Geminiviruses of the genera *Begomovirus* and *Curtovirus* utilize three replication modes: complementary-strand replication (CSR), rolling-circle replication (RCR) and recombination-dependent replication (RDR). Using two-dimensional gel electrophoresis, we now show for the first time that maize streak virus (MSV), the type member of the most divergent geminivirus genus, *Mastrevirus*, does the same. Although mastreviruses have fewer regulatory genes than other geminiviruses and uniquely express their replication-associated protein (Rep) from a spliced transcript, the replicative intermediates of CSR, RCR and RDR could be detected unequivocally within infected maize tissues. All replicative intermediates accumulated early and, to varying degrees, were already present in the shoot apex and leaves at different maturation stages. Relative to other replicative intermediates, those associated with RCR increased in prevalence during leaf maturation. Interestingly, in addition to RCR-associated DNA forms seen in other geminiviruses, MSV also apparently uses dimeric open circular DNA as a template for RCR.

Maize streak virus (MSV) is the type member of the genus *Mastrevirus* in the family *Geminiviridae* (Stanley et al., 2005) and is widely distributed in sub-Saharan Africa (Shepherd et al., 2009). All geminiviruses (reviewed by Jeske, 2009) express a replication-associated protein (Rep; Fig. 1, ORFs C1/C2) essential for replication. Whilst in begomoviruses, topocuviruses and curtoviruses, Rep is expressed from a single intronless (C1) ORF, in mastreviruses it is expressed from a spliced transcript (Fig. 1; ORF C1C2). In addition to Rep, mastreviruses also express a variant of this protein called RepA (Fig. 1; ORF C1) that is translated from the approximately 80% of *rep* gene transcripts that remain unspliced.

After transfer into the cell nucleus, geminivirus genomes are replicated by host polymerases, using two distinct replication modes: complementary-strand replication (CSR) and rolling-circle replication (RCR) (reviewed by Jeske, 2007). In begomoviruses and curtoviruses, recombination-dependent replication (RDR) has been identified as a third replication mode that utilizes viral covalently closed circular DNA (cccDNA) as template molecules (Alberter et al., 2005; Jeske et al., 2001; Jovel et al., 2007; Morilla et al., 2006; Pilartz & Jeske, 2003; Preiss & Jeske, 2003). Mastreviruses might behave differently from members of the other genera in the family because they are the only geminiviruses to express RepA. Furthermore, they lack genes encoding transcriptional activator and replicational enhancer proteins that are found in begomoviruses and curtoviruses. Although neither of these proteins is absolutely required for RDR (Jeske et al., 2001), the possibility remains that they may enhance the efficiency of the process.

Here, we use one-dimensional (1D) and two-dimensional (2D) gel electrophoresis, in combination with Southern blotting and hybridization as described previously (Jeske, 2007; Preiss & Jeske, 2003), to monitor the accumulation of MSV replicative intermediates in maize tissues at varying developmental stages. Detailed descriptions of the meth-
ods, as well as the 2D blots at higher resolution (Supplementary Figs S1–S4), are provided in the supplementary material (available in JGV Online).

After agroinfection of maize with cloned MSV-A DNA (Owor et al., 2007), total nucleic acids were isolated from different sections of leaves five and six, shoot apices and emerging immature leaf laminae and sheaths (Fig. 1a, d). DNA from the different tissues was separated on 1D agarose gels in the presence of chloroquine, in order to differentiate single-stranded (ss) DNA from double-stranded (ds) DNA and to resolve the monomeric topoisomers of cccDNA of viral origin after Southern blotting and hybridization (Fig. 1b).

After establishing the infection in shoot apices (Fig. 1b, SA), the relative amounts of the main species of open circular (oc) DNA and ssDNA remained essentially constant, but an increasing gradient of heterogeneous viral DNA – represented by the signal smear in the background of the lanes – was observed reproducibly for the three leaf samples L#5 A, B and L#6 A (Fig. 1b). As reported previously (Jeske et al., 2001), these signal smears bury the secrets of replicational dynamics, which can be unravelled by 2D electrophoresis (Figs 2 and 3).

We performed 16 2D blot hybridizations of eight independent biological samples, and show representative blots in Figs 2 and 3 to demonstrate the most interesting features. They reveal that mastreviruses produce the same replication products as have been seen previously in similar experiments with begomoviruses and curtoviruses. These include ssDNA, ocDNA, cccDNA and linear heterogeneous-length dsDNA (hdsDNA) molecules in both monomeric and multimeric states, as well as several true replicative DNA intermediates of CSR, RCR and RDR.

