A rep-based hairpin inhibits replication of diverse maize streak virus isolates in a transient assay

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Maize streak disease, caused by the A strain of the African endemic geminivirus, maize streak mastrevirus (MSV-A), threatens the food security and livelihoods of subsistence farmers throughout sub-Saharan Africa. Using a well-established transient expression assay, this study investigated the potential of a spliceable-intron hairpin RNA (hpRNA) approach to interfere with MSV replication. Two strategies were explored: (i) an inverted repeat of a 662 bp region of the MSV replication-associated protein gene (rep), which is essential for virus replication and is therefore a good target for post-transcriptional gene silencing; and (ii) an inverted repeat of the viral long intergenic region (LIR), considered for its potential to trigger transcriptional silencing of the viral promoter region. After co-bombardment of cultured maize cells with each construct and an infectious partial dimer of the cognate virus genome (MSV-Kom), followed by viral replicative-form-specific PCR, it was clear that, whilst the hairpin rep construct (pHPrepΔI662) completely inhibited MSV replication, the LIR hairpin construct was ineffective in this regard. In addition, pHPrepΔI662 inhibited or reduced replication of six MSV-A genotypes representing the entire breadth of known MSV-A diversity. Further investigation by real-time PCR revealed that the pHPrepΔI662 inverted repeat was 22-fold more effective at reducing virus replication than a construct containing the sense copy, whilst the antisense copy had no effect on replication when compared with the wild type. This is the first indication that an hpRNA strategy targeting MSV rep has the potential to protect transgenic maize against diverse MSV-A genotypes found throughout sub-Saharan Africa.

INTRODUCTION

Resistant plant varieties have long proven to be the most effective and convenient control option for many plant diseases, including the economically devastating maize streak disease, caused by the single-stranded geminivirus maize streak virus (MSV; genus Mastrevirus, family Geminiviridae; reviewed by Martin & Shepherd, 2009; Shepherd et al., 2010). However, due to a multitude of factors such as farmers’ selection of seeds for the next season’s planting, inconsistent vector pressures (and hence inoculum pressures) across maize-growing regions, variability in virus pathogenicity and differences in the resistance characteristics of maize grown in different areas, apparent resistance breakdown is frequently experienced. This means that there is a continual need for novel sources of resistance to add to the already existing pool of control options.

In recent years, genetic engineering has provided a relatively fast and precise tool for the development and implementation of new resistance strategies against plant viruses, such as those based on expression of virus-derived constructs (Prins et al., 2008). Our laboratory has done just this for MSV resistance, by showing that constitutive expression of a dominant-negative mutant of the MSV replication-associated protein gene (rep) confers significant protection against MSV infection to both suspension cultured cells and regenerated transgenic maize (Shepherd et al., 2007a, b). In order to obtain a greater degree of protection, however, we have also investigated in
this study the application of a natural antiviral defence system, used to protect plants against virus infection, by engineering plants to express dsRNA for viral genes.

The use of innate antiviral defences to protect crops against viruses dates back to 1929, when McKinney (1929) found that tobacco plants infected by a mild strain of tobacco mosaic virus were protected against infection by a more severe strain of the same virus. Termed cross-protection, the use of mild virus strains to protect against more severe strains has been used to control some serious viral diseases, including those caused by Citrus tristeza virus (Muller & Costa, 1968), tomato mosaic virus (Fletcher, 1978) and papaya ringspot virus (Yeh et al., 1988). Such a strategy has also been evaluated as a control option for the geminivirus East African cassava mosaic virus-Uganda (Owor et al., 2004). In most cases, this cross-protection probably works via the induction of natural plant antiviral silencing pathways (RNA-mediated cross-protection; Ratcliff et al., 1999; Chellappan et al., 2004a).

