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Tissue Specificity of *Zea mays* Infection by Maize Streak Virus

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Maize streak virus (MSV) is a single-stranded DNA virus and type member of subgroup I of the Geminiviridae, members of which have been considered generally as viruses limited to phloem tissues. We have studied this characteristic and the extent of MSV penetration into the shoot apical meristem of maize using immunohistochemical and in situ hybridization techniques. This approach has been used to investigate the suggestion that host cell division might be required for the replication of MSV. The results show that, within the shoot apex, MSV is present only in the vascular tissues and does not invade the apical meristem. In mature leaves, virus is located only in areas of the leaf displaying the characteristic chlorotic streak symptoms of infection and, in contrast to the situation in apical and stem tissues, it is no longer restricted to the vasculature. Viral coat protein and both positive and negative strands of the DNA genome were found in mesophyll, vascular-associated parenchyma, and bundle sheath cells of the leaf. MSV was not usually found in nonphotosynthetic tissues outside of the vasculature. Localization of both double-stranded viral DNA and transcripts encoding proteins involved in virus replication, identified cells in which virus replication was active. Further spatial comparison with the distribution of transcripts of histone H2b, an S-phase specific gene, implied that host DNA replication was not essential for viral replication.

Additional keywords: symptomatology; virus invasion; histone 2b; cell cycle.

Maize streak virus (MSV) is the type member of subgroup I of the Geminiviridae (Mayo and Martelli 1993) and possesses a monopartite genome of single-stranded (ss), circular DNA. Transcription of the viral genome is bidirectional from a double-stranded (ds) template. Products derived from the virion sense are encoded by two major transcripts (Morris-Krsinich et al. 1985). The virion-sense genes (V1 and V2) are necessary for systemic spread of the virus although they are both dispensable for replication (Boulton et al. 1989a; Lazarowitz et al. 1989); V1 and V2 encode the movement protein and coat protein, respectively (Boulton et al. 1993). By analogy with their counterparts in another subgroup I geminivirus, wheat dwarf virus (WDV), the two complementary-sense genes (C1 and C2) are believed to be essential for viral replication (Schalk et al. 1989). Geminivirus DNA replication is thought to occur in the nuclei via a double-stranded (ds) intermediate using a rolling-circle mechanism (Saunders et al. 1991; Koonin and Ilyina 1992; Laufs et al. 1995). Furthermore, it has been suggested that coincident host cell division may be necessary for geminivirus replication (Townsend 1986). For MSV, virus replication in protoplasts was correlated with the onset of cell division in a small proportion of the population (Boulton et al. 1993). More specifically, Accotto et al. (1993) demonstrated for the related digitaria streak geminivirus (DSV) that cellular DNA replication (the S-phase of mitosis, rather than actual cell division) was important in viral replication. In contrast, however, a geminivirus vector based on a replicon from WDV initiated replication independently of cell division (Timmermans et al. 1992).

MSV is transmitted into the phloem sieve tubes of graminaceous host plants via the leafhopper vector *Cicadulina mbila*. In common with other geminiviruses (Harrison 1985; Francki et al. 1985) subsequent invasion of the plant might be expected to be limited to the phloem-associated cells. However, based on the brief time necessary for virus acquisition by insects (Storey 1938), Francki et al. (1985) suggested that MSV could be an exception to this rule. Aggregates of virions have been seen in the nuclei of a variety of cell types, including bundle sheath and mesophyll cells (Pinner et al. 1993).

The symptoms of MSV on maize plants develop as chlorotic streaks along leaf veins. The development and extent of these symptoms can be correlated with the age of the leaf at the time of inoculation (Peterschmitt et al. 1992) and, in common with many other plant viral infections (see for example, Leisner et al. 1993), appears to be related to phloem translocation and the demands of sink and source tissues for photoassimilate.

To clarify the pattern of tissue invasion shown by MSV and to determine whether MSV is capable of invading the shoot apical meristem, we have correlated host vegetative development with virus distribution using the techniques of immunocytochemistry and in situ hybridization. Our results demonstrate that MSV did not invade the shoot apical meristem of maize, and although a vascular limitation is seen in the stem and shoot-tip, this restriction is lost in the tissