003).

To investigate further the role of MSV RepA in viral replication, and in particular the importance of an intact pRBR binding domain, we analysed the viral replicative intermediates of three mutants of the MSV-A isolate MSV–Kom (from Komatiepoort, South Africa; Schnippenkoetter et al., 2001): one which cannot express RepA alone due to the removal of the rep intron (MSV–KomΔI), and two mutants deficient in pRBR-binding, MSV–KomRb− and MSV–KomRb−C(601)A (Shepherd et al., 2005).

Results and discussion

MSV DNA forms in maize suspension cells or maize plants

To discriminate viral DNA forms and replicative intermediates in maize cells, one-dimensional (1D; Fig. 1A) and two-dimensional (2D; Fig. 1B–D) gel electrophoresis followed by Southern blot hybridisation was applied as previously described (Jeske et al., 2001; Erdmann et al., 2010). Wild-type MSV–Kom (hereafter referred to as wt) replicated to considerably lower levels in suspension cells than in systemically infected plants and consequently different amounts of total DNA (400 ng versus 30 ng, Fig. S2) had to be loaded on gels to yield similar overall signal strengths after hybridisation. Under these conditions, no

Fig. 1. (A) 1D gel electrophoresis of total nucleic acids containing ~100 pg of viral DNA as determined by qPCR. Nucleic acids were separated for 19 h at 0.7 V/cm on a chloroquine-containing 1.4% agarose gel, transferred to a nylon membrane, and hybridised with a full-length MSV probe. 100 pg and 10 pg of the respective cloned and restricted full-length linear double-stranded DNA (lin) show the different sizes of the constructs as hybridisation standards (H100, H10). Viral DNA forms such as open circular (oc), single-stranded (ss) and circular covalently closed (ccc) DNA as well as multimeric (m) forms are indicated. Exposure time: 30 min. (B–D) 2D gel electrophoresis of wt (B) and the intronless rep mutant, MSV–KomΔI (C, D) DNA from suspension cells at 4 days post bombardment (dpb). DNA samples (B—1.3 μg, C—1.6 μg, D—6.5 μg) were separated on 0.5% agarose gels for 19 h at 0.7 V/cm for the first dimension (horizontal), and then on chloroquine-containing 1.4% agarose gels for 19 h at 1.5 V/cm for the second dimension (vertical) and prepared for hybridisation as described above. DNA forms are linear DNA (lin), heterogeneous linear double-stranded DNA (hds), single-stranded DNA, open circular (oc) and circular covalently closed DNA (ccc), as well as their dimeric forms (2oc and 2ccc respectively), smaller cccDNA of defective DNA (<1ccc) and relaxed cccDNA (rccc). Intermediates of rolling circle replication (RCR), recombination-dependent replication (RDR) and complementary-strand replication (CSR) are indicated. The unknown intermediate x is typical for MSV replication.
Expression of RepA has a profound effect on MSV replication modes

Viral DNA titres, quantified by real-time quantitative PCR (qPCR), were vastly reduced if the intronless rep mutant, pKomΔI (hereafter referred to as ΔI), was used to inoculate suspension cells (Fig. 2). In addition, 1D gel electrophoresis showed that ssDNA was below the detection limit (Fig. 1A). Because the 92-nt rep intron is missing, the mutant was slightly smaller than wt (Fig. 1A), which accordingly resulted in a shift in the mobilities of the oc and cccDNAs relative to those of the wt.

Due to the low titre of viral DNA in suspension cultures, it was challenging to optimise its extraction and gel separation to visualise the true replicative intermediates using 2D gel analysis. Although still close to the limit of detection, we succeeded unequivocally here for the first time in presenting the replicative intermediates of wt and mutant viruses under these conditions.

The ΔI mutant was able to replicate in suspension cells to a certain extent, as shown by both qPCR (Fig. 2) and the accumulation of monomeric ccc and ocDNA after 1D gel analysis (Fig. 1A). To control for input plasmid DNA levels that may be detectable by qPCR, a non-replicating MSV mutant, MSV-PstI (described by Owor et al. (2011)), was examined in parallel. This mutant does not express Rep (Fig. 2; Rep−), preventing replicational release from the inoculated plasmid. The remaining DNA levels of the Rep− mutant in BMS cells detected 4 days after bombardment were just 1% of the wt virus, whereas the ΔI mutant generated 7-fold more DNA than this control (p < 0.0001, Mann-Whitney test), confirming replicative release and true autonomous replication of viral monomeric circular dsDNA. Nevertheless, ssDNA was not detected in the 1D blot (Fig. 1A) and replicative intermediates indicating CSR and RCR were missing in the 2D analysis, both of which were clearly resolved for the wt (Fig. 1B, C). Loading four times more DNA for the ΔI mutant on 2D gels, RDR intermediates, but no RCR intermediates became visible (Fig. 1D). Notably, wt as well as mutant MSV produced relatively high amounts of defective DNA in suspension culture which is seen in the smaller ccc forms (Fig. 1B and C; < 1ccc).

In summary, the ΔI mutant replicates in suspension cells predominantly, if not exclusively, by RDR.

Replicative intermediates of pRBR-binding mutants

An MSV-KomRb− triple mutant (nts A(601)C, A(602)T and G (604)A with reference to the rep ORF start) was engineered previously to abolish pRBR binding to RepA (Shepherd et al., 2005). Within 10 days of inoculating plants with this mutant, a one-nucleotide reversion (C(601)A) was consistently detected (Shepherd et al., 2005) which rendered the triple mutant a double mutant. The single-nt reversion did not restore pRBR-binding activity, but possibly repaired an adversely affected genomic DNA secondary structure (Shepherd et al., 2006).

It is still uncertain why the C(601)A reversion occurred with such a high frequency, in that it restored neither symptoms (Shepherd et al., 2005) nor virus titres to wt levels in infected plants (Fig. 2). Although a previous semi-quantitative PCR assay revealed no difference in virus titres between wt viruses and the two pRBR mutants in suspension cells (Shepherd et al., 2005), the quantitative real-time PCR assay (Fig. 2) now shows 2.7- or 2-fold lower virus titres in cells infected with the triple mutant (MSV-KomRb−) or reversion mutant (MSV-KomRb−C(601)A) than those with wt virus. Whereas the difference between the two mutants was not significant (p = 0.59, Mann Whitney test), the differences between the mutants and the wt virus were supported statistically (p < 0.0001, Wilcoxon signed rank test).

In plants, the reduction of virus titres compared with the wt was even higher than in suspension cells (7.4-fold and 6.4-fold reduction for the triple mutant and the reversion mutant, respectively), again without significant differences between the mutants (p = 0.8; Mann Whitney test). This indicates that replication of the mutants is less impaired in suspension cells (~40% of the wt level) than in plants (~15% of the wt level).

To further investigate the role of the pRBR binding motif for MSV replication in plants, the replicative intermediates of wt and the two pRBR− mutants were compared after 2D gel electrophoresis (Fig. 3A–C). To compensate for the differences in virus titres so as to allow unbiased identification of qualitative differences of viral DNA forms, it was necessary to expose the films for different times (15 min for the triple mutant, 1 min for the reversion mutant and the wt) in order to yield similar signal strengths for oc and ssDNA (both taken as internal reference points).

Most of the major DNA forms (Fig. 3A–C; ss, oc, lin, ccc; monomeric and multimeric) were present in wt, triple mutant and reversion mutant blots, supporting previous findings
(McGivern et al., 2005; Shepherd et al., 2005) that an intact pRBR-binding site is not absolutely required for MSV replication. However, the efficiency of replication as represented by the arcs of replicative intermediates (Fig. 3A–C; CSR, RCR, RDR) was reduced in the triple mutant even when the blot was exposed for 15 × longer than for both the wt and revertant. Conversely, at the same exposure time as the wt, RCR and RDR arcs of the revertant were more wt-like, although CSR appeared to be reduced. No RDR arc was detectable for the triple mutant (Fig. 3B), even after prolonged exposure (data not shown).

Some peculiar features appeared in 2D electropherograms for the mutants. A complex spot arrangement (Fig. 3B; Z) at the position of trimeric cccDNA may hint at knotted DNA forms and a low resolution of replicative intermediates for the triple mutant. The reversion mutant revealed heterogeneous ocDNA (Fig. 3C, hoc), which has been observed so far only for DNA satellite replication (Alberter et al., 2005). Both mutants lacked the DNA form indicated by spot X that was present for wt (Fig. 3A) as described previously (Erdmann et al., 2010). Spot X is located on two arcs, representing the CSR on 2ss templates and the end of RCR. It may, therefore, indicate stalled processing during one of the two replication modes.

In the reversion mutant, 2cccDNA and RDR signals were stronger than in the triple mutant, more closely resembling the wt. Lines for the replicational intermediates, CSR and RCR, were also more intense in the reversion mutant than in the triple mutant. These results are consistent with the previous notion that the mutants might change the secondary structure of the viral DNA or complementary sense RNA transcripts, in addition to their DNA-binding and oligomerisation site (Fig. S1)( Horvath et al., 1999). Since MSV Rep and RepA contain the same DNA-binding and oligomerisation site (Singh et al., 2008). Oligomerisation of Rep is also important for the formation of three different Rep–DNA complexes in the monocot-infecting Wheat dwarf mastrevirus (WDV; Castellano et al., 1999). Since MSV Rep and RepA contain the same DNA-binding and oligomerisation site (Fig. S1) (Horvath et al., 1998; Missich et al., 2000), hetero-oligomerisation between Rep and RepA might be important in MSV for either the DNA binding affinity of Rep/RepA hetero-oligomers, or the specificity with which these hetero-oligomers bind DNA. In this scenario, the absence of RepA in the ΔI mutant could abolish RCR and thereby directly prevent the formation of ssDNA.

In contrast to MSV, RepA-deficient mutants of WDV (Collin et al., 1996) and the dicot-infecting Bean yellow dwarf mastrevirus (BeYDV; Liu et al., 1998; Hefferon and Dugdale, 2003) replicated to higher titres in cell culture than did wt viruses. BeYDV ssDNA

Possible functions of RepA in MSV replication

RepA may exert a direct or indirect role on replication modes. In addition to its function as a site-specific endonuclease, it is likely an activator of V-sense gene expression in MSV (Munoz-Martin et al., 2003; Hefferon et al., 2006) and the lack of RepA in the ΔI mutant abolishes the V1 ORF expression, leading to a destabilisation of ssDNA. The absence of ssDNA is typical of CP-deficient mutants (Boulton et al., 1993; Liu et al., 1997, 1999a, 2001; Hefferon and Dugdale, 2003). As geminiviral CPs bind ds- and ssDNA in a sequence non-specific manner and contain a functional nuclear localisation site (Liu et al., 1999a; Unseld et al., 2001, 2004; Hehnle et al., 2004), RCR might depend on encapsidation of the viral ssDNA occurring concomitantly with replication (Malik et al., 2005). A lack of RepA would possibly also affect expression of the MP (expressed from the V2 ORF). Although there may not be a direct role of MP in RCR, an interaction between MP and CP may be required for efficient sequestration by CP of geminiviral ssDNA. The lack of RCR intermediates in the absence of RepA may indicate a tight connexion between RCR and genome packaging, since complete Rep is able to initiate RCR of other geminiviruses (Elmer et al., 1988; reviewed by Laufs et al. (1995), Bisaro (1996)).

Alternatively, Rep–RepA hetero-oligomerisation may be important for RCR in mastreviruses. The RCR initiation process in Mungbean yellow mosaic India begomovirus (MYMIV) is based on complex oligomerisation of the Rep protein (Singh et al., 2008). Rep mediates partial denaturation of double-stranded viral DNA that is essential for the formation of the ori hairpin structure (Singh et al., 2008). Oligomerisation of Rep is also important for the formation of three different Rep–DNA complexes in the monocot-infecting Wheat dwarf mastrevirus (WDV; Castellano et al., 1999). Since MSV Rep and RepA contain the same DNA-binding and oligomerisation site (Fig. S1) (Horvath et al., 1998; Missich et al., 2000), hetero-oligomerisation between Rep and RepA might be important in MSV for either the DNA binding affinity of Rep/RepA hetero-oligomers, or the specificity with which these hetero-oligomers bind DNA. In this scenario, the absence of RepA in the ΔI mutant could abolish RCR and thereby directly prevent the formation of ssDNA.
accumulated in cultured cells infected with intronless rep mutants that cannot express RepA (Liu et al., 1998). Thus, the initiation of replication or stabilisation of ssDNA by encapsidation does not depend on RepA in these two other mastrevirus species. Although all these experiments were performed in cultured cells, they differ according to their respective hosts (wheat for WDV, tobacco for BeYDV, maize for MSV), and therefore, it is difficult to compare the viral DNA replication and replicative DNA forms of the three mastreviruses. It remains possible that RepA function may vary in either a virus- or host-dependent manner.

For example, inoculating maize leaves biolistically with two reporter constructs containing respectively the MSV and WDV LIRs, Munoz-Martín et al. (2003) compared the effect of WDV RepA, Rep and pRBR binding mutants of Rep and RepA on the virion-sense promoters of WDV and MSV. In contrast to the WDV promoter, the MSV promoter had basal activity in the absence of RepA, but transcription from both promoters was enhanced upon WDV RepA co-expression with the reporter constructs. This upregulation was reduced, to different extents for both promoters, if RepA was mutated in the pRBR-binding domain.

The full-length WDV Rep did not promote transcription (WDV) or even decreased it (MSV), presumably indicating a switch from transcription to replication, since the reporter constructs contained the origin of replication. Correspondingly, the WDV Rep mutant deficient for pRBR binding restored some of the transcription of the MSV reporter, but not of the WDV reporter. In these experiments, none of the MSV Rep variants was tested, making it difficult to compare these results with the data presented here.

In addition to the particular virus-host combination, the tissue tropism of the virus within an individual host may be an important determinant of RepA function. Although most geminiviruses are phloem-limited (reviewed by Wege (2007)), there are some well-known exceptions to this rule. Most notable here are MSV in maize (Lucy et al., 1996) and Tomato golden mosaic virus (TGMV) in Nicotiana benthamiana (Nagar et al., 1995; Wege et al., 2001), which invade mesophyll cells as well. Generally the meristems of geminivirus-infected plants have been found to be virus-free (Horns and Jeske, 1991; Lucy et al., 1996). Meristem cultures can therefore be used successfully to raise ACMV-free cassava (Kharga and Gamborg, 1975) and Abutilon mosaic virus (AbMV)-free Abutilon plants (unpublished data). Correspondingly, AbMV titres declined rapidly in callus and suspension cultures which had been established from systemically infected plants (Song and Jeske, 1994). Apart from possibly explaining the overall reduction of virus titres (whether for wt or mutant) that we observed in cell culture compared with in plants, these results indicate a complex interaction of cell cycle control and virus replication with different outcomes for different tissues.

McGivern et al. (2005) showed that MSV mutants unable to bind pRBR were restricted to vascular tissues. A similar change of tissue tropism was observed for the begomovirus TGMV and its pRBR binding mutants (Kong et al., 2000). Both reports suggest that the binding of pRBR to Rep may be more important to reactivate the cell cycle in mesophyll than in phloem tissues. The limited number of infected cells may, therefore, explain the lower titres of both pRBR mutant viruses in systemically infected plants (Fig. 2). In suspension culture, the difference in titres between wt and pRBR mutant viruses was smaller than in plants (Fig. 2), probably because host replication factors are already more abundant in actively dividing cells and pRBR binding is less important under these conditions. In addition, a reduction in viral replication efficiency is likely to have a greater effect in whole plants due to an impaired rate of movement as a result of lower viral titres.

Conclusion

Lower than wt replication levels were observed for both pRBR mutants of MSV (Fig. 2), indicating that pRBR binding is certainly important for efficient viral replication in plants and/or the invasion of mesophyll cells. Such binding is, however, not essential either for the formation of the major replicative intermediates (Fig. 3B, C), or for systemic plant infection (Shepherd et al., 2005). Thus, we can conclude, for MSV at least, that pRBR-binding may serve to simply enhance replication. As with ACMV (Bruce et al., 2011), there may be additional factors required for the activation of cell-cycle specific genes in terminally differentiated cells that are still functional in the pRBR mutants, as has been discussed for initiation of re- replication by ACMV Rep in fission yeast, which lacks homologues of the RB protein family (Kittelmann et al., 2009).

The occurrence of mostly wt-like replicative intermediates in cells infected with both pRBR mutants indicates that the absence of ssDNA accumulation in cells infected with the RepA-deficient mutant, Δl, is not simply attributable to a lack of viral-mediated pRBR binding during Δl mutant infection. It is therefore apparent that one or more of the other likely RepA activities, such as its role in V-sense gene transactivation, and/or its interaction with the replication initiation complex, are far more important during MSV replication than is its pRBR binding activity.

Materials and methods

Inoculation of maize suspension cells

For replication assays in maize suspension cells, a partial dimer (1.1 mer) of the MSV-Kom genome (pKom602; Schnippenkoetter et al. (2001); GenBank accession no. AF003952.1) was used. The construction of a MSV-Kom mutant lacking the intron in rep (pKommA1) has been described (Shepherd et al., 2005). Black Mexican sweet (BMS) suspension-cultured cells were subcultured at a 1:3 dilution 3 days prior to bombardment. Four hours before bombardment, 1.0 mL packed volume of actively dividing cells was plated onto solid media. An aliquot of between 9 and 21 μg of plasmid DNA for each cloned virus was precipitated onto 1 μL of 60 mg/mL gold suspended in 50% glycerol (L of 60 mg/mL gold suspended in 50% glycerol) according to the protocol of Dunder et al. (1995) and as described by Shepherd et al. (2005). After allowing 4 days for replication, three plates of cells per construct were combined for DNA extraction (see below).

Agroinfection of maize

Three-day old maize seedlings (Zea mays L cv. Golden Bantam) were agroinoculated with clones of MSV-Kom (wt), MSV-KomRb (triple mutant) and MSV-KomRb C401A (reversion mutant) as by Shepherd et al. (2006). Total DNA was extracted at 11 days post inoculation (dpi) from the middle portion of fully-expanded leaf number three according to Erdmann et al. (2010).

Total DNA extraction from plants and suspension cells

Total nucleic acids were extracted from plants as described by Erdmann et al. (2010), but with adding N-ethylmaleimide (NEM) to the extraction buffer to block topoisomerases (Jeske et al., 2001). For extraction from BMS cells, more starting material was used because a greater amount of DNA was required to detect replicative forms in blots, and solution volumes were increased accordingly. The solution volumes and changed parameters used for extraction from BMS are given in square brackets below.
Fresh plant material (200 mg) or BMS cells [600 mg] were shock-frozen in liquid nitrogen and ground to a fine powder. 500 μL [2.5 ml] extraction buffer (100 mM Tris–HCl pH 7.0; 100 mM NaCl; 1 mM Na-EDTA; 1% SDS; 100 mM dithiothreitol; 10 mM NEM) and 500 μL [2.5 ml] phenol/chloroform/isoamylalcohol (PCI; 25/24/1) were added to the frozen material and agitated for 20 min before centrifugation at 12,000×g [2400×g] for 10 min. The upper aqueous phase was transferred to a new tube and extracted twice with 500 μL [2.5 ml] PCI and once with CI, before adding 3 M Na-acetate (pH 4.8) to a final concentration of 0.3 M to the aqueous phase. Nucleic acids were precipitated for at least 30 min with isopropanol (−20 °C), pelleted, washed with 70% ethanol, dried and eluted in TE-buffer (10 mM Tris–HCl pH 8; 1 mM Na-EDTA).

Agarose gel-based measurements of DNA concentrations were performed on ethidium bromide (EtBr)-containing 0.8% agarose gels, using the GeneTools densitometry from Syngene (Synoptics, Cambridge, UK), with known concentrations of Hyperladder VI 10-48.5 kb (Bioline Ltd, London, UK) as references.

1D and 2D gel-electrophoresis and Southern blotting

Nucleic acids were separated by 2D agarose gel electrophoresis as described (Jeske et al., 2001), except that a horizontal slab gel replaced the tube gel for the first dimension (Erdmann et al., 2010). Samples were separated on chloroquine-containing gels (20 μg/ml) for 19 h at 0.7 V/cm in 1D gels, or at 1.5 V/cm in the second dimension of 2D gels. DNA was transferred to a positively charged nylon membrane (Amersham Hybond N+; GE Healthcare, Freiburg, Germany) by alkaline blotting (Chomczynski and Qasba, 1984). Full-length MSV probes were generated from pkom602 by PCR (Primers: 5′-GGATCCACAGAAGCTTGGC-3′ and 5′-ATGATC-GATATCCGACTTGGATCC-3′, each 100 μM) using SuperTherm PCR kit (Promega, Mannheim, Germany). One hundred pg of the gel-purified PCR product (PCR DNA and GelBand Purification Kit; GE Healthcare) was then used in a 50 μl DIG-labelling PCR reaction (PCR DIG Probe Synthesis Kit; Roche, Mannheim, Germany). The labelled product was diluted in 40 ml hybridisation solution (DIG easy Hyb; Roche). Hybridisation and detection was performed as described (Jeske et al., 2001; Erdmann et al., 2010). Film exposure times are indicated in the figure legends of each blot. Images were documented using an Epson Perfection V700 scanner operated by Epson SilverFast 6.5.

Quantitative PCR

For quantitative real-time PCR (qPCR), a Rotor gene RG-3000A device (Corbett Research, Sydney, Australia) and SYBR Green I were used to determine viral titres. In parallel determinations for each sample, 10 ng and 1 ng total DNA as template and primer pairs of viral rep and maize 18S genes (Rep Realfw: 5′-TTGCCTGTGCAAGGGATTCT-3′ and rev: 5′-CCCTGGGATCTATTCCCTTA-3′ or 18S Real fw: 5′-CAGGATACGGCTGTTACT-3′ and rev: 5′-GGTAA-GTTTCCCGGTGGTG-3′) were used.

Viral DNA was quantified using a Sensimix DT mix (Quantace, Cambridge, UK) according to the manufacturer’s instructions in a final volume of 25 μl with 0.2 μM primers and 4 μM MgCl2. Rep primers amplified a 125-nt product of the C terminus of the rep gene. To normalise the amount of viral to plant DNA, the Zea mays 18S small subunit ribosomal RNA gene (GenBank accession no. AF168884) was amplified. Standard curves containing 10–105 fg of cloned MSV rep and 1–500 ng host DNA served as references (Owor et al., 2011). An initial denaturation step at 95 °C for 10 min was followed by 40 cycles of [95 °C for 10 s, 60 °C for 15 s and 72 °C for 20 s].

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Appendix A. Supplementary materials

Supplementary materials associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.04.012.

References