It is now possible, however, to engineer plants in which these silencing pathways are activated constitutively without the need for prior infection by a mild virus strain. The cross-protection-like responses of these engineered plants can result in resistance phenotypes ranging from delayed symptom development, less severe symptoms and reduced/delayed DNA/RNA accumulation to complete resistance against symptomatic infection (Angell & Baulcombe, 1997; Ratcliff et al., 1999; Chellappan et al., 2004a; Owor et al., 2004; Di Nicola-Negri et al., 2005, 2010; Zrachya et al., 2007).

The geminivirus rep gene is an ideal target for silencing because of its indispensable role in virus replication and its high degree of sequence conservation among even distantly related viruses (Hanley-Bowdoin et al., 1999; Vanderschuren et al., 2007a; Shepherd et al., 2009). The choice of the rep gene as a target for silencing is further strengthened by studies with other geminiviruses in which the silencing of rep was shown to be effective at reducing virus replication in cassava, tomatoes and beans (Chellappan et al., 2004a; Zhang et al., 2005; Abbary et al., 2006; Fuentes et al., 2006; Bonfim et al., 2007; Ramesh et al., 2007; Vanderschuren et al., 2009). In addition, short interfering RNAs (siRNAs) derived from the geminivirus bidirectional promoter were shown to reduce virus titres (Pooggin et al., 2003; Dogar, 2006; Vanderschuren et al., 2007b) and possibly to direct methylation of the viral intergenic region (Dogar, 2006).

Due to the large amounts of time, effort and expense needed to engineer resistant crop plant genotypes, evaluation of new transgene-based resistance strategies often requires a fast and efficient ‘model’ system that will generate information in the shortest time possible. One such model system that has proven to be an invaluable tool for evaluating and comparing different MSV resistance strategies utilizes transient expression of transgenes by black Mexican sweet (BMS) cells in suspension culture (Shepherd et al., 2005, 2007a), combined with quantification by real-time PCR of MSV DNA accumulation during transient infection. In light of the growing need to diversify sources of maize streak disease resistance (reviewed by Shepherd et al., 2010), we evaluated the efficacy of two potentially silencing-inducing hairpin RNA constructs (i.e. inverted repeats separated by an intron that, when expressed and spliced, should form hairpin RNA) – one containing inverted repeats of a portion of the MSV rep gene and the other containing inverted repeats of the MSV long intergenic region (LIR) – in inhibiting the replication of diverse MSV genotypes in BMS cells.

RESULTS

Hairpin repΔI662 completely inhibits cognate virus replication

Replication of isolate MSV-Kom (see Methods) after co-bombardment with various constructs based on pHANNIBAL, a gene silencing vector, was analysed by conventional replicative-form-specific PCR. Whilst virus was undetectable in BMS cells co-bombarded with the infectious MSV-Kom clone pKom602 and the hairpin rep construct pHPrepΔI662, there was detectable virus replication in BMS cells bombarded with pKom602+pHANNIBAL and with pKom602 alone. These two treatments reflected wild-type virus replication rates, and viral DNA titres determined for all other construct combinations were compared against these (Fig. 1a).

To determine whether the observed inhibition of virus replication was potentially due to an hpRNA-based mechanism, two additional constructs were co-bombarded separately with pKom602: repΔI662 in the sense orientation only (pSenserepΔI662) and repΔI662 in the antisense orientation only (pAntirepΔI662). Surprisingly, whilst the antisense repΔI662 had no effect on virus replication, replication in the presence of repΔI662 in the sense orientation was lowered in comparison with wild-type replication (Fig. 1b).

No PCR product was amplified from MSV-Pst1, a non-replicating mutant of MSV-Kom (Fig. 1a), indicating that input plasmid (i.e. the MSV-Kom-containing plasmid bombarded into BMS cells) was undetectable and therefore had no discernible influence on our assay. Thus, detected amplicons were a result of virus replication following replicational release of unit-length virus genomes from input plasmid molecules.

In comparison with rep-based constructs, MSV-Kom replication was not affected by sense, antisense or hairpin LIR constructs. Virus replication in the presence of all LIR constructs was the same as that in the presence of pHANNIBAL alone (Fig. 1c).

