Vectors based on maize streak virus can replicate to high copy numbers in maize plants

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The genome of maize streak virus (MSV) consists of one molecule of circular, single-stranded DNA of 2.7 kb. A reporter gene (bar) coding for phosphinothricin acetyltransferase was inserted into the small non-coding region of the MSV genome. The recombinant bar-containing MSV vectors were introduced into maize seedlings via agroinfection. The chimeric viral DNA was found to replicate to high copy numbers in maize leaves resistant to the application of the herbicide Basta. This establishes the usefulness of MSV as an efficient replicating vector in cells of maize plants.

Maize streak virus (MSV) is a member of the geminivirus group of plant viruses and infects the monocotyledonous plant maize. The MSV genome consists of one molecule of circular single-stranded (ss) DNA of about 2.7 kb. Viral transcription and DNA replication are accomplished via double stranded (ds) DNA replicative forms in the plant cell nucleus. Sequence analysis of the MSV genome reveals four open reading frames (ORFs): V1, V2, C1 and C2 (Davies & Stanley, 1989; Mullineaux et al., 1984; Fig. 1). The proteins encoded by the virion sense ORFs V1 and V2 have been identified in MSV-infected maize tissue as putative movement protein and coat protein, respectively (Boulton et al., 1993; Morris-Krsinich et al., 1985; Mullineaux et al., 1988). Mutagenesis studies revealed that the proteins encoded by ORFs V1 and V2 are both required for systemic spread of the virus and symptom development in plants, although neither is required for viral DNA replication (Boulton et al., 1989; Lazarowitz et al., 1989; Shen & Hohn, 1991). Viral DNA replication requires the two complementary sense ORFs, C1 and C2 (Lazarowitz et al., 1989; Shen & Hohn, 1991).

Geminiviruses as replicating vectors have been reviewed recently (Timmermans et al., 1994). For geminiviruses infecting monocots, replicating vectors were mainly constructed based on the genome of wheat dwarf virus (WDV), a close relative of MSV, and studied in monocot protoplasts (Hofer et al., 1992; Matzeit et al., 1991; Mullineaux, 1992; Timmermans et al., 1992; Ugaki et al., 1991). Lazarowitz et al. (1989) reported the replication in inoculated leaves of maize plants of MSV vectors in which ORF V2 or ORFs V1 + V2 have been replaced by the chloramphenicol acetyltransferase (CAT) or hygromycin phosphotransferase (Hph) genes. However, no Hph and only weak CAT activity was detected. We have previously shown that insertion of the β-glucuronidase (GUS) gene (under the transcriptional control of the CaMV 35S promoter and the nopaline synthase terminator) into an intact MSV genome did not interfere with replication of the chimeric viral genome. It was also demonstrated that viral replication led to an increase in GUS activity in infected maize plants (Shen & Hohn, 1994). Due to the destructive nature of the assay for GUS activity, we have not been able to study the copy number of MSV vectors in maize plants. In the present study, we replaced the GUS gene in the MSV vectors with the herbicide resistance gene bar from Streptomyces hygroscopicus (De Block et al., 1987), which codes for phosphinothricin acetyltransferase (PAT). We show that agroinfection of maize plants with MSV-bar resulted in leaf tissues resistant to the application of the herbicide Basta and that a high copy number (up to more than 500 molecules per cell) of MSV-bar DNA could be found in these tissues.

The bar gene used here was under the transcriptional control of the CaMV 35S promoter and terminator and was inserted into the non-coding, small intergenic region (SIR) of MSV in the binary vector pCMSV (Shen & Hohn, 1991), resulting in pMSV-bar (Fig. 1). pMSV-D-bar was obtained by deletion of the 1 kb BamH1–NcoI fragment covering the V1 coding sequence and part of the V2 coding sequence of MSV (Fig. 1). Agroinfection (Grimsley et al., 1987) was used to infect maize seedlings.

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**Fig. 1.** Schematic representation of the binary vectors used for agroinfection. The coding sequence of the *bar* gene is shown as a black box, the 35S promoter as a triangle and the 35S terminator as a filled circle. The open boxes represent the MSV genome. The ORFs V1, V2, C1 and C2 as well as the large intergenic region (LIR) and the small intergenic region (SIR) are indicated. pCMSV contains a 1.6-mer of the MSV genome in the T-DNA of pCIB200 (Shen & Hohn, 1991). pMSV-bar contains the *bar* gene inserted in the AsnI site of the SIR of pCMSV. pMSV-D-bar differs from pMSV-bar by the deletion of the region *Bam*HI–*Nco*I which covers the ORFs V1 and V2. LB, left border of T-DNA; RB, right border of T-DNA; B, *Bam*HI; Bg, *Bg*II; N, *Nco*I; X, *Xho*I.

**Fig. 2.** Maize plants agroinoculated with pMSV-D-bar or mock-inoculated after application of herbicide Basta. Four days after inoculation, plants were sprayed with 0.2% Basta + 0.05% SDS. This treatment was repeated three times at 3 day intervals. The picture was taken 10 days after the last treatment.

*Agrobacterium* cells containing MSV or *bar*-containing derivatives of MSV were injected into the coleoptilar node of 3-day-old maize seedlings and inoculated seedlings were transplanted into soil. Inoculation with pCMSV containing the wild-type MSV genome resulted in 100% of plants showing viral symptoms. As expected (Shen & Hohn, 1994), no viral symptoms were observed on the plants inoculated with pMSV-bar (0/80) or pMSV-D-bar (0/80).

