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Reciprocal exchanges involving virion and complementary sense genes, the LIR and SIR between the two clones of *maize streak mastrevirus* result in ameliorated symptoms in maize

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Research Article

Keywords: Mastrevirus, Virulence determinants, Genetic analysis, Zea mays

Posted Date: March 29th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1497660/v1

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Abstract

A genetic analysis of *maize streak mastrevirus* (MSV) virulence was conducted using two infectious DNA clones derived from isolates collected in Kenya. Virulence was tested on the susceptible Pioneer hybrid 3379 using vascular puncture inoculation of kernels. Marked differences in the severity of chlorotic streaks and stunting of seedlings were associated with mild [pMSV-KL (mild)] and severe [pMSV-Km(severe)] parental infectious MSV DNA clones. To identify determinants conditioning these differences, chimeric clones were constructed from parental clones employing restriction endonuclease fragments. Clone identities were confirmed by restriction mapping. Putative virulence determinants were identified for genomic fragments encoding the replication initiator (Rep and RepA), movement (MP) and coat (CP) protein ORFs and for those containing non-coding long (LIR) and short intergenic regions (SIR). Recombinant clones containing LIRs plus the 5'–terminus of Rep/RepA (first 189 nt) ORFs from pMSV-Km(Severe) and pMSV-KL(Mild) reciprocally exchanged displayed intermediate symptoms. Complementary replacement of the Rep/RepA ORF resulted in symptoms indistinguishable from those of parental clones. The recombinant clone constructed with LIR, Rep and RepA genome sequences from pMSV-Km(Severe) and MP, CP and SIR sequences from pMSV-KL(Mild) showed significantly more severe symptoms and accumulated higher concentrations than pMSV-Km(Severe). In contrast, the reciprocal clone showed significantly milder symptoms and lower viral titers than pMSV-KL(Mild). Viral accumulation was correlated with the intensity of leaf chlorosis and the degree of plant stunting. These data support the hypothesis that MSV virulence is a polymorphic trait involving virion and complementary sense genes (LIR and SIR).

Introduction

*Maize streak mastrevirus (*MSV), the causative agent of maize streak disease, is a major pathogen of maize (*Zea mays* L.) throughout sub-Saharan Africa and the Islands of Mauritius and Reunion (Shepherd et al., 2010; Emeraghi et al., 2021). MSV is a species and the type member of the genus *Mastrevirus* in the family *Geminiviridae* (Shepherd et al., 2010). Other genera of the latter are the genus *Begomovirus* and the genus *Curtovirus*. Species of the mastreviruses have a single-component, single-stranded, circular DNA genome approximately 2.7 kb in length and characteristic gemininate particles. Like all members of the family *Geminiviridae*, transcription of the MSV genome is bidirectional with transcripts that are independently controlled and initiated from the noncoding long intergenic region (LIR) (Willment et al., 2007). The noncoding short intergenic region (SIR) serves as the origin of replication of complementary-sense DNA and for polyadenylation and termination of transcription (Palmer and Rybicki, 1998; Rojas et al., 2018). Open reading frames V1 and V2 are transcribed from the virion sense strand, whereas C1 and C2 are expressed from the complementary strand. Four functional proteins are encoded from these ORFs. These proteins include a replication-initiator protein [Rep protein (~ 41.3 kDa)] from a C1/C2 ORF splice event], replication-associated protein [RepA protein (31.5–31.6 kDa)] translated from an unspliced RNA of the C1 ORF (Palmer and Rybicki 1998), a movement protein [MP (10.9 kDa); V1 ORF] and a coat protein

[CP (26.9–27.0 kDa); V2 ORF]. Natural vectors of mastreviruses are various leafhopper species. Several *Cicadulina* species transmit MSV (Shepherd et al., 2010; Emeraghi et al., 2021).

MSV exists as various isolates that differ in the severity of the symptoms they incite on maize and their ability to infect resistant maize (Monjane et al., 2011; Sime et al., 2021). Plants infected by severe isolates show marked chlorosis of the entire leaf lamina, leading to necrosis, stunting and producing poor cobs, whereas mild isolates cause only limited streaks or spots, little stunting and no marked cob effects (Bosque-Pérez, 2000; Shepherd et al., 2010; Emeraghi et al., 2021). Chlorotic streaks on leaves were reported to be due to the destruction of chloroplasts (Willment et al., 2007).

Infectious clones have been described for MSV isolates from various regions of Africa (Martin & Shepherd, 2009; Shepherd et al., 2010). Comparison of the sequences obtained for a number of symptomatically distinct clones showed extensive homology (> 95%), suggesting that few differences in the viral genomes were responsible for the differences in symptom severity (Varsani et al., 2008). Determinants for host range, onset of symptoms and symptom type (severity of chlorosis and streak length) were mapped to the genomic region involving the 5' terminus of the C1 ORF (Rep/RepA protein) and the LIR of the Nigerian clone of MSV-N (Boulton et al. 1991). Streak width was determined by the virion-sense portion of the genome. Boulton et al. (1991) reported that a single base substitution (nt-2473) (“A” for the severe clone and “G” for the mild clone in the LIR region affected the symptom severity and host range of the MSV-N clone. Studies have suggested that the location of the determinants of virulence may vary for different MSV isolates and could be scattered across different regions of the MSV genome (Martin & Rybicki, 2002).

Several previous studies used chimeric geminivirus clones to identify determinants of various biological phenotypes (Boulton et al., 1991; Martin and Rybicki, 2002). These scientists used chimeric constructs of MSV to identify the virulence determinants of near sequence homologous mild and severe symptom clones of various African isolates (Martin and Rybicki, 2002). Similarly, pseudo-recombinants and recombinants between different begomoviruses (Unseld et al., 2000) were constructed to study their symptom determinants.

In the present attempt to identify viral *genes* involved in virulence and possible interactions, chimeric clones were constructed that contained portions of the genomes of a mild (pMSV-KL) and severe (pMSV-Km) clone derived from field-collected isolates from Kenya. The original isolates were obtained from maize-infected plants in the Kenyan Lake Victoria region, named MSV-KL, and from the wild grass Setaria verticillata, named MSV-Km. Upon cloning, pMSV-Kl incited narrow, discontinuous, mild chlorotic streaks accompanied by mild stunting, while pMSV-Km caused long, wide, severe chlorotic streaks (yellowish white) accompanied by significant stunting of infected plants. These chimeric pMSV genomes derived from pMSV-KL and pMSV-Km were evaluated for virulence on a susceptible maize hybrid under controlled, reproducible environmental conditions.

Materials And Methods

Construction of dimeric clones

Total genomic DNA was isolated from MSV-infected maize leaves, and viral DNA was separated from host nucleic acids by agarose gel (0.8%) electrophoresis. The double-stranded replicative form of the MSV DNA was excised from the agarose and purified using Geneclean II according to the manufacturer's instructions (Bio101, Inc., La Jolla, CA). Tandem dimeric copies were constructed in the bacterial transformation vector pUC19 after digestion with *Bam*H1. The recombinant plasmids were maintained either in *E. coli* strain DH5a at − 80°C or as viral DNA in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA buffer (TE) at 4°C. These infectious clones were the source of the chimeras used in the present study and were designated pMSV-KL and pMSV-Km.