Hairpin repΔI662 is effective against highly divergent MSV genotypes

Having determined that the hairpin repΔI662 construct completely inhibited cognate MSV-Kom replication, its
effect was tested on the following virus isolates representing the complete spectrum of severe disease-causing MSVs across Africa, whose maximum divergence is 4.62% at the nucleotide level: the severe southern and East African isolates MSV-MatA and MSV-Gat; the severe Réunion isolate MSV-Reu; and the Ugandan isolates MSV-UMpi11, MSV-UKas75 and MSV-UKab82 (see Methods). These Ugandan isolates belong to the most widespread and common maize-infecting MSV lineages found in the most extensive survey yet undertaken of MSV in Uganda (Owor et al., 2007). Replication of all six isolates was inhibited (replicating virus DNA was either undetectable or only barely detectable) when co-bombarded with pHrepΔI662 (Fig. 2), indicating that a rep-hairpin-based resistance mechanism could potentially be effective against the entire range of MSV-A isolates found throughout sub-Saharan Africa. Supporting this view is the fact that there are a number of stretches of high sequence identity shared by all eight isolates in the targeted 662 bp region (see Supplementary Alignment S1, available in JGV Online), a pre-requisite for a successful hairpin-mediated resistance strategy.

Relative quantification of virus replication using real-time PCR

As conventional PCR demonstrated that both the hairpin repΔI662 and sense repΔI662 constructs inhibited MSV replication, we decided to quantify the degree of replication inhibition more accurately by real-time PCR.

For each run, viral DNA concentrations in all samples were calculated using primers specific to the viral rep and endogenous maize primers (specific to the Zea mays 18S small subunit rRNA gene). A relative quantification was obtained by calculating the ratio of viral rep DNA to maize 18S DNA (expressed as fg virus per ng maize DNA). A ratio of the concentration of pKom602 + construct to the concentration of pKom602 alone (wild type) was then calculated. Thus, a ratio of 1 indicated no difference in virus replication compared with that of wild type.

The effect of each construct on virus replication can be seen in Fig. 3. This box-and-whiskers plot, arranged on a logarithmic scale, shows the range of values obtained with each virus/construct combination, as well as the median (horizontal line). Each replicate consisted of a separate
Fig. 3. Vertical box-and-whiskers plots summarizing real-time PCR data on all bombarded construct combinations. The plots show the sample minimum and maximum, the lower quartile (25th percentile; bottom of box), the median (50th percentile; horizontal line in box) and the upper quartile (75th percentile; top of box). The whiskers indicate the 10th–90th percentile: any data points outside of this are shown as dots (these may be considered outliers). The y-axis (on a log_{10} scale) shows the ratio of pKom602 + construct to pKom602 alone (wild type). Thus, a ratio of 1 indicates no inhibition of virus replication compared with wild type (e.g. see the median of pKom602 + pHANNIBAL). A value of <1 indicates a reduction in virus replication. The numbers of replicates used to construct the plots (i.e. the number of bombarded samples) were as follows: pKom602 + pHANNIBAL, 30; pKom602 + pAntirepΔI662, 24; pKom602 + pSenserepΔI662, 24; pKom602 + pHPrepΔI662, 11; pHANNIBAL alone, 3; pHPrepΔI662 alone, 3; non-bombarded control, 6; pMSV-PstI, 9.

plate of bombarded BMS cells, ranging from three to 33 plates per construct combination (three replicates for both pHANNIBAL and pHPrepΔI662 bombarded alone to check primer specificity, and 33 replicates for pKom602 co-bombarded with pHANNIBAL). Although a wide variation was seen among replicates (which is to be expected in this type of assay), the differences between constructs were great enough and the number of replicates high enough for accurate statistical analyses.

Although virus replication in the presence of pHANNIBAL appeared slightly higher than when pKom602 was bombarded alone [mean ratio vs pKom602 (wild type)=1.52; median ratio=1.04], this was not significant (P=0.17). In agreement with conventional PCR, the antisense repΔI662 construct had no significant effect on virus replication (mean ratio vs wild type=1.24; median=0.79; P=0.2), whilst the sense repΔI662 construct significantly inhibited virus replication (mean ratio vs wild type=0.16; median=0.09; P<0.0001).

The greatest degree of inhibition was achieved by the hairpin repΔI662, again confirming the conventional PCR results (mean ratio vs wild type=0.0072; median=0.0065; P<0.0001). Comparing the mean ratios of the sense and hairpin repΔI662 datasets, the hairpin repΔI662 construct was 22 times more inhibitory towards virus replication than the sense repΔI662 construct. The difference between the two constructs was highly significant (P<0.0001).

Confirming the specificity of the rep primers for pKom602, no product was amplified from BMS cells bombarded with pHANNIBAL or pHPrepΔI662 alone. This showed that the primers could amplify neither the vector nor the repΔI662 construct in either orientation.

In addition, a non-replicating MSV-Kom construct, pMSV-PstI (Shepherd et al., 2005), was used to determine whether there was amplification of the input (bombarded) plasmid in the absence of virus replication. There was indeed low-level amplification (mean ratio of pMSV-PstI: pKom602=0.01; median=0.01; P<0.0001). In other words, levels of pMSV-PstI still detectable in BMS cells 4 days after bombardment were 100 times lower than levels of pKom602 (even though the same amounts of the two plasmids were used to bombard the BMS cells). This difference was due to the non-functional rep of pMSV-PstI not allowing replicational release of the viral genome from the plasmid backbone. Although the value of 0.01 was higher than the mean ratio of the pKom602 + pHPrepΔI662 dataset (0.007), the difference between the pMSV-PstI and MSV-Kom + pHPrepΔI662 datasets was not significant (P=0.08). This indicated that the amplification seen in samples bombarded with pKom602 + pHPrepΔI662 was probably from residual input plasmid and not from virus replication. It is therefore possible that pHPrepΔI662 achieved 100% replication inhibition when bombarded at a 1:1 ratio with pKom602.

Conversely, the difference in amplification between pMSV-PstI and MSV-Kom + pSenserepΔI662 was significant (P<0.0001), indicating that the virus was replicating in the presence of pSenserepΔI662, albeit at lower levels than the wild type.
DISCUSSION

Gene silencing has been used successfully since the 1990s, albeit inadvertently in some cases, to provide resistance to some economically devastating plant virus diseases (reviewed by Fuchs & Gonsalves, 2007). Given that it is a natural defence system that reduces the accumulation of viral DNA (Covey & Al-Kaff, 2000; Voinnet, 2001; Waterhouse et al., 2001; Chellappan et al., 2004b; Buchmann et al., 2009; Rodriguez-Negrete et al., 2009), gene silencing is an attractive option for the development of novel MSV resistance genes. Previous studies have demonstrated the inhibition of geminivirus replication via targeting of either the rep gene (Vanitharani et al., 2003; Chellappan et al., 2004b; Varma & Praveen, 2006; Bonfim et al., 2007) or the bidirectional promoter sequences in the viral intergenic region (Pooggin et al., 2003; Vanderschuren et al., 2007) for silencing. Because of their potential to induce silencing by the formation of dsRNA, we used pHANNIBAL-based constructs containing inverted repeats of a portion of the MSV intronless rep gene and the entire MSV LIR. The 662 bp portion of MSV-Kom rep was chosen because it contains putative siRNA-generating sequences for eight MSV isolates, which, at the time of the design of the construct, were the only available full-genome sequences in GenBank.

To rapidly assay the effect of these constructs on MSV replication, we used a well-established transient gene expression system combining particle bombardment of maize cells in suspension culture and viral replicative-form-specific PCR. This system has been used previously to screen for other MSV resistance genes (Shepherd et al., 2007a), as well as to determine the size of the MSV minimal replication origin (Willment et al., 2007).