To evaluate the expression of the *bar* gene, the
Table 1. Resistance to Basta application of plants agroinfected with vectors carrying the bar gene

<table>
<thead>
<tr>
<th>Binary vector</th>
<th>No. of inoculated plants</th>
<th>No. of plants with green sectored leaves*</th>
<th>No. of plants surviving the Basta application*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (mock-inoculation)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pMSV-bar</td>
<td>120</td>
<td>27</td>
<td>7</td>
</tr>
<tr>
<td>pMSV-D-bar</td>
<td>120</td>
<td>30</td>
<td>11</td>
</tr>
</tbody>
</table>

* Scoring was done 18–21 days after inoculation.

inoculated plants were sprayed with an aqueous solution containing 0.2% Basta (equivalent to 0.4 g/l glufosinate-ammonium; Plüss-Stauffer AG, Oftringen, Switzerland) and 0.05% SDS. The first treatments were performed 4 days after inoculation, when the second leaves started to appear. The treatment was repeated two or three times, at intervals of 3 days. One week after the final Basta treatment (18–21 days after inoculation), all the mock-inoculated plants died. However, about 25% of the plants inoculated with Agrobacterium containing the constructs pMSV-bar or pMSV-D-bar had first and/or second leaves which remained partially or totally green and 5–10% of the plants survived the Basta treatments and continued to grow (Fig. 2; Table 1). This observation is in agreement with our previous results in which the GUS gene was used as reporter. There the expression of the GUS gene exhibited a marked plant-to-plant variation, with only few plants expressing the GUS gene in more than three or four leaves (Shen & Hohn, 1994). This in turn implies that only leaves expressing the herbicide resistance gene to a certain level were able to partially or totally survive the treatment. All plants surviving Basta treatment became sensitive to further Basta treatment later (40 days after inoculation). This result confirms our previous conclusion (Shen & Hohn, 1994) that MSV carrying the reporter genes was not able to spread systemically in plants and infect newly developing leaves. In addition, DNA molecules carrying the bar gene might be degraded or the expression of the bar gene may become repressed in the infected leaves at later stages.

Although replication of MSV vectors carrying the CAT, Hph or GUS genes has been detected in maize

Fig. 3. Southern blot showing replication of MSV-bar and MSV-D-bar in maize tissues that had survived the herbicide treatment. Equal amounts (5 μg) of plant DNA samples, undigested (lane 8) and digested with NcoI which linearizes MSV-bar and MSV-D-bar (lanes 4–7), were separated on 1% agarose gels by electrophoresis, blotted onto Zeta-probe nylon membranes (Bio-Rad) and hybridized with a 32P-labelled bar probe. The bar probe was produced from the isolated BglII fragment located at the 5'-end of the coding sequence of the bar gene (Fig. 1), using a random primer labelling kit (Boehringer Mannheim) and [α-32P]dATP (Amersham). Lanes 1 and 2 contain pMSV-D-bar digested with XhoI, which liberated MSV-D-bar from the plasmid. Lane 3 contains pMSV-bar digested with XhoI, which liberated MSV-bar from the plasmid. The nature of the faint bands migrating faster than the prominent bands in lanes 1, 5 and 7 has not been identified. The amounts of plasmid DNA loaded are equivalent to 500, 50 and 250 copies per maize genome for 5 μg maize DNA, respectively, for lanes 1, 2 and 3. Lane 4 contains DNA of an uninfected maize plant. Lane 5 contains DNA isolated from a mixture of green leaf tissue of four plants inoculated with pMSV-bar. Lanes 6 and 7 contain DNA isolated from two individual plants inoculated with pMSV-D-bar. Lane 8 contains DNA isolated from a mixture of green leaf tissue of four plants inoculated with pMSV-D-bar. Lin (a) indicates the linear dsDNA form of MSV-bar. Oc (b), Lin (b) and Ccc (b) indicate open circular, linear and covalently closed circular dsDNA forms of MSV-D-bar.
are shown in Fig. 3) isolated from a total of 42 plants. It is difficult to estimate the copy number of bar-containing MSV vectors since the selection for herbicide resistance may still allow viral DNA-free cells to survive due to the non-cell-autonomous nature of the resistance. Nevertheless, a range of less than 50 (lane 6) to more than 500 (lane 8) molecules per cell, on the average, could be roughly estimated for 12 DNA samples (four of them are shown in Fig. 3) isolated from a total of 42 plants. This is comparable to the estimated copy number of dicot-infecting geminivirus vectors obtained in transgenic plants (Hayes et al., 1988, 1989; Kanevski et al., 1992). However, the estimated copy number for MSV vectors obtained here is much lower than that for WDV vectors tested in maize protoplasts, where the copy number was approximately 2500–30000 (Timmermans et al., 1992). This difference may reflect an underestimation of the copy number of our MSV vectors of the replicative activity of the two different viruses, but it may also be due to the difference between protoplasts and plant cells.

Our results suggest that the bar gene may be a useful reporter for the study of viral replication in plants. The observation of high copy numbers of vectors based on MSV establishes the usefulness of this viral genome as a transient gene expression vector in maize plants.

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References


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