Characterization of virulence using vascular puncture inoculations (VPI)

To confirm previously reported virulences of pMSV-Km and pMSV-KL (Ngwira 1997), dimeric clones were used to infect the susceptible Pioneer hybrid P3379 using VPI of kernels as described previously (Redinbaugh et al. 2001). The infected plants were maintained in pots in a quarantine greenhouse or growth chambers at the Ohio Agricultural Research Center (OARDC), Wooster. Fifty seeds were inoculated by VPI for each MSV clone. Five symptomatic plants per clone from each inoculation were randomly selected for further disease assessment. The proportion of leaf area covered by chlorotic spots was determined visually on a scale of 0–5, where 0 was no symptoms and 5 was very severe symptoms (Shepherd et al., 2005). In addition, the heights of the five infected plants were measured as the distance from the soil stem collar to the tip of the fourth leaf. Data from three replications in a completely randomized design were recorded at 7-day intervals from 14 days to 28 days after VPI.

Construction of recombinant chimeric clones

Recombinant chimeric clones were constructed utilizing conserved restriction endonuclease sites in the viral genomes and multiple cloning sites (MCSs) of pUC19 to exchange DNA fragments between pMSV-Km(Severe) and pMSV-KL(Mild) using standard cloning techniques. Prior to cloning procedures, the orientation of the genomic MSV inserts in pUC19 was determined by digestions with a set of restriction enzymes suitably located in the genomes of MSV and pUC19 (Redinbaugh et al. 2001). Only clones of similar orientation were chosen for the construction of recombinant clones, thus ensuring that fragments in the same orientation were exchanged. The appropriate fragments for the construction of recombinant clones were obtained from monomeric forms of the parental clones in pUC19 (~ 5.3 kb). Alternatively, fragments were also excised directly from the parental dimeric clones, also in pUC19. To obtain parental DNA monomers from the dimeric clones, infectious clones of the latter were first digested using unique viral restriction enzyme sites (viz., *Nsi*1, *Xho*1 and *Bgl*II) (Fig. 1). The resulting 5.3 kb parental monomeric clones were recovered by gel electrophoresis and religated at 14°C overnight, and the ligation mixtures were used to transform *E. coli* cells (viz., DH5alpha or XL1Blue). Colonies harboring putative recombinant

clones were screened by restriction enzyme analysis of DNA template prepared using the Wizard Miniprep kit (Promega, Madison, WI) according to the manufacturer's instructions.

To construct recombinant chimeric clones pMSV-Km(KL_{LIR}) and pMSV-KL(Km_{LIR}), a reciprocal exchange of a 0.5 kb DNA fragment was made following *Nsi*1 and *Xba*1 restriction digestion (Figs. 1 and 2). The *Nsi*1 site (Position 2187) is located at the 5'-terminus of the C1 ORF, while the *Xba*1 site is located within the MCS of pUC19. Thus, *Nsi*1 and *Xba*1 digestion ensured an exchange of viral sequences between nts 2187–2687 containing the 5' end of the C1 ORF and the entire LIR of MSV. Similarly, chimeric clones pMSV-Km ($KL_{MP:\ CP}$) and pMSV-KL ($Km_{MP:CP}$) containing MP and CP ORFs and the SIR were made by exchanging *Eco*R1-*Bgl*II DNA fragments (Fig. 2). The recombinants pMSV-Km(KL_{Rep}) and pMSV-KL(Km_{Rep}) were constructed from pMSV-KL(Km_{LIR}) and pMSV-Km(KL_{LIR}) (Fig. 2) by insertion of an *Eco*R1-*Bgl*II fragment from the parental pMSV-Km(Severe) and pMSV-KL(Mild), respectively (Fig. 2). Thus, pMSV-Km ($KL_{\ Rep}$) and pMSV-KL ($Km_{\ Rep}$) represent reciprocal exchanges in which the C2 ORF and most of the C1 ORF (627 of 816 nts.) were derived from one parental clone, with the remainder of the genome derived from the other parent (Fig. 2).

The presence of clone-specific restriction enzyme sites permitted construct verification of recombinant genomes after each cloning or subcloning procedure in *E. coli* using the appropriate endonucleases (Figs. 1 and 2). The infectious dimers of the chimeric clones were constructed as *Bam*H1 inserts in the pUC19 vector. *Bam*H1-digested MSV DNA was eluted following agarose gel electrophoresis. To obtain tandem dimeric repeats in pUC19, *Bam*H1-digested MSV DNA was ligated at 14°C overnight into an appropriately digested and dephosphorylated pUC19 vector at a ratio of 1:6. Ligation mixtures were used to transform competent cells of the commercially available HB101 strain of *E. coli* (Gibco) according to the manufacturer's instructions. Colonies harboring putative dimeric chimerical clones were screened using minipreparations and analyzed by restriction enzyme digestion.

Maxipreparations of plasmids containing dimeric chimerical DNAs were performed using "Wizard Maxiprep" silica-based anion-exchanger columns from Promega (Madison, WI) according to the manufacturer's instructions. Isolated plasmid DNA was diluted in 10 mM Tris, pH 8.0, 1 mM EDTA to 0.2 g l^{-1} and used as inoculum to infect maize hybrid P3379 by VPI. Disease severity incited by each clone was assessed on five randomly selected infected plants as described above, and the time of first appearance of symptoms was recorded. In addition, the percent leaf area covered by chlorotic spots in symptomatic plants was estimated for leaf three using a digital image camera (Sony Digital Mavica, MVC-FD91) and quantified using Image Pro Plus software (version 3.0 from Media Cybernetic, L.P.) (Martin and Rybicki 1998). Symptoms on plants infected with recombinant MSV DNA constructs were compared with control plants infected with parental mild and severe clones, mock-inoculated and uninfected, respectively. Three leaves were measured, and the results were averaged.

Analysis of MSV genome sequences and coat proteins in infected plants

Total plant DNA was extracted from plants infected with parental and recombinant DNA clones using a modified version of the CTAB extraction protocol. The appropriate MSV genome region was amplified by PCR, and the degenerated DNA fragments were processed using the Wizard PCR Preps DNA purification system from Promega (Madison, WI) according to the manufacturer's instructions. The constructs were verified using the enzymes EcoRV, AflIII and Ban1, which produced typical digestions (data not presented). To confirm the expected exchanged fragments, sequences of recombinant viral DNAs were determined and compared to sequences of the original cloned MSV DNA.

