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Transmission of viral RNA and DNA to maize kernels by vascular puncture inoculation

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Abstract

Vascular puncture inoculation (VPI) is an effective technique for transmission of maize viruses without using arthropods or other biological vectors. It involves using a jeweler's engraving tool to push minuten pins through a droplet of virus inoculum toward the major vascular bundle in the scutellum of germinating kernels. Here, VPI is shown to be useful for introducing RNA and DNA viral genomes into maize. Maize dwarf mosaic potyvirus (MDMV) virions, MDMV genomic RNA, foxtail mosaic potexvirus (FoMV) genomic RNA and maize streak geminivirus (MSV) DNA were introduced into kernels by VPI, and infection rates determined. At high concentrations, both MDMV virion and genomic RNA preparations produced 100% infection of susceptible maize. However, MDMV genomic RNA was transmitted with about 100-fold lower efficiency than virions. FoMV genomic RNA and MSV DNA were transmitted at lower efficiency than the MDMV RNA, and the highest transmission rates were about 50%. Ribonuclease A pretreatment prevented genomic MDMV and FoMV RNA transmission, but not MDMV virion transmission indicating the viral RNA was the infectious entity. Proteinase K (ProK) pretreatment reduced transmission of MDMV RNA suggesting that integrity of the viral genomic protein bound covalently to the viral RNA may be important for efficient transmission. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Maize dwarf mosaic virus; Foxtail mosaic virus; Maize streak virus; Vascular puncture inoculation

1. Introduction

Vascular puncture inoculation (VPI) is a powerful technique developed to facilitate mechanical transmission of maize viruses (Louie, 1995). It involves using a jeweler's engraving tool to push minuten pins through a droplet of virus inoculum toward the major vascular bundle in the scutellum

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of germinating maize (*Zea mays* L.) kernels. It was proposed that viruses enter cells through openings in cell walls and the cell membrane created by the vibrational wave energy generated from the engraver's tool. The technique permits efficient, vector-free transmission of all maize viruses tested to date including most of the principal maize viruses that occur worldwide. VPI also was critical in studies to transmit and characterize a new virus, maize necrotic streak virus (MNeSV), for which no biological vector has been identified (Louie et al., 2000). In addition, recent work suggests that VPI may be useful for inoculating viruses into other grain seeds such as wheat, barley and rice, as well as into soybeans (R. Louie, unpublished results).

To date, viruses have been transmitted from extracts of infected leaves and from purified virions by VPI (Louie, 1995; Louie et al., 2000; Madriz-Ordenana et al., 2000). Because it is possible that nucleic acids or proteins may also enter maize cells through the same openings created by the engraving tool, genomic RNAs for maize dwarf mosaic potyvirus (MDMV) and foxtail mosaic potexvirus (FoMV) and maize streak geminivirus (MSV) DNA were examined for their ability to infect germinating maize kernels after VPI. Preliminary reports of this study have been presented (Ngwira et al., 1995; Ngwira, 1997; Redinbaugh et al., 2000).

2. Materials and methods

2.1. Virus source, maintenance and isolation

MDMV strain A (MDMV) was collected in Ohio and maintained in *Sorghum bicolor* 'Sart' as previously described (McDaniel and Gordon, 1989). MDMV virions were purified according to Shulka et al. (1989), frozen in liquid nitrogen, and stored at −80 °C until use. Virion protein concentration was determined by the dye-binding method using bovine serum albumin (BSA) as a standard (Redinbaugh and Campbell, 1985).

FoMV was obtained in freeze-dried *Chenopodium quinoa* leaves from Dr J.B. Bancroft (University of Western Ontario, London, Ont., Canada) under United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) permit 44076. For culture and maintenance, the virus was transmitted mechanically by leaf rub inoculation (RI) or VPI to *Z. mays* 'Oh28' and *Hordeum vulgare* 'Barsoy' using extracts from infected leaves (Bancroft et al., 1991; Louie, 1995).

MSV was obtained from infected *Setaria verticillata* leaves from Kenya imported under USDA-APHIS permit 43266 (Njuguna, 1996). The virus was transmitted mechanically to and maintained in *Z. mays* (Pioneer hybrid 3379, P3379) by VPI.

2.2. Viral RNA isolation

Genomic RNA for MDMV and FoMV was isolated from partially purified virions. Virions were isolated by preparing an extract from infected leaves, extracting with chloroform, and centrifuging at $100\,000 \times g$ through 30% sucrose according to the method of Shulka et al. (1989). The resulting pellet was resuspended in 25 mM Na-citrate, pH 7.0, 4 M guanidine thiocyanate, 1.5% (w/v) Na-lauryl sarcosine and 0.1 M 2-mercaptoethanol. RNA was subsequently isolated by centrifugation through CsCl according to Chirgwin et al. (1979). The RNA pellet was resuspended in water, extracted with phenol:chloroform, precipitated with Na-acetate and ethanol, and dissolved in diethylpyrocarbonate (DEPC)-treated water (Sambrook et al., 1989).

2.3. Maize streak virus clones

For construction of MSV containing plasmids, total nucleic acids were isolated from MSV-infected maize leaves according to the method of Hughes et al. (1991,1992), except that the extraction buffer included 1% (v/v) 2-mercaptoethanol. After two extractions with phenol:chloroform, nucleic acids in the aqueous extract were precipitated with Na-acetate and ethanol (Sambrook et al., 1989). The nucleic acids were collected by centrifugation at $24\,000 \times g$ for 10 min, resuspended in 10 mM Tris–HCl, pH 8, 50 mM NaCl and 1 mM ethylene diamine tetra acetic acid

(EDTA), then treated with 1 mg/ml RNAse A for 1 h at 37 °C. After phenol:chloroform extraction and Na-acetate and ethanol precipitation as above, the DNA was resuspended in 10 mM Tris–HCl, pH 8, and 1 mM EDTA. Viral DNA was separated from host nucleic acids by agarose gel (0.8%) electrophoresis in 40 mM Tris–acetate, pH 8.2, 1 mM EDTA and 0.5 μg/ml ethidium bromide (Sambrook et al., 1989). 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, USA). The purified DNA was linearized by digestion with *BamHI*, ligated into the *BamHI* site of pUC19 and transformed into *Escherichia coli* DH5α (Sambrook et al., 1989). MSV DNA-containing plasmids were identified through a combination of restriction endonuclease digestion and sequence analysis (Howell, 1984; Lazarowitz, 1988). An infectious clone was obtained by inserting a head-to-tail dimer of the 2.7 kb MSV genome into pUC19 (Howell, 1984; Lazarowitz, 1988). Plasmid DNA was isolated using Wizard Maxi-Preps (Promega, Madison, WI, USA), and sequenced by primer walking using an ABI 377 DNA Sequence Analyzer and Big Dye Terminator chemistry (PE Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were analyzed using DNA Strider (Dr C. Marck, Service de Biochimie et de Genetique Moleculaire, Gif-sur Yvette, France) and MacVector 6.5 (Oxford Molecular Group, Great Britain). The sequence of MSV-Km DNA was deposited in Genbank (Accession # AF395891).

2.4. Virus transmission and assay

Nucleic acid concentrations were calculated from the absorbance at 260 nm assuming 50 μg DNA/absorbance unit and 40 μg RNA/absorbance unit in a 1 cm light path (Sambrook et al., 1989). The amount of MDMV RNA in virions was estimated from the protein concentration assuming a virion content of 5% RNA and 95% protein (Shulka et al., 1989; Brunt et al., 1996). Prior to VPI, serial dilutions of each inoculum were made to the concentrations indicated in the figures. Virions were diluted with 10 mM Tris–citrate, pH 7.2, RNA with DEPC-treated water, and plasmid DNA with 10 mM Tris, pH 8, and 1 mM EDTA. Where indicated, virions and/or RNA were treated for 10 min at 37 °C with 0.1 or 1 μg/μl ribonuclease A (Sigma, St. Louis, MO, USA) or 1 μg/μl Proteinase K (ProK; Gibco BRL, Rockville, MD, USA) prior to inoculation.

The maize lines used for testing inoculation efficiency were selected for susceptibility to the respective viruses (Louie, 1995; Changa, 1998; Redinbaugh et al., unpublished results). 'Spirit' sweet corn was used for MDMV inoculations, 'Seneca Chief' sweet corn for FoMV, and P3379 for MSV. For each inoculum dilution, 25 kernels were inoculated per experiment. The specific conditions for pretreatment and inoculation of maize kernels were based on optimal conditions previously identified for transmission of maize white line mosiac virus, maize rough dwarf fijivirus (MRDV) and MSV from leaf extracts (Louie, 1995, 1998; Changa, 1998). Kernels were soaked in tap water at 30 °C for 2 h. P3379 kernels were then incubated on moist paper towels at 22 °C for 4 h prior to inoculation, while Seneca Chief and Spirit kernels were inoculated immediately. For VPI, five minuten pins affixed to the flattened end of 10-gauge copper wire were mounted on an engraving tool (Ideal Industries Inc., Sycamore, IL, USA). The vibrating engraving tool was used to push the minuten pins through 2–3 μl inoculum placed on top of kernels and into the scutellum along the length of the embryo (Louie, 1995). After a 2 day incubation on moist paper towels at 30 °C, the kernels were planted in autoclaved greenhouse soil and transferred to a greenhouse. All plants were scored for symptom appearance for 2–3 weeks postinoculation.

For mechanical transmission of MDMV RNA by leaf RI, 2 μl RNA solution mixed with an equal volume of a suspension of bentonite (5 mg/ml water) and a small amount of 600 mesh silicon carbide was rubbed onto primary and secondary leaves of 14- to 20-day old test plants using a gloved thumb and forefinger.

Leaves of inoculated plants were tested for the presence of virus using Western blot analysis using a 1:2000 dilution of MDMV or MSV antisera as described (Sambrook et al., 1989). Reverse

transcription-polymerase chain reaction (RT-PCR) was carried out using the Access RT-PCR according to the manufacturer's instructions (Promega). Forward and reverse primers corresponded to nt 3118–3139 and 3599–3620 of the foxtail mosaic virus (FoMV) cDNA (Genbank Accession M62730), respectively. Virions in sap of infected leaves were viewed using a Philips 201C electron microscope after negative staining with phosphotungstic acid.

3. Results

3.1. Transmission of maize dwarf mosaic potyvirus RNA

To test the utility of VPI for transmission of viral RNAs, transmission of the easily mechanically transmissible potyvirus, MDMV, was compared with transmission of its RNA. After inoculation of virions or genomic RNA to 'Spirit' sweet corn kernels, mosaic symptoms typical of MDMV infection developed on all leaves of up to 94% of inoculated plants (Fig. 1). MDMV capsid protein was detected in symptomatic leaves (SL) by Western blot analysis (data not shown). Thus, inoculation of maize with MDMV RNA and virions appeared to incite a similar disease of maize.

The relative efficiencies of MDMV RNA transmission by VPI and leaf RI, and VPI of purified MDMV virions were tested using serial dilutions of virion or genomic RNA solutions (Fig. 1A). Infection rates increased in plants inoculated with increasing concentrations of either the virion or genomic RNA with a pattern similar to that commonly observed for mechanical transmission of plant viruses (Matthews, 1991). Transmission of the virion was clearly more efficient than transmission of the RNA, with 100-fold more MDMV RNA being required to produce infection rates similar to those of virion-inoculated plants. The MDMV genomic RNA also produced typical mosaic symptoms in seedlings after leaf RI. However, transmission by leaf RI was significantly greater than zero only for 290 ng viral RNA. In contrast, VPI with this amount of RNA resulted in 94% infection.