In this study, replicative-form-specific PCR showed that the MSV-Kom-derived repΔI662 hairpin interfered with MSV-Kom replication to the extent that viral DNA levels were undetectable by conventional PCR. In addition, the hairpin construct was able to reduce replication of diverse isolates belonging to the MSV-A group—the only strain known to cause severe maize streak disease in maize (Martin et al., 2001) – with nucleotide sequence identities differing by as much as 4.62%. Although the diversity of these isolates appears low, this must be put in the context that all African MSV-A strains differ by only ~3% at the nucleotide level (Varsani et al., 2008). These isolates were therefore chosen because of their relatively high diversity within the MSV-A group and because they all cause severe disease in maize. In addition, the three Ugandan isolates chosen represent the most frequently occurring (and therefore most contemporarily relevant) MSV-A1 lineages found in East Africa (Owor et al., 2007; Varsani et al., 2008).

Quantification by real-time PCR indicated that rep amplicons detected in samples bombarded with pKom602 + pHPrepΔI662 (a 139-fold reduction in replication compared with wild type) were probably input plasmid, with fluorescence in these samples being no different from that of samples bombarded with a non-replicating MSV-Kom mutant (pMSV-PstI). This indicated that, when bombarded at a 1:1 weight ratio, virus replication was completely inhibited by the repΔI662 hairpin. It is worth mentioning here that the promoter in pHANNIBAL driving expression of the sense and antisense repΔI662 RNA is the dicot-specific cauliflower mosaic virus (CaMV) 35S promoter. It is possible that, for cases where viral load is extremely high, a monocot-specific promoter such as the maize ubiquitin promoter may be a better option for higher-level expression. Nevertheless, in this assay, CaMV 35S was certainly adequate.

The presence of the sense and antisense repΔI662 fragments together in pHANNIBAL (inverted repeats separated by an intron) led to significantly greater virus replication inhibition than that achieved by the sense or antisense versions alone. However, both replicative-form-specific conventional PCR and real-time PCR showed that there was also a reduction in virus replication when pKom602 was co-bombarded with pSense repΔI662, albeit to a much lesser extent (22-fold less) than that achieved by the hairpin. It is unclear why the sense version of repΔI662 should interfere with virus replication.

It is not surprising that the antisense construct pAnti repΔI662 did not interfere with virus replication. Despite the fact that, theoretically, an antisense transcript could form dsRNA with the sense transcript from the virus, it has been shown previously that antisense MSV rep is ineffective at reducing viral loads, even when expressed from the maize ubiquitin promoter (Shepherd et al., 2007a).

Whilst targeting of the bidirectional promoter has been successful with dicot-infecting geminiviruses such as African cassava mosaic virus (ACMV) (Dogar, 2006; Vanderschuren et al., 2007b) and Vigna mungo yellow mosaic virus (Pooggin et al., 2003), it did not appear to work for MSV. None of the MSV LIR constructs (hairpin, sense or antisense LIR) reduced replication of MSV-Kom. With ACMV, it is thought that targeting the viral promoter region may activate a natural host defence mechanism [transcriptional gene silencing (TGS)] against DNA viruses that replicate in the nucleus, whereby siRNA-mediated methylation of the viral promoter region reduces transcriptional activity of the promoter (Dogar, 2006; Vanderschuren et al., 2007b; Raja et al., 2008). It has also been shown that the begomovirus AL2 protein (also known as the transcriptional activator protein or TrAP) and the related curtovirus L2 protein suppress TGS by inactivating adenosine kinase, which is required for efficient production of the methyltransferase cofactor, S-adenosyl methionine (Buchmann et al., 2009). However, neither TGS nor suppression of TGS has been associated with an MSV infection of maize.