Viral accumulation was determined by an F(ab)2-Protein A ELISA as previously described (Todd et al., 2010). Leaf tissue (0.5 g) from the sixth leaf was taken from plants infected with parental and recombinant viral clones four weeks after VPI, and ELISA was used to determine the relative concentrations of viral CP. Leaves from mock-inoculated plants were used as controls. The reactions were read at 405 nm using a spectrophotometer, and the mean absorbencies observed among treatments were compared using SPSS (SPSS Inc. Version 6.0).

Data analysis

Data for plant height, disease severity and percent chlorotic leaf area were subjected to an analysis of variance utilizing repeated measures taken at 1, 2 and 3 weeks after planting. The analysis was implemented using SAS PROC MIXED PC version 8.01 [SAS Institute Inc., Cary, NC]. The Tukey method of mean adjustment for multiple comparisons was used to separate means at $p = 0.05$.

Results

Symptom phenotypes incited by recombinant clones

The severity of symptoms and stunting incited on inoculated P3379 hybrid maize plants by infectious dimeric clones, pMSV-Km and pMSV-KL, differentiated these clones into severe and mild symptomatic clones (Table 1). Specifically, clone pMSV-Km incited long, wide and severe chlorotic streaks (yellowish white) accompanied by significant stunting of infected plants. In contrast, plants infected with clone pMSV-KL developed narrow, discontinuous, mildly chlorotic (light green) streaks accompanied by mild stunting statistically indistinguishable from both mock and uninoculated plants (Figs. 3 and 4). More severe symptoms were observed on thirteen different maize inbred lines inoculated with MSV-Km, as measured by AUDPC, and disease incidence was lower for MSV-Kl (Table 1).

The LIR and 5' end of the Rep A ORF

To test the contribution of the LIR plus the 5' end of the RepA ORF to virulence, VPI inoculation of recombinant clones [pMSV-Km(KL_{LIR}] resulted in intermediate symptom expression in infected plants. The pMSV-Km(KL_{LIR}) exhibited symptoms similar to those of the severe parental clone pMSV-Km(Severe) (Fig. 3; Table 1). Plants infected with this chimeric clone expressed long, wide and sometimes discontinuous moderately severe chlorotic streaks accompanied by intermediate stunting (Fig. 3;

Table 1). The latent period for symptom expression of this clone was indistinguishable from that of pMSV-Km(Severe), with symptoms appearing commonly 40 days after planting (Table 1). In contrast, plants infected with pMSV-KL(Km_{LIR}) had a shorter latent period and exhibited slightly longer and wider streaks than those of the mild parental pMSV-KL(Mild) (Fig. 3).

The SIR, complementary and virion-sense sequences

Similarly, the four chimeric clones pMSV-Km (KL_{Rep}), pMSV-KL (Km_{Rep}), pMSVKm ($KL_{MP:CP}$) and pMSV-KL($Km_{MP:CP}$) (Fig. 4) were constructed to assess the role of the SIR and complementary- and virion-sense sequences in MSV virulence. Plants infected with pMSV-Km (KL_{Rep}) expressed significantly milder chlorotic streaks and stunting than those infected with pMSV-Km (Severe) (Fig. 4; Table 2). However, the latent period for symptom expression by this clone was not different from that of pMSV-Km(Severe) (Table 2). Furthermore, symptoms incited by pMSV-Km (KL_{Rep}) were intermediate and significantly different compared to those of pMSV-KL(Mild) (Table 2). In contrast, pMSV-KL(Km_{Rep}) incited symptoms similar to those caused by pMSV-Km(Severe) (Table 2). As with pMSV-Km (KL_{RepA}) and pMSV-Km (Severe), the latent periods for symptom expression by pMSV-KL (Km_{Rep}) and pMSV-KL (Mild) were not significantly different.

Chimeric clones involving the MP and CP ORFs plus the SIR showed marked effects on the severity of chlorosis and stunting. The chimera pMSV-Km($KL_{MP:CP}$) incited more severe leaf symptoms than the parental pMSV-Km(Severe) (Fig. 3; Table 2). However, the latent periods for these two clones were the same at 4.5 days. In contrast, plants infected with pMSV-KL($Km_{MP:CP}$) exhibited a significant reduction in chlorosis and stunting compared to the parental pMSV-KL(Mild) clone. The disease symptoms of this clone also appeared 1.5 days later than those of pMSV-KL(Mild).

Accumulation of virions of parental and recombinant clones

To test whether the altered virulence resulted in changes in the amount of virus particles in the plants, the titer was assessed. The results showed that viral accumulation in P3379-infected plants, as assessed by the amount of CP antigen, was generally correlated with disease severity (Fig. 5). Maximum virus accumulation among all clones was observed in plants infected with pMSV-Km($KL_{MP:CP}$), which expressed the most severe symptoms, whereas the lowest accumulation occurred with pMSV-KL($Km_{MP:CP}$), which expressed the mildest symptoms.

Discussion

The genetic basis of MSV symptom severity was investigated by constructing recombinant clones containing portions of genomic regions exchanged between the symptomatically distinct clones pMSV-Km(Severe) and pMSV-KL(Mild). A determinant of symptom severity was mapped to the N*si*1-B*am*H1 fragment containing the LIR and 5' terminus of the Rep/RepA (first 189 nt) gene. Previously, a mutation in the promoter of the Rep/RepA genes (TATA to TGTA) located in the LIR was shown to reduce the severity of chlorosis and length of streaks, increase latency, and restrict the host range of clone pMSV-Ns (Boulton

et al., 1991). It was speculated that the mutation in the promoter affected the transcription of the complementary sense genes, resulting in decreased viral replication. However, in another study, when the LIR sequences of pMSV-KL(Mild) and pMSV-Km (Severe) were aligned, both were found to contain a Rep/RepA promoter with a TATA sequence (Edema, 2001). To test whether other mutations within the LIR and 5' end of the Rep/RepA ORFs accounted for the differences in MSV symptom severity between these two clones, VPIs were performed in the present study on maize hybrid P3379 kernels using chimeric clones pMSV-Km (KL_{LIR}) and pMSV-KL (Km_{LIR}), in which the LIR plus the 5'-terminus of the Rep A/Rep ORF were exchanged between the severe and mild parental clones. The two chimeric clones exhibited intermediate symptoms relative to those of the recipient parental clones. The intermediate phenotype indicates that LIR alone was only partially responsible for symptom severity. Thus, our results are consistent with previous studies. Nevertheless, three different studies to date have implicated the LIR in MSV symptom severity, but to varying degrees (W. H. Schnippenkoetter et al., 2001). The findings of these studies suggest that the determinants located in the LIR differ between MSV clones with differing symptom severities.