To verify that the infection rates observed in Fig. 1A resulted from transmission of the MDMV RNA and not residual contaminating virions, RNA and virion solutions were pretreated with ribonuclease A (RNase) prior to VPI (Fig. 1B). Treatment of the isolated RNA (290 ng) with 1 mg/ml RNase completely abolished infectivity, and 0.1 mg/ml RNase pretreatment reduced infec-

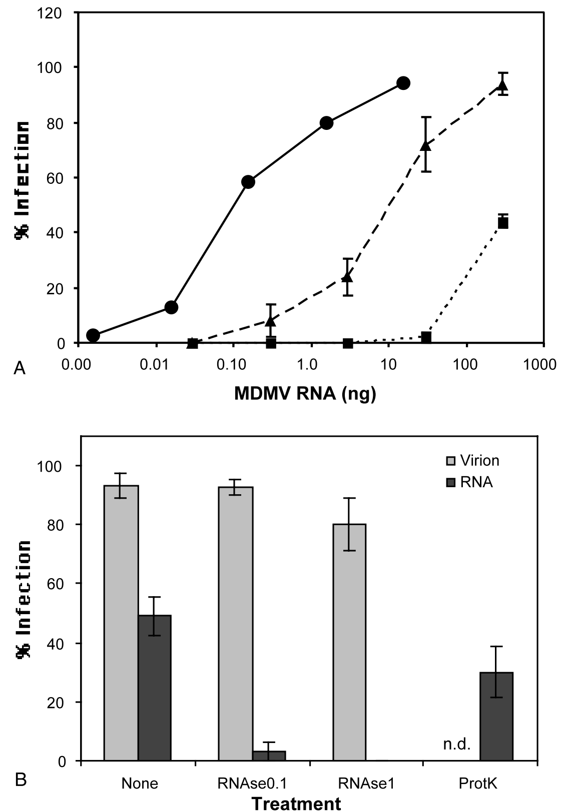

Fig. 1. VPI of maize dwarf mosaic virus (MDMV) into germinating maize kernels. In panel A, maize ('Spirit') kernels were vascular puncture inoculated with 2 μl of a suspension containing the indicated amount of RNA in purified MDMV virions (●). In addition, MDMV RNA solution (2 μl) was mechanically inoculated into kernels by VPI (▲) or into seedling leaves by leaf RI (■). Panel B shows the effect of ribonuclease and proteinase treatment on transmission of MDMV by VPI. The MDMV virion (light gray) and RNA (dark gray) solutions were pretreated with 0.1 μg/μl ribonuclease A (RNase0.1) or 1 μg/μl ribonuclease A (RNase0.1), and MDMV RNA was pretreated with 1 μg/μl ProK prior to VPI of maize kernels. The data presented are the mean ± S.E. for five experiments, n.d., not done.

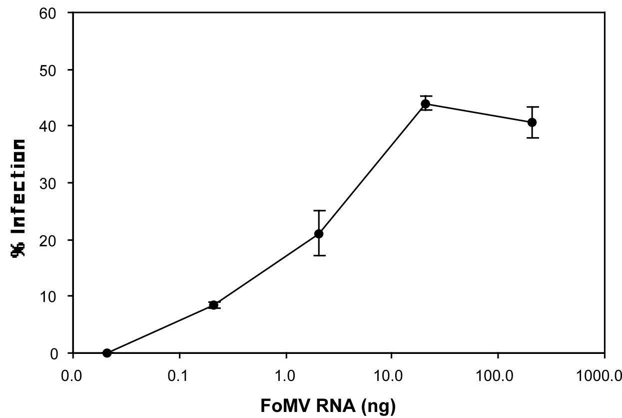

Fig. 2. VPI of FoMV RNA into germinating maize kernels. Maize ('Seneca Chief') kernels were inoculated with 2 μl solution containing the indicated amount of FoMV RNA. The data are the mean ± S.E. for four experiments.

tivity by 97%. In contrast, similar treatment of virions with 0.1 or 1 mg/ml RNase reduced infectivity by only 8 and 20%, respectively. Under the conditions used, the RNase treatments eliminated the genomic RNA band on ethidium bromide-stained denaturing agarose gels (Sambrook et al., 1989), but had no effect on the size or apparent concentration of MDMV capsid protein observed on sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE; data not shown). A 10 min treatment of isolated MDMV RNA with ProK, a broad spectrum protease (Campbell and Gerard, 1994), reduced infectivity of the RNA by about 40% (Fig. 1B).