The left upper spots of ssDNA in Figs 2 and 3 represent linear DNA, because the track of linear CSR intermediates starts here and ends at the spot of linear dsDNA (Figs 2 and 3; compare CSR l with c). Remarkably, the CSR track was overprinted by several spots, indicating the accumulation of incomplete intermediates and implying the frequent interruption of complementary-strand DNA synthesis (Figs 2 and 3). Although evidence of CSR interruption has been detected before for other geminiviruses (Jovel et al., 2007; Preiss & Jeske, 2003), it has never been seen to this extent.

Another feature differentiating replicative intermediates of MSV from those of other geminiviruses is higher ratios of linear : circular ssDNA and higher amounts of the corres-

Fig. 1. Sampling of leaf material and 1D gel detection of MSV DNA by Southern blot hybridization. (a) Schematic drawing of the investigated plants and the genome structure of MSV: in plant A, leaf 5 lamina had emerged from the whorl, the developing sheath was still enclosed inside the whorl and its ligule was not yet visible. Only the tip of leaf 6 had emerged from the whorl. In plant B, only the lamina tip of leaf 5 had emerged from the whorl; the rest of leaves 5 and 6 were enclosed inside the whorl. (b) Samples from leaves 5 (L#5) and 6 (L#6) were collected either from the lamina or from the sheath as depicted below the gel images (d) as well as from shoot apices (SA, buried in the whorl in the sketch). As a loading control, genomic DNA in a parallel, ethidium bromide-stained gel is shown (c), and after Southern blotting, viral DNA was detected by a full-length MSV DNA probe (b). For comparison, hybridization standards (HS) were loaded. Viral DNA with multimeric (mult), monomeric open circular (oc), linear (lin), covalently closed circular (ccc) and single-stranded (ss) conformations is indicated.
ponding CSR intermediates. These linear molecules may result from replication defects, nuclease attack during plant defence and/or residual enzymic activity during sample preparation.

Although it is clear that most CSR is initiated on monomeric circular ssDNA molecules (tracks initiated at point ‘ss’ in Figs 2 and 3), faint tracks ending at point ‘2oc’ in the longer exposure of Fig. 2 and in Fig. 3 indicate that dimeric circular ssDNA molecules are also templates of CSR. The presence of heterogeneous-length ocDNA (hocDNA; see arc crossing spots labelled as oc and 2oc in Figs 2 and 3) indicates that CSR utilizes intermediate-length templates, as well as monomeric and dimeric templates. Such hocDNA has only been seen before in 2D gel analyses of the monopartite begomovirus tomato leaf curl virus and its satellites (Alberter et al., 2005).

The replicative intermediates produced when heterogeneous circular ssDNA acts as a CSR template cannot be assessed, because they form fields of hybridization signals rather than discrete arcs or lines (Preiss & Jeske, 2003). All of the MSV 2D blots showed clear evidence of RDR intermediates, indicated by faint arcs originating on dimeric cccDNA spots (labelled RDR in Figs 2 and 3). As described elsewhere (Jeske et al., 2001), similar intermediates originating on monomeric cccDNA spots could not be resolved in our 2D gels, because they are overprinted by the prominent tracks formed by the hdsDNA molecules that are produced as a result of RDR.

Three previously undescribed DNA forms are indicated by X, YR and Z in Figs 2 and 3. Spot X is located exactly at a point where the track representing RCR intermediates is crossed by the track representing CSR intermediates. Therefore, it cannot be determined whether spot X is indicative of stalled RCR or stalled CSR. The straight line of YR runs parallel to RCR intermediates, and may therefore represent RCR intermediates nicked at a single site on the template strand. It remains to be determined why such large amounts of ssDNA as represented by the YR track have accumulated here. The spots labelled Z (Fig. 3) lie on the vertical trajectory of ocDNA and may represent ocDNA molecules that are bound covalently to Rep.

For the first time in geminiviruses, we show that dimeric ocDNA (2oc in Fig. 3) may also serve as a template for RCR. Despite being initiated from a dimeric template, the relative shortness of the straight line representing the RCR intermediates (labelled ‘RCR at dimeric oc template’ in Fig. 3) indicates that only monomeric progeny ssDNA molecules are the probable product of this replication mode.

In summary, the intermediates of MSV replication that we have detected convincingly imply that, as with other geminiviruses, multitasking occurs during mastrevirus replication.

To monitor the different DNA intermediates during leaf development with the highest possible resolution, we had to optimize the gel conditions and exposure times. Therefore, the comparison of the blots for different develop-
Developmental stages of the leaves does not allow an absolute quantification of DNA forms. However, the relatively constant amount of ocDNA (Fig. 1) allowed a rough estimation of the concentration of viral DNA loaded onto different 2D gels.