A determinant of symptom severity was identified on the BglII-Nsi1 fragment, which contains the entire C2 ORF and most of the RepA ORF. Clones carrying this fragment had more severe symptoms and higher levels of viral CP antigen in leaf tissues. The symptom severity of pMSV-Km ($_{KL\ RepA}$) was similar to that of the donor clone pMSV-KL(Mild) and that of pMSV-KL ($_{Km\ RepA}$) was similar to that of the donor pMSV-Km(Severe). The correlation between symptom severity and CP antigen accumulation suggested that symptom development is related to virus accumulation. This interpretation is consistent with the function of the RepA and C2 gene products RepA and Rep in MSV replication (Palmer and Rybicki 1998). RepA and/or Rep have also been reported to activate transcription of the virion MP and CP genes (Roja et al., 2018). It is possible that the mild phenotype of pMSV-KL(Mild) results from lower Rep or RepA protein replicative activities. Further experimentation is needed to fine-map the determinants within the Rep/RepA ORFs that contribute to these activities and symptom severity.

An unexpected finding was that the most severe symptoms induced by the chimeric clone pMSV-Km (KL_{MP}) were due to sequences from pMSV-KL (Mild). Conversely, pMSV-KL ($Km_{MP:CP}$) induced the mildest symptoms and accumulated to relatively low concentrations. Because the MP sequences of pMSV-Km (severe) and pMSV-KL (mild) are identical, the effects were apparently due to differences in either the CP or the SIR or both. While the CP is multifunctional and is involved intracellularly and in conjunction with the MP in intercellular movement and in genome encapsidation (Zhang et al. 2001), the SIR is bifunctional as the origin of replication of the complementary-sense DNA and for polyadenylation and termination of transcription (Willment et al., 2007).

While it may be unclear how sites within CP and/or SIR would modulate this enhancement of MSV symptom severity, these results show that the increase in severity is linked to an increase in viral CP antigen. The extent of viral invasion of host tissues may have contributed to this increased concentration through enhanced CP interaction with the MP to facilitate intercellular movement. Furthermore, CP effects on ds to ssDNA conversions may have been accelerated. For the SIR, mutations at the polyadenylation

signal that possibly reduced the processing efficiency and/or stability of viral mRNAs were previously suggested to affect the virulence of MSV (Palmer and Rybicki, 2001). The data presented here are consistent with the hypothesis that genomes of naturally occurring strains of MSV contain multiple nucleotides found in all ORFs and noncoding regions that determine the symptom severity of MSV. In contrast, (Boulton, et al., 1991) concluded that a single nucleotide found in the LIR regulated symptom severity.

Although the chimera containing the MP genome fragment of pMSV-KL(Mild) enhanced the symptom severity of pMSV-Km(Severe), this fragment from pMSV-Km(Severe) diminished the mild symptoms of pMSV-KL(Mild). Thus, the fragment caused opposite effects when substituted in the severe and mild clones. This effect is perhaps surprising because it might have been expected that the fragment from the severe clone would have enhanced symptom expression in the mild clone and *vice versa* for the severe clone. Given that the known functions of the genome components of the fragment are concerned with cell-to-cell movement (MP) and ingress and egress of the nucleus as well as encapsidation (CP) and genome replication (SIR), it could be expected that these components from the severe clone would be better suited for performing these functions than those from the mild clone. Since the evidence suggested that this effect was not the case, other effects of the components may have been involved. The evidence may indicate that a compatible interaction between the components of the fragment and the remainder of the recipient genome and host factors determine the symptom outcome. Thus, for the mild clone fragment, these functions were better realized than by its own components, whereas with the severe clone fragment, the realization was less than for its own components. In other words, the level of the effect of these components is dependent on their interaction with the components in the remainder of the genome. Speculation on the nature of these interactions may be premature since there is yet no evidence that such interactions are conditioning symptom severity.

Another possibility is that these fragments have differential effects on the host plant response to the clone. For example, one or more of the fragment components may affect the plant's ability to suppress viral genome replication through a posttranscriptional gene silencing (PTGS) mechanism. Thus, the severe clone fragment would have less of an effect on this mechanism than the mild clone. Bisaro, 2006 showed that PTGS is mediated by small interfering RNAs (siRNAs) and microRNAs (miRNAs). However, to our knowledge, no comparable demonstration has been reported for MSV or mastrevirus proteins. The enhanced and diminished symptom severities of the chimeras were obtained repeatedly among plants inoculated with the chimeras within an experiment and between experiments and hence were highly reproducible. The latter indicated that the effects were inherited traits of the chimera genomes. The effects of host plant genotype and environment on symptom severity were not assessed. However, an earlier study (Redinbaugh et al., 2001) found no differences in the relative symptom severities of MSV isolates collected in Kenya on a range of maize genotypes from highly susceptible to moderately resistant to resistant, suggesting again that symptom severity is a genetically regulated viral trait and not primarily determined by the host genotype. Additionally, it was observed in one case in the present study when maize plants inoculated with these chimeras or their parental clones were temporarily placed under higher light and temperature that fostered more vigorous plant growth, and symptom severities were

ameliorated correspondingly for all clones, indicating that growth conditions may influence symptom severities but not relative severities.

The enhanced symptom severity of pMSV-Km ($KL_{MP:CP}$) suggests a possible mechanism for increased severity arising in natural MSV populations. Naturally occurring mixed infections have been observed (Owor et al., 2007). It would also require the recombination event along with successful establishment and proliferation of the recombinant genome within the population to the extent that its effect would be expressed by the population. Current evidence to support these proposed events exists (Owor et al., 2007). Evidence for genome sequence heterogeneity within natural MSV populations exists (Martin et al., 2015; van der Walt et al., 2008), and the present findings demonstrate the existence of sequences with the potential to generate enhanced recombinant severity. However, evidence for the existence of such sequences together within a natural MSV population is lacking. Evidence for recombination among MSV and mastrevirus genomes was presented in earlier studies (Owor et al., 2007), although none of the putative recombinant sequences were as long as those involved in the present report. Additionally, none of the recombinants appeared to incite more severe symptoms on maize than closely related nonrecombinant viruses (Martin et al., 2002). Whether the putative recombination altered symptom severity was not reported for the recombinant genomes or for recombinant MSV genomes in mixed natural populations.

While increased disease severity involving recombination among sequences of MSV genomes has not been reported for field-collected isolates, recombination appears widespread among some naturally occurring begomoviruses (Shi et al., 2014) and may have led to the emergence of several serious begomovirus diseases (Varsani et al., 2008). Of special interest for Africa is the recombination involving begomovirus species *East African cassava mosaic virus* (EACMV) and ACMV and their infection of cassava (*Manihot esculenta* Crantz) in East (Uganda) and West Africa (Cameroon) Africa (Pita et al., 2001). The recombinations involved both pseudo-recombinants between two strains of EACMV, EACMV-UG2 (DNA A) and EACMV-UG3 (DNA B), and interspecific recombination between EACMV-UG2 DNA-A and DNA-A of ACMV within the CP ORF of the former genome involving a 459 nt fragment. However, it seemed unlikely that this recombinant was responsible for the increased disease severity (Munoz et al., 1997). Rather, the latter appeared due to combined infections of EACMV and ACMV in a synergistic response of enhanced symptom severity. Furthermore, the recombinant was identified in both mild and severe EACMV-UG2 strains (Pita et al., 2001), thereby making it further unlikely that the recombination affected symptom severity in the synergistic response with ACMV.