3.2. Transmission of foxtail mosaic potexvirus RNA

Some single-stranded (SS) positive sense RNA viruses, including MDMV, have a viral genomic protein covalently bound to 5′ end of the genome (Shulka et al., 1994). Others, such as FoMV, have a 5′ cap structure rather than a protein (Bancroft et al., 1991). Transmission of FoMV RNA was tested to determine if a viral genomic protein is required for efficient transmission of viral RNA genomes by VPI. Inoculation with more than 0.2 ng FoMV RNA into 'Seneca Chief' kernels incited systemic mosaic symptoms on inoculated plants (Fig. 2). Leaf RI with extracts from SL produced 36 ± 5 and 42 ± 19% infection of barley and maize seedlings, respectively, indicating the SL contained infectious virus. In addition, RT-PCR assays indicated that FoMV RNA was present in SL from inoculated plants (data not shown).

Similar to MDMV, VPI with increasing amounts of the FoMV RNA resulted in increased infection rates; however, the rate leveled off at about 40% infection after inoculation with 20 ng RNA (Fig. 2). The rate of transmission was 0% for FoMV RNA pretreated with either 0.1 or 1 mg/ml RNase A ($n = 4$). These data indicated that RNA was the infectious agent and that there was no contamination of the viral RNA with virions.

3.3. Transmission of maize streak geminivirus DNA

Transmission of cloned DNA from the monopartite geminivirus MSV was tested to determine if VPI would also be effective for transmission of viral DNA. A maize streak virus (MSV) clone was constructed using *BamHI* digests of the double-stranded, replicative form of MSV isolated from infected leaf tissue. The sequence of the cloned MSV DNA (about 2.7 kb) was more than 97% identical to that of the cloned Kenyan MSV isolate (Howell, 1984). A head-to-tail dimer of the viral DNA sequence inserted into pUC19 (pMSV-Km; about 8 kb) was used for testing the efficiency of viral DNAs by VPI.

VPI of maize kernels with as little as 0.2 ng of plasmid DNA produced infection rates that were significantly higher than zero (Fig. 3). Infected seedlings had streaking and stunting symptoms characteristic of MSV infection. Extracts prepared from symptomatic seedlings inoculated with the MSV DNA contained a protein of about 27 kDa that reacted with MSV antisera indicating the MSV coat protein was present (Fig. 4A). In addition, SL contained discrete DNAs corresponding to the approximately 3 kb viral open circular (OC), linear, supercoiled and single stranded (SS) DNA species (Fig. 4B). These are the forms of monomeric MSV DNA normally found in infected tissue. Because plasmid DNA was used as the inoculum, DNA was the infectious agent.

As with the MDMV and FoMV genomic RNAs, VPI with increasing amounts of plasmid between 0.24 and 240 ng DNA produced increasing rates of infection (Fig. 3A). However, the infection rate at 240 ng DNA was greater than that observed at 2.4 μg DNA. The germination rates for maize kernels inoculated with MSV DNA were also monitored (Fig. 3B). Although the germination rates for MSV DNA inoculated kernels were somewhat variable, seeds inoculated with 2.4 μg plasmid DNA germinated at a lower rate than those inoculated with less DNA.