In conclusion, we have shown that targeting of rep using an hpRNA strategy was an extremely effective means of...
The rep LIR could not be effectively targeted by a similar strategy. Furthermore, the MSV-Kom-derived hairpin construct, pHPrep\textsc{rep}\textsc{Δ}662, effectively inhibited the replication of MSV-A genotypes representing the entire breadth of known MSV-A diversity. These viruses included diverse virus genotypes from Uganda (including an isolate belonging to the specific MSV-A\textsubscript{3} lineage accounting for 60\% of all MSV infections sampled throughout Uganda in 2005; Owor et al., 2007), southern Africa and the Indian Ocean island of La Réunion. As the rep\textsc{Δ}662 inverted repeat was 22-fold more effective at inhibiting virus replication than the same sequence in the sense-only orientation whilst in the antisense-only orientation it did not inhibit replication at all, it is possible that the replication inhibition by pHPrep\textsc{rep}\textsc{Δ}662 was due to the formation of dsRNA. This could potentially have been processed into siRNAs, which may then have targeted the homologous viral rep RNA for degradation.

This is the first study to show that a hairpin dsRNA-based strategy could be applied to the engineering of resistance in maize to MSV. Supporting the view that our transient expression results will translate into success in transgenic maize, we have previously demonstrated the successful application of another MSV-Kom rep-based construct (\textsc{pre}p\textsuperscript{I–219RB–}) that was initially tested using the same transient assay system used here, and is currently being used productively in the development of transgenic MSV-resistant maize genotypes (Shepherd et al., 2007b).

**METHODS**

**Generation of inverted-repeat (hairpin) constructs.** A gene silencing vector, pHANNIBAL (Helliwell & Waterhouse, 2003), incorporating the Gateway technology (www.invitrogen.com) was obtained from Commonwealth Scientific and Industrial Research Organization, Australia. Eight rep gene sequences from diverse isolates [MSV-A\textsubscript{3} KE_MtKA-1997, MSV-A\textsubscript{3} RE_Reu2-1997 (MSV-Rep), MSV-A\textsubscript{3} KE_Ken –1983, MSV-A\textsubscript{3} ZW_MatC-1998, MSV-A\textsubscript{1} ZW_MatA-1994 (MSV-MatA), MSV-A\textsubscript{1} ZW_MatB-1996, MSV-A\textsubscript{1} ZA_MakD-1998 and MSV-A\textsubscript{1} ZA_Kom-1989 (MSV-Kom)] were analysed for potential siRNA-generating sequence stretches using the siRNA-designing program IMGENEX (http://www.imgenex.com/siRNA_resources.php). For this analysis, we used ‘intronless’ rep sequences (rep\textsc{Δ}I, simulating a spliced C1/C2 product), because it was assumed that the most likely target for post-transcriptional RNA silencing would be mature mRNA, i.e. spliced transcripts.

MSV-Kom, which provided sequences previously developed to produce MSV resistance-inducing transgenes (Shepherd et al., 2007a, b), was selected as the template for design of the hairpin constructs. A 662 nt sequence stretch in MSV-Kom rep\textsc{Δ}I (rep\textsc{Δ}I662; nucleotide coordinates 58–719, with 1 being the first nucleotide of the rep\textsc{Δ}I ATG) was chosen to form the hairpin, as this encompassed at least three predicted siRNA-forming sequences from all eight isolates.

The rep\textsc{Δ}I662 construct and the LIR from MSV-Kom were inserted as Xho\textendash;EcoRI fragments for the ‘sense arm’ and as Xba\textendash;ClaI fragments for the ‘antisense arm’ into pHANNIBAL. Two primer pairs incorporating Xhol/EcoRI sites (sense) and XbaI/ClaI sites (antisense) (see Supplementary Table S1, available in JGV Online) were used to amplify a 662 bp product containing the portion of rep predicted to form siRNAs from an intronless rep construct, pSKRep\textsc{Δ}I (Shepherd et al., 2005), and a 315 bp product containing the LIR from an infectious MSV-Kom clone, pKom602 (Schnippenkoetter et al., 2001). Amplification conditions for both rep\textsc{Δ}I662 and the LIR consisted of initial denaturation at 95 °C for 1 min, followed by 30 cycles of 95 °C for 45 s, 57 °C for 30 s and 72 °C for 3 min, and a final extension at 72 °C for 7 min. Amplified products were cloned into pGEM-T Easy (Promega) and the insert sequenced to ensure the absence of PCR artefacts.