The relatively high ratios of ssDNA : dsDNA in the shoot apex (Fig. 2, left panel) may indicate that, in this tissue at least, ssDNA is imported from infected older leaves and converted by CSR to oc dsDNA – the form of MSV DNA that has the next highest prevalence in the shoot apex. The presence of linear hdsDNA demands a more cautious interpretation. Either both hdsDNA and ssDNA can be transported, or extended replication has already taken place in these young tissues. To examine these possibilities, the blot was highly overexposed (Fig. 2, right upper panel): this revealed that, even at this early developmental stage, all known replicative intermediates were present. Whilst CSR intermediates were most prevalent in these blots, those of RCR were least prevalent.

It is important to stress here that, despite our efforts to discriminate tissues at different developmental stages, the DNA analysed may be derived from asynchronously infected cell populations and, thus, may stem from cells supporting different stages of virus multiplication. It is also almost certain that all replication products and intermediates became progressively more detectable as leaves emerged and expanded, simply because viral DNA concentrations increased. Despite these potentially confounding effects, clear differences were observed in the relative abundance of certain replication products and intermediates when sheath and lamina samples were compared (Figs 2 and 3). Dimeric cccDNA (labelled 2ccc) was reproducibly more abundant in sheath tissues than it was in lamina tissues. On the contrary, RCR intermediates were considerably more prominent in lamina tissues than they were in sheath tissues. This latter trend was especially marked in samples from leaf tips, which even displayed obvious evidence of RCR on dimeric oc templates (Fig. 3).

We have shown that, although mastreviruses have a smaller inventory of genes than other geminiviruses, they also utilize multiple pathways of replication. Starting with all replication modes in the shoot apex, MSV RCR increases in prominence during the differentiation of infected cells. Given that monomeric ssDNA molecules are both the products of RCR and the encapsidated MSV genomes that accumulate within infected tissues in preparation for transmission (Lucy et al., 1996; Pinner et al., 1993), this time course makes good biological sense.

Replicative intermediates increased in prevalence within a single, fully differentiated symptomatic leaf, from its base to its tip. During the earliest stages of a natural geminivirus infection, no viral genes can be expressed and CSR must rely completely on pre-existing host replication enzymes. The very first rounds of CSR could thus be accomplished even within fully differentiated cells with constitutively expressed host DNA repair polymerases (Anderson et al., 2008). As an infection proceeds within a plant, geminiviral DNA continuously invades new cells, creating secondary multiplication sites. In maize, the fifth leaf primordium is the youngest in which MSV DNA is detectable (Lucy et al., 1996). At this stage, the virus is restricted to the vascular tissues. After the emergence of leaf lamina from the whorl, the corresponding leaf sheath is still undergoing proliferative cell division throughout its length. Accordingly, the mitosis-specific histone H2b is expressed in the apical meristem, developing vascular traces, leaf primordia and young expanding leaves (Lucy et al., 1996).

Our results indicate that RCR, which yields the monomeric ssDNA molecules needed for packaging and viral transmission, plays a subordinate role to CSR in young differentiating tissues, as demonstrated by reduced ratios of RCR:CSR intermediates relative to the leaf lamina in both the shoot apices and leaf sheaths that we analysed. Circular ssDNA genome-length molecules produced by RCR may...
have several fates (reviewed by Gutierrez, 2002): they may be (i) converted into dsDNA via CSR, (ii) sequestered for transport into neighbouring cells or (iii) encapsidated into virions. At least for the mature cells in leaf lamina tips, it is conceivable that the most important fate of RCR products is encapsidation. These virions then accumulate to form paracrystalline inclusions within infected cell nuclei that may be taken up by leafhopper vectors (Pinner et al., 1993). The late accumulation of RCR intermediates therefore fits well with the ultrastructurally observed accumulation of MSV virions in older maize tissues (Pinner et al., 1993).

The data presented here may also explain why mastreviruses are so prone to recombination, which has been suggested as a major driving force for the evolution and epidemic emergence of all geminiviruses (Padidam et al., 1999). The high abundance of linear ssDNA accumulating already during the early stages of geminivirus systemic infections would be lost from the pool of replicating molecules without adequate repair mechanisms. Therefore, it is conceivable that geminiviruses have evolved the capacity for RDR to solve this problem efficiently. A natural consequence of RDR is that, if two related geminivirus genomes enter the same nucleus (Morilla et al., 2004), it is very probable that RDR will produce a wide variety of chimaeric genomes.

With this report on MSV, we complete a cycle of investigations on geminivirus that show the general flexibility and multitude of replication pathways available to these economically important plant pathogens. However, the recent demonstration that recombination patterns are broadly conserved amongst ssDNA virus families – from prokaryote-infecting microviruses through to vertebrate-infecting circoviruses (Lefevre et al., 2009) – suggests that the degrees of replicational multitasking found in geminiviruses might also exist in these other families.

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