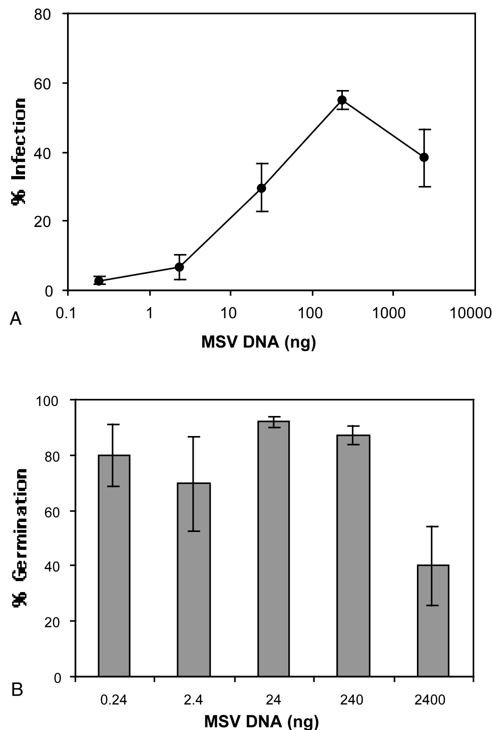

Fig. 3. Transmission of MSV DNA by VPI. Maize kernels were inoculated with 3 μl of solution containing the indicated amount of plasmid DNA carrying a head-to-tail dimer of the MSV genome. The infection rates for germinated seedlings (panel A), as well as the germination rates (panel B) are shown. Kernels were inoculated by pushing the pins through the inoculum three times on each side of the embryo. Data presented are the mean ± S.E. for five experiments.

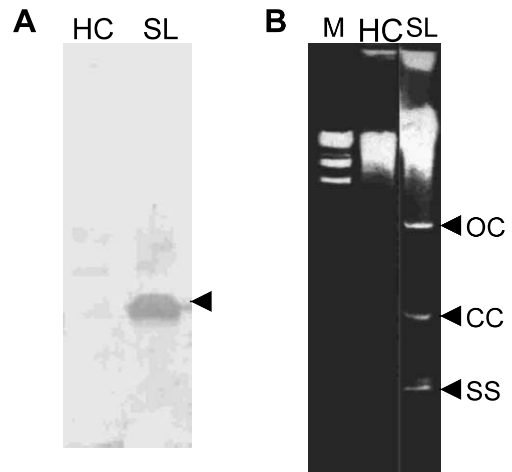

Fig. 4. Accumulation of MSV in plants inoculated with viral DNA. SL and from maize plants vascular puncture inoculated with MSV and leaves from healthy control (HC) plants were tested for the presence of MSV virion protein by Western blot analysis (panel A). The arrow at the right of the figure indicates the migration of the 27 kDa marker. In panel B, an ethidium bromide stained agarose gel of DNA isolated from symptomatic (SL) and HC leaves is shown. Lane M contains HindIII digested lambda DNA. The arrows at the right of the panel indicate the migration positions of the OC double-stranded, the closed circular (CC) double-stranded and SS forms of the MSV genomic DNA.

4. Discussion

These results extend the utility of VPI to include transmission of isolated viral genomic RNA and cloned viral DNA. The pattern of infection relative to virion or nucleic acid concentration was typical of that seen for other virions and viral RNAs (Fraenkel-Conrat and Singer, 1999; Matthews, 1991). For all three viruses, inoculation of kernels with the nucleic acid produced symptomatic plants, and extracts from plants showing FoMV and MSV symptoms were shown to be infectious. In addition, either viral nucleic acid (FoMV and MSV) or virion protein (MDMV and MSV) was detected in tissues infected systemically. Ribonuclease treatment substantially decreased or eliminated the infectivity of MDMV and FoMV genomic RNA, while similar treatment of MDMV virions had little effect on infectivity. These data, combined with the fact that the density of MDMV and FoMV virions (~ 1.3

g/cm^3) is substantially lower than that of the CsCl solution (1.7 g/cm^3) through which the RNAs were sedimented, indicate that the intact genomic RNA was the infectious agent. Because plasmid DNA was used for MSV inoculations, the infections could not have arisen from virion contamination. These data indicate that native virus was produced after VPI with the genomic RNA or DNA, and that the nucleic acid, not contaminating virion, was the infectious agent.

Although infection rates approached 100% after VPI with MDMV RNA, the maximal infection rates leveled off or decreased at high concentrations of FoMV RNA and MSV DNA. It is possible that some contaminant reduced transmission in the less-dilute nucleic acid preparations. In the case of MSV, where high concentrations of plasmid DNA also significantly reduced the germination rate, it is possible that a severe infection of embryo cells at high concentrations of MSV DNA was responsible for the reduced the kernel germination rate. If this is the case, then the observed infection rates are underestimates. In addition, the length of the preinoculation incubation, number of inoculations, and maize genotype influence the rate of virus transmission by VPI (Changa, 1998; Louie, 1998). Similarly, adjustment of the inoculation conditions may allow for increased transmission of the FoMV RNA and MSV DNA.