Nevertheless, opportunities for recombination exist. Natural isolates of MSV are known to consist of a mixture of genetically diverse but related virus genomes (van der Walt et al., 2008) and mixed infections where different mastreviruses co-infecting the same host have been described (Willment et al., 2007). The data presented in the latter study demonstrating that the genomes of MSV-Tas and MSV-VM are likely a mosaic of DNA originating from at least two different viral species (Martin and Shepherd, 2009) would seem to support these possibilities. Furthermore, the findings reported here of dimeric constructs of

recombinant clones expressing more severe or milder symptoms suggest a possibility for a natural recombinant with greater or lesser symptom severity.

It is not known whether the sequences that caused the enhancement of MSV symptom severity could be supplied in *trans* rather than by recombinational events between putative mild and severe parental genomes. Others have found that MSV infection can be achieved by complementation of deficient MSV movement (Palmer and Rybicki, 2001) or replication functions in *trans* (Palmer and Rybicki 2001). These reports allow the inference that enhancement of symptom severity due to determinants could act in *trans*. However, the present study did not try to reproduce this enhanced or decreased symptom severity in *trans* using infectious clones of pMSV-KL(Mild) and pMSV-Km (Severe) in combined infections. Further investigations will be necessary to better understand the viral population dynamics in combined infections by heterologous MSV genomes (Owor et al., 2007).

While this study characterized one of the genetics of symptom severity, it is not at all certain that there is a genetics of symptom severity. Rather, the genetics characterized in this study may be of various vital viral life cycle functions, such as genome replication and intra- and intercellular movement, and their effect in turn on symptom severity. However, it is possible that these genetics directly affect the host plant, resulting in the expression of differing symptom severities, which in turn facilitate the vital functions mentioned above. In other words, these genetic elements might create the host plant environment as manifested in symptom severity that permits the virus to replicate and systemically infect the plant.

Declarations

The authors declare that they have no conflicts of interest.

ACKNOWLEDGMENTS

We thank Margret Redingburg, Dave Fulton, Kristen Willie and John Abt for invaluable technical support and Prof. N. H. Gordon (Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106) for advice on statistical analyses. The first author was on student fellowship from the Rockefeller Foundation. MSV was held and manipulated under APHIS and MUA permits. 43266 and 99R0033, respectively.

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Tables

Table 1

Virulence1,2 of parental and chimeric Maize streak virus (MSV) clones constructed by a reciprocal exchange of the long intergenic regions (LIR) and the adjacent 5'-terminus of the Replication associated

protein (RepA) open reading frame (ORF) of the mild and severe symptom parental clones

Clone	Plant height	Disease severity	Percent chlorotic	Latent period
MSV-Km (Severe)	32.6a	4.9a	56.7a	4.0a
MSV-KL (Km_{LIR})	42.3bc	3.9b	38.4b	4.5a
MSV-Km (KL_{LIR})	39.5b	4.1b	53.5a	4.0a
MSV-KL (Mild)	46.4c	3.0c	26.1b	6.3b
TE buffer control	54.1d	0.0d	0.0c	-

* Means with the same letter are not significantly different at P=0.05 using Tukey's method for multiple comparison of means.

Table 2

Virulence[1,2] of parental and chimeric *Maize streak virus* (MSV) clones constructed by a reciprocal exchange of the 3' terminus of the replication initiator gene (Rep), designated 3'-terminus RepA protein + C2 ORFs (open reading frames), and the movement (MP) and coat (CP) protein ORFs plus the short intergenic region (SIR), designated MP + CP ORFs + SIR, of mild [pMSV-KL(Mild)] and severe [pMSV-Km(Severe)] parental clones

Clone	Plant height	Disease severity	Percent chlorotic	Latent period
pMSV-Km(Severe)	28.6a	4.6a	66.7b	4.5a
pMSV-Km (KL_{MP})	27.0a	4.7a	80.1a	4.5a
pMSV-Km (KL_{Rep})	44.0c	4.1b	44.9c	4.5a
pMSV-KL(Mild)	50.0d	2.6d	31.2d	5.5ab
pMSV-KL(Km_{MP})	54.0e	1.7e	24.9d	7.0b
pMSV-KL (Km_{Rep})	37.2b	3.0c	59.8b	5.5ab
TE buffer control	53.4e	0.0f	0.0e	-

*Means with the same letter are not significantly different at P=0.05 using Tukey's method for multiple comparison

Figures

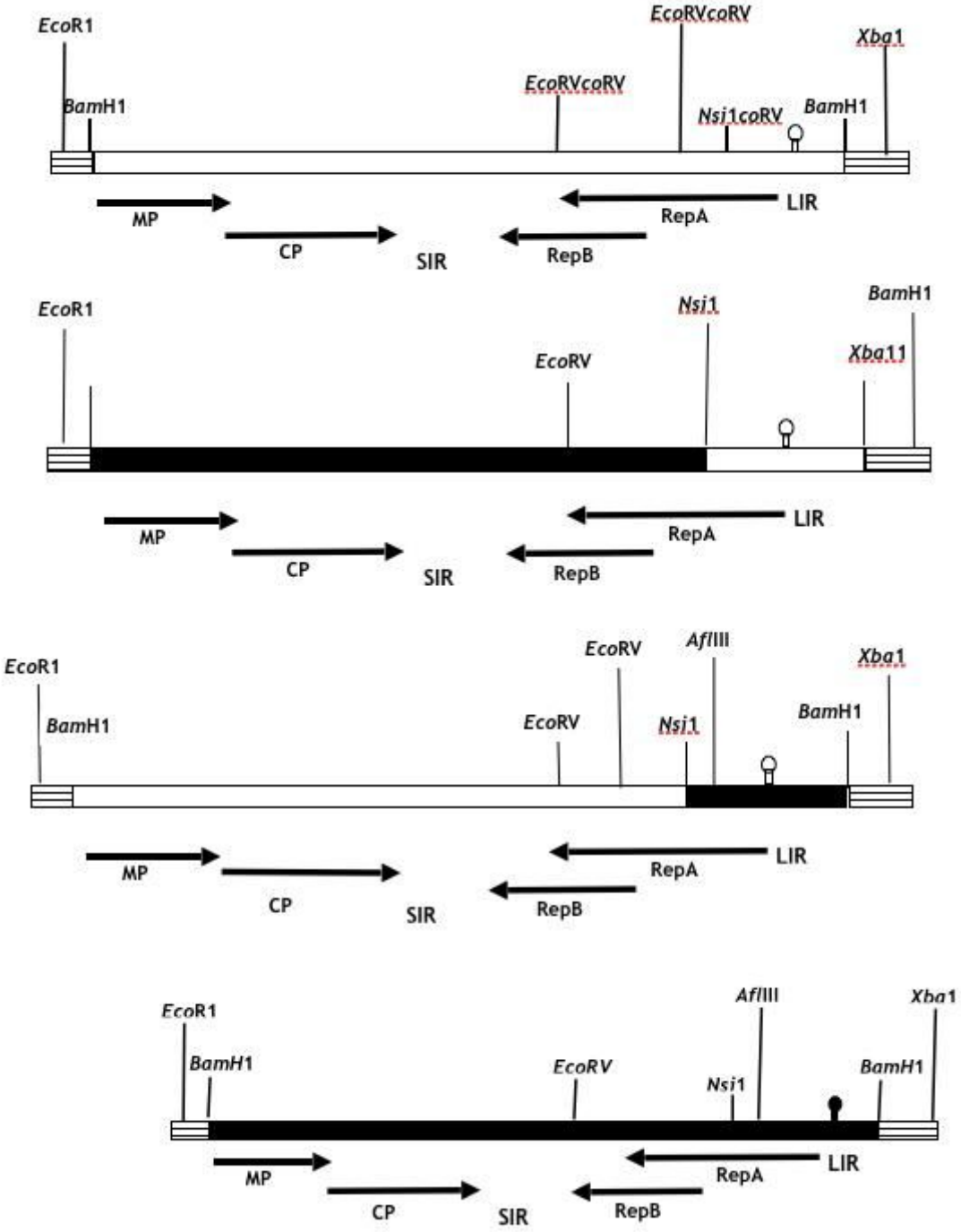

Figure 1

Schematic representation of the parental and chimeric clones in which the *Nsi*1-*Bam*H1 fragments were exchanged. pMSV-Km (Severe) and pMSV-KL (Mild) are shown as filled and open boxes, respectively. The hatched boxes are pUC19 DNA. Restriction sites used in the cloning are shown on the respective clones. A genetic map of MSV is shown at the top and bottom of the figure.

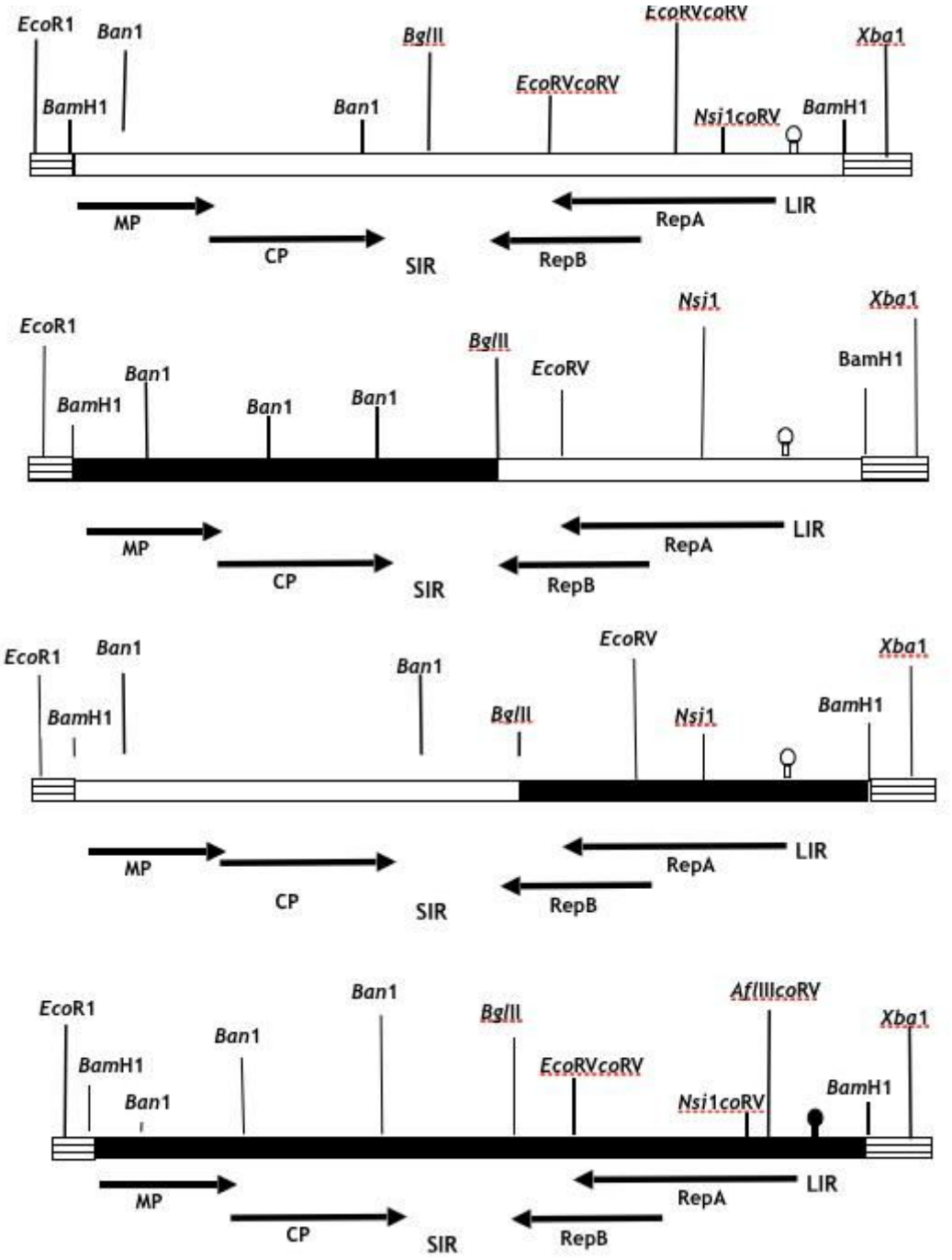

Figure 2

Schematic representation of the parental and chimeric clones in which the *Bgl*II-Nsi1 and *Bam*H1-*Bgl*II fragments were exchanged. pMSV-Km(Severe) and pMSV-KL (Mild) are shown as filled and open boxes, respectively. The hatched boxes are pUC19 DNA. Restriction sites used in the cloning are shown on the respective clones. A genetic map of MSV is shown at the top and bottom of the figure.

