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

Wen-Hui Shen† and Barbara Hohn*

Friedrich Miescher Institute, PO Box 2543, CH-4002 Basel, Switzerland

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, 1994).

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; 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reported the replication in inoculated leaves of maize plants of MSV vectors in which ORF V2 or ORFs V1 + V2 have been replaced by the chloramphenicol acetyltransferase (CAT) or hygromycin phosphotransferase (Hph) genes.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 BamHI–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.

* Author for correspondence. Fax +41 61 6973976.

† Present address: Institut de Biologie Moléculaire des Plantes du CNRS, Université Louis Pasteur, 12 rue du Général Zimmer, 67084 Strasbourg Cedex, France.
**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
inoculated plants were sprayed with an aqueous solution containing 0.2% Basta (equivalent to 0.4 g/l glufosinate-ammonium; Plüss-Staufer 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

### 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 sectoral 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.
plants previously (Lazarowitz et al., 1989; Shen & Hohn, 1994), analysis at the DNA level could not be correlated to activity of these reporter genes in the same plant tissue. The unique activity assay for herbicide resistance represents an advantage of the bar gene over previously used reporter genes. The tissues resistant to Basta treatment were used to assess the replication activity of MSV-bar and MSV-D-bar in cells of maize plants. Total DNA was isolated 20–40 days after inoculation from leaves or whole shoots resistant to Basta treatment, using the method described by Burr & Burr (1981). DNA samples were analysed by Southern blot (Southern, 1975) using a 32P-labelled bar probe. Three hybridization signals corresponding to the different dsDNA forms of replicating MSV-constructs were observed with undigested representative DNA samples (Fig. 3, lane 8). Digestion of the DNA samples with Neol resulted in the linear forms corresponding to MSV-bar (lane 5) or MSV-D-bar (lanes 6 and 7).

A big plant-to-plant variation in the amount of MSV-D-bar DNA was found when whole shoots were used for DNA isolation (Fig. 3, lanes 6 and 7). However, when DNA samples were prepared from mixtures of first and second leaves of plants partially resistant to Basta treatment, the amount of MSV vector DNA was relatively constant from sample to sample (data not shown) and it was also higher than in DNA prepared from whole shoots (Fig. 3, lane 8). These differences may reflect differences in viral replication and the resultant differences in the proportion of infected versus uninfected cells in different tissues. Based on the relatively constant fraction of viral DNA in DNA samples of leaf mixtures, the relative copy numbers of MSV-D-bar were found to be several fold higher than those of MSV-bar (Fig. 3, lanes 5 and 8). This may be because the genome of MSV-D-bar is 1 kb smaller than that of MSV-bar. A correlation between genome size and copy number has been previously noticed for vectors based on other geminiviruses (Ward et al., 1992). This observation of higher copy number with smaller genome size of MSV vectors may explain our 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.

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).

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References


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