The number of viral genomes needed to achieve 50% infection for the MDMV virion and RNA, the FoMV RNA and MSV DNA were calculated from the data in Figs. 1–3 and the molecular weights of the inoculated nucleic acids (Table 1). On a genome basis, VPI of MDMV virion was about 100-fold more effective than VPI of MDMV RNA. However, 100-fold more MDMV RNA was required to produce 50% infection of maize by leaf RI than by VPI. The increased efficiency of VPI relative to leaf RI may help obviate some of the problems reported recently for transmission of maize virus RNAs synthesized in vitro (Choi et al., 1999; Robertson et al., 2000).

MDMV RNA was transmitted more than 20-fold more efficiently than FoMV RNA. Although both MDMV and FoMV infect similar cell types and tissues and both genomes are polyadenylated, the 5′ end of the potyvirus genomes have bound covalently viral genomic proteins, while potexvirus genomes have a 5′ cap structure (AbouHaidar and Bancroft, 1978; Sonenberg et al., 1978; Shulka et al., 1994; Brunt et al., 2000). Both the viral genomic protein and 5′ cap are thought to stabilize the viral RNA against degradation by intracellular ribonucleases suggesting the viral genomic protein is more effective for protection than the cap structure. This notion is bolstered by the fact that transmission of MDMV RNA is reduced by pretreatment with ProK. Alternatively, degradation of the viral genomic protein may reduce translation efficiency, and hence the infectivity of the viral genome (Leonard et al., 2000).

Previously, MSV DNAs were transmitted from binary Ti-plasmid vectors carrying tandem dimeric copies of the genome in *Agrobacterium tumefaciens* through ‘agroinoculation’ or ‘agroinfection’ (Grimsley et al., 1987; Grimsley and Bisaro, 1987). Several bipartite geminiviruses can be transmitted from *E. coli* plasmid DNA using a biolistic procedure (Garzon-Tiznado et al., 1993), but this technique has not been tested for MSV. However, VPI of MSV DNA may be similar to biolistic inoculation of geminiviruses in that both methods provide a mechanism for entry of DNA

Table 1
Transmission efficiency for maize virus RNA and DNA

Inoculum	Inoculation method	Virus molecules for 50% infection ($\times 10^{-6}$)[a]
MDMV virion	VPI	2.7
MDMV RNA	VPI	270
FoMV RNA	VPI	6300
MSV DNA	VPI	13 000
MDMV RNA	RI	23 000

Virions and nucleic acids were introduced into maize kernels by VPI or maize seedlings by leaf RI.

[a] The viral nucleic acid (ng) required to produce 50% infection was estimated from the data presented in Figs. 1–3, and moles of virus were calculated using molecular weights of 3.06, 5.23 and 2.0×10^6 Da for the MDMV RNA, MSV DNA and FoMV RNA, respectively, where 50% infection was not achieved (i.e. FoMV), the amount of nucleic acid producing the highest infection rate was used.

into plant cells. Because MSV may also be transmitted from pSPORT and pPZP201 binary (Hajdukiewicz et al., 1994) plasmids by VPI, the nature of the plasmid appears to have little affect on transmission (R. Edema et al., unpublished results). The ease and low cost of VPI make it an ideal inoculation method for studies of MSV gene function and virulence.

VPI was shown previously to be effective for transmission of all tested maize viruses (Louie, 1995). Recently, VPI was instrumental in the isolation and characterization of MNeSV, a new virus that is not transmitted by RI and for which no vector is known (Louie et al., 2000). Thus, the technique is useful for the study of new virus isolates, particularly when the vector is unknown. This study indicates that viral nucleic acids also may be transmitted to maize by VPI with relatively high efficiency. VPI of viral RNA and DNA ability will facilitate experiments that require development and transmission of infectious virus clones, as well as studies of genetically modified viruses. In addition, the relatively high rates of MSV transmission and the consistency of symptoms produced after VPI with MSV DNA suggest that VPI may be useful for identification and characterization of virus resistant maize germplasm.

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