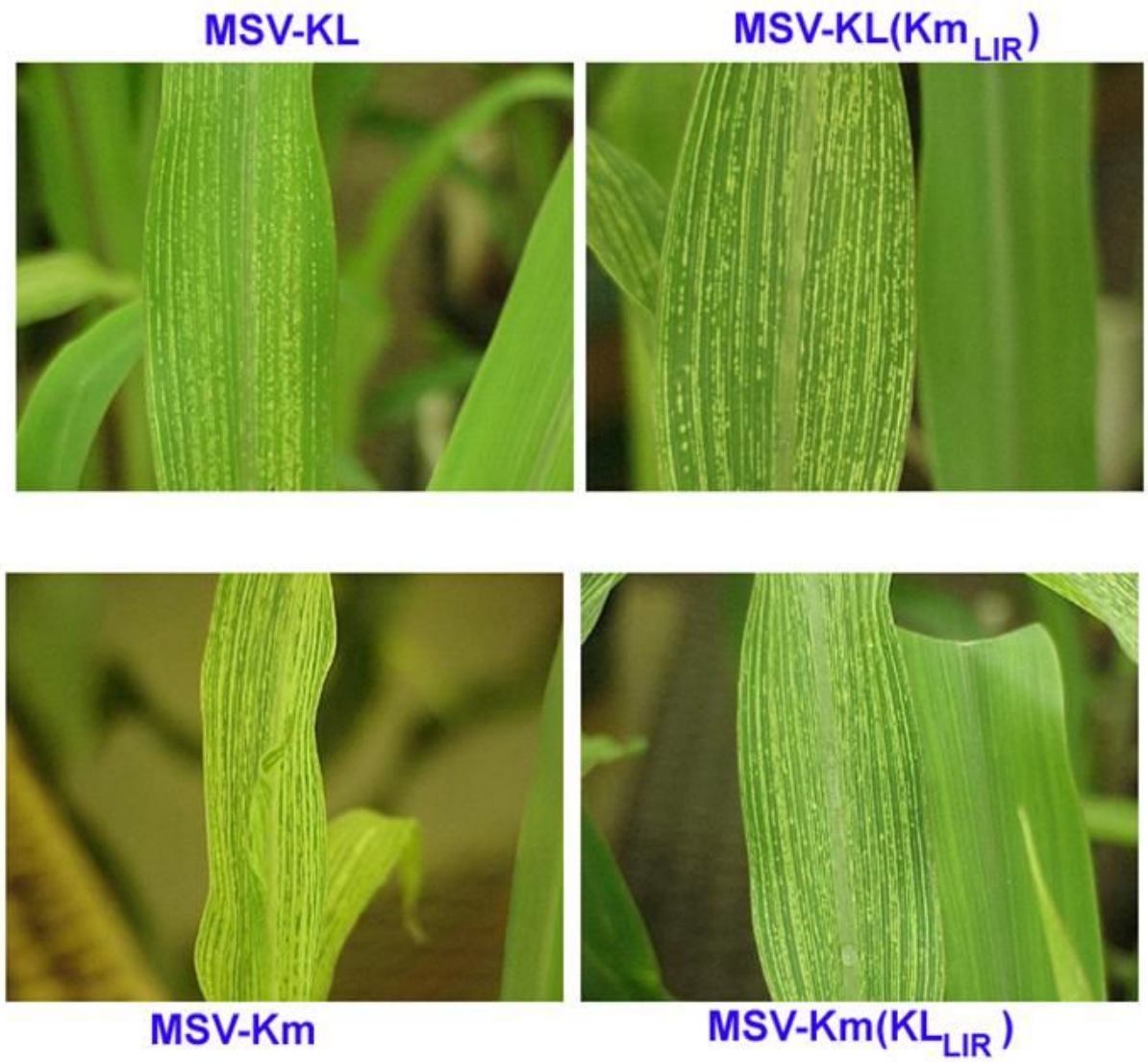

Figure 3

Symptoms observed on leaves of Pioneer maize hybrid P3379 infected with parental and chimeric *Maize streak virus* (MSV) clones constructed by complementary exchanges of the non-coding long intergenic region (LIR) plus the 5'-terminus of the Replication associated protein (RepA) open reading frame (ORF) between parental clones. Infections resulted from vascular puncture inoculation (VPI) of kernels and leaf symptoms were photographed 3 weeks after planting of inoculated kernels. Plates: A, parental clone pMSV-KL(Mild); B, recombinant clone pMSV-KL(Km_{LIR}); C, recombinant clone pMSV-Km (KL_{LIR}; and D, parental clone pMSV-Km(Severe). A genetic map of each clone appears to the right of each panel. The open and filled arrows denote open reading frames (ORFs) of parental clones pMSV-Km(Severe) and pMSV-KL(Mild), respectively, and the direction of transcription of the ORFs. Non-coding regions are shown as boxes with the short intergenic region designated SIR. The rightward transcribed ORFs are the CP coat and MP movement proteins. The leftward C2 ORF is expressed as a spliced product with the RepA ORF.