The antisense and sense arms were inserted into the XhoI/EcoRI and XbaI/ClaI sites of pHANNIBAL to make the hairpin constructs pHPrep\textsc{rep}\textsc{Δ}I662 and pHPLIR. For sense-only and antisense-only versions of the constructs, pSense\textsc{rep}\textsc{Δ}I662 and pSenseLIR (sense) and pAnti\textsc{rep}\textsc{Δ}I662 and pAntiLIR (antisense) were made by inserting just the sense or antisense PCR product into the relevant sites of pHANNIBAL. Cloning of the LIR in the sense and antisense orientations, respectively, corresponded to insertion of the LIR in the virion-sense and complementary-sense orientations.

**Transient replication assays.** BMS cells subcultured 3 days prior to bombardment were used to assay for MSV replication as described by Palmer et al. (1999), with modifications by Shepherd et al. (2005). A 1 : 1 weight ratio of pKom602 and the test construct (pHPrep\textsc{rep}\textsc{Δ}I662, pSense\textsc{rep}\textsc{Δ}I662, pAnti\textsc{rep}\textsc{Δ}I662, pHPLIR, pSenseLIR, pAntiLIR or pHANNIBAL) was mixed together and precipitated onto 1 μm gold particles according to the protocol of Dunder et al. (1995). The PDS-1000/He Biolistic particle bombardment delivery system (Bio-Rad) was used to deliver the DNA-coated gold into BMS cells as described by Shepherd et al. (2005). For each precipitation, 1.6 μg pKom602 and 1.6 μg test construct were mixed and pipetted into an Eppendorf tube containing 50 μl gold (60 mg ml\textsuperscript{−1}) suspended in 50 % glycerol. In each experiment, at least two precipitations were prepared for each construct combination, and these were pipetted separately to show reproducibility. Three plates of BMS cells were bombarded per precipitation. Thus, there were six biological repeats per construct combination per experiment. Each experiment was then repeated three times to give a total of 18 biological replicates. For the negative control, a non-bombarded BMS plate was included in each experiment. As a plasmid-input control, BMS cells were also bombarded with a non-replicating MSV-Kom construct (pMSV-Psr; Palmer & Rybicki, 2001). This was to determine whether the primers used subsequently to quantify MSV replication could be used to detect the bombarded (input) plasmid after 4 days of cell growth and division.

In addition to the cognate MSV-Kom (the South African MSV isolate from which the rep constructs were derived), MSV-Reu (from La Réunion island, off the east coast of Africa), MSV-MatA (from Zimbabwe, southern Africa), MSV-A\textsubscript{1} KE_Gat-1998 (MSV-Gat; from Kenya, East Africa) and three isolates from Uganda in East Africa (Owor et al., 2007), MSV-A\textsubscript{1} UG_Mpi11-2005 (MSV-Umpi11), MSV-A\textsubscript{1} UG_Kas75-2005 (MSV-Ukas75) and MSV-A\textsubscript{1} UG_Kab82-2005 (MSV-Ukab82), were co-bombarded at a 1 : 1 weight ratio with pHPrep\textsc{rep}\textsc{Δ}I662 or pHANNIBAL. All bombarded and non-bombarded (control) plates were incubated at 25 °C in the dark for 4 days, after which total DNA was extracted as described by Shepherd et al. (2005).

**PCR analysis**

**Conventional PCR.** As a first step in determining the effect of the silencing constructs on virus replication, viral replicative-form-specific PCR was used as described by Shepherd et al. (2005). Total DNA samples at a starting concentration of 100 ng ml\textsuperscript{−1} were used as template for amplification using degenerate primers 215–234 and 1770–1792 (Willment et al., 2001), designed to amplify a 1305 kb...
portion of the MSV genome from replicative-form DNAs but not from the linear cloned MSV DNA provided as input.

**Real-time PCR.** Virus titres in bombarded BMS cells were quantified using real-time PCR. With the aid of Primer3 software (http://frodo.wi.mit.edu/primer3/), primers were designed to amplify a 128 bp region of the MSV-Kom rep gene: RealRep (F) and RealRep (R) (Supplementary Table S1). The amplicon encompassed nt 934–1061 of the MSV-Kom rep (nt 842–969 of the intronless rep) all co-ordinates are relative to the first nucleotide of the rep start codon. As repΔ62 in the pHANNIBAL constructs (which were co-bombarded with MSV-Kom) contained nt 58–719 of the intronless rep, these primers could not amplify repΔ62 and were therefore specific for MSV-Kom. To confirm this, the primers were tested using a BMS cell sample that was bombarded with pHPrepΔ62 alone (no pKom602) as template.

A second set of primers, Maize185 (F) and Maize185 (R), was used to amplify a 173 bp product from the Zea mays 18S small subunit rRNA gene (GenBank accession no. AF168884). The concentration of BMS cell DNA in each sample that was determined with this control was used to normalize the data from different replicated runs. For each sample, MSV-Kom rep- and maize 18S-specific PCRs were performed in the same run but as separate reactions. Also included in each run were rep and 18S standard curves. For the rep standard curve, 50 ng BMS DNA µl⁻¹ was spiked with tenfold dilutions of pKom602 plasmid in order to simulate replicated virus in a BMS genomic background. Plasmid concentrations were 1000, 100, 10, 1, 0.1 and 0.01 pg µl⁻¹. For the 18S standard curve, BMS genomic DNA was gel quantified and diluted to 100, 50, 25, 10, 5 and 1 ng µl⁻¹. These concentrations were chosen because in general the test/unknown sample concentrations fell within these ranges.

Real-time PCR was performed using a Rotorgene 6000 PCR machine (Corbett Research) and the DNA minor groove-binding fluorescent dye SYBR Green I provided in the Sensimix dT mix (Quantace), according to the kit instructions. Each 25 µl reaction mix consisted of 13 µl Sensimix, 50 mM MgCl₂ added to a variable final concentration of 0.2 µM each primer to a final concentration of 0.2 µM and 1 µl template DNA of varying concentration (see below).

It was determined from several optimization experiments that final concentrations of 3 and 7 mM MgCl₂ were optimal for the rep and 18S primers respectively. In addition, due to wide variations in the MSV-Kom titres present in different samples (e.g. BMS bombarded with MSV-Kom alone had high virus titres, whilst BMS bombarded with MSV-Kom+ hairpin construct contained extremely low virus titres), different amounts of template DNA were used. Having first approximated virus titres using conventional PCR, three different starting concentrations of template were used for real-time PCR according to the following rule of thumb: samples with high virus titres: 10 ng genomic DNA; samples with medium virus titres: 50 ng genomic DNA; samples with low/undetectable virus titres: 100 ng genomic DNA. This was in order to have the most efficient amplification with the lowest background fluorescence. The variable amounts of starting template were normalized by calculating the amount of virus in fg (determined from the rep standard curve) present per ng genomic DNA (calculated from the 18S standard curve).

Cycling conditions were an initial hold at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 15 s.

Data were analysed using the computer program Rotor-Gene, version 6. Data were used only if amplification efficiencies calculated by the program were >80% and Pearson’s correlation coefficient, r², of the standard curves was >0.99 (rep and 18S standard curves were included in each run, rather than importing a previously performed standard curve). Automatic threshold levels were usually used, although sometimes they were manually adjusted if necessary. For each run, melting curve analysis was also performed.

**Statistical analysis.** Real-time PCR data were imported from Rotor-Gene version 6 into Microsoft Excel 2007 for calculation of the virus titres present in each sample. Further statistical analyses (Mann–Whitney tests and box-and-whiskers plots) were carried out using GraphPad, version 5.

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