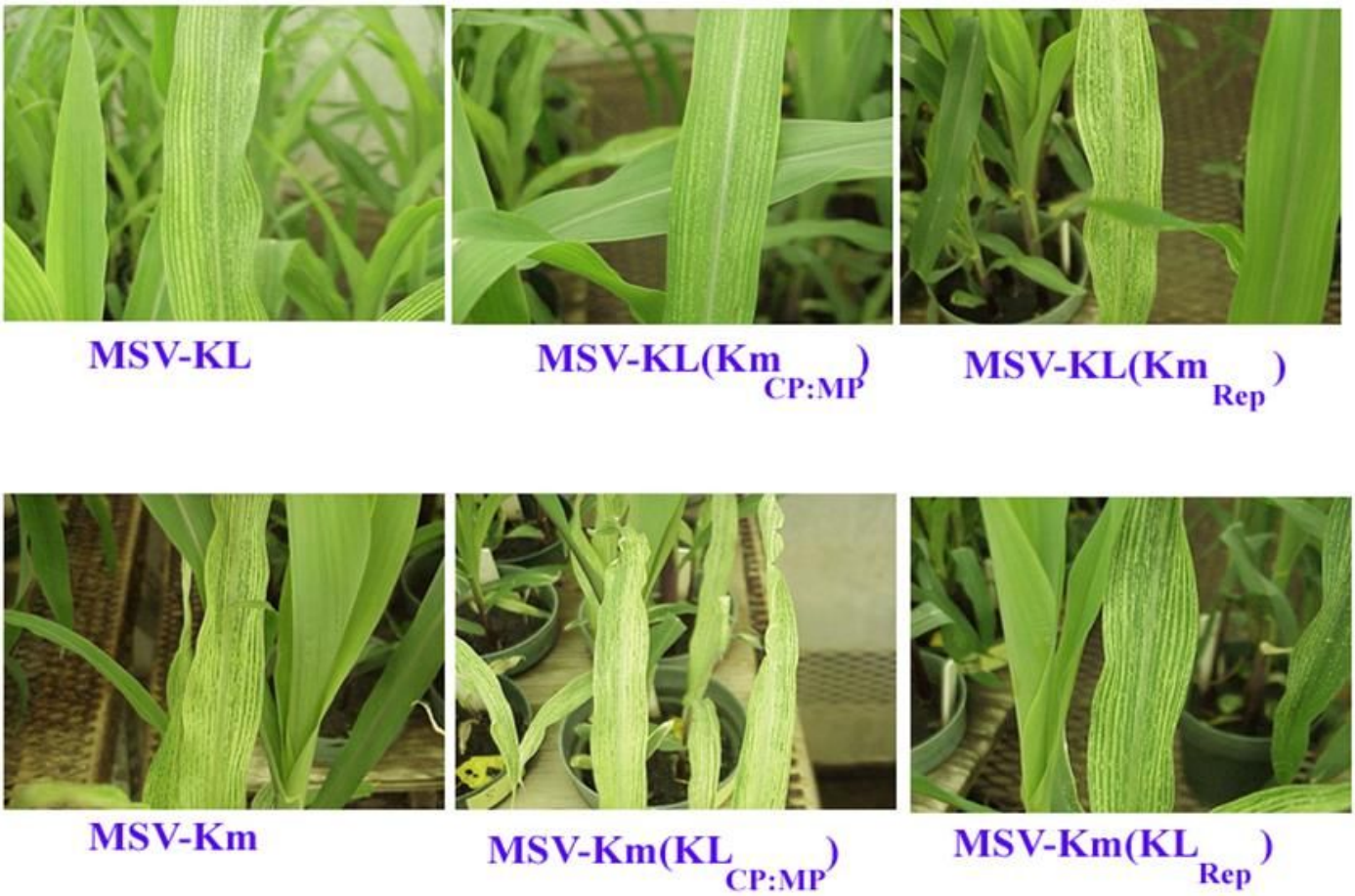

Figure 4

Symptoms observed on leaves of Pioneer maize hybrid P3379 infected with parental and chimeric MSV clones constructed by complementary exchanges of the 3'-terminus of the Replication associated (RepA) protein plus the C2 open reading frames (ORFs) between parental clones and of the movement (MP) and coat (CP) protein ORFs plus the short intergenic region (SIR) also between the parental clones. Infections resulted from vascular puncture inoculation (VPI) of kernels and leaf symptoms photographed 3 weeks after planting of inoculated kernels. Plates: A, parental clone pMSV-KL(Mild); B, recombinant clone pMSV-KL (Km_{Rep}); C, recombinant clone pMSV-KL ($Km_{MP:CP}$); D, recombinant clone pMSV-Km ($KL_{MP:CP}$); E, recombinant clone pMSV-Km (KL_{RepA}); and F, parental clone pMSV-Km(Severe). A genetic map of each clone appears to the right of each panel. The open and filled arrows denote ORFs of parental mild and severe clones and the direction of transcription of the ORFs. Non-coding long intergenic region (LIR) and SIR are shown as boxes.

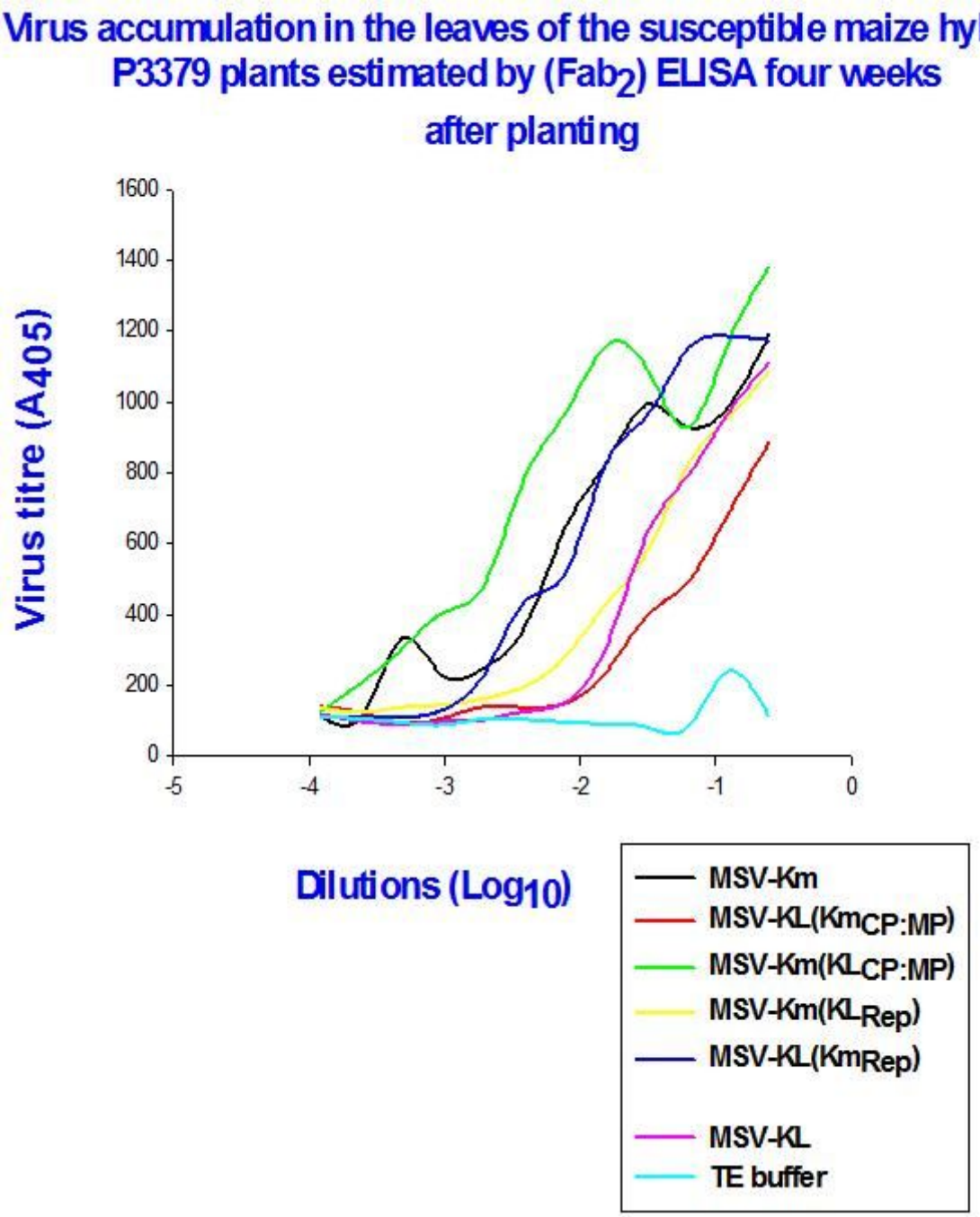

Figure 5

Maize streak virus (MSV) accumulation associated with infection of the susceptible maize Pioneer hybrid P3379 by various MSV clones following vascular puncture inoculation (VPI) of kernels. Virus accumulation was estimated by the relative amount of the virus coat protein (CP) antigen from the sixth leaf of infected plants collected 4 weeks after VPI. F $(ab)_2$-Protein A ELISA was used to estimate relative concentrations of viral CPs. Seedlings were infected with parental clones pMSV-KL(Mild) or pMSV-Km(Severe) or with recombinant clones pMSV-KL ($Km_{LIR+5'\text{-terminus RepA ORF}}$); pMSV-Km($KL_{LIR}$), pMSV-KL ($Km_{Rep}$); pMSV-Km($KL_{Rep}$); pMSV-KL ($Km_{MP:\,CP}$) or pMSV-Km ($KL_{MP:\,CP}$). Recombinant clone designations are explained in legends to Figs. 2 and 3. Each data point is a mean of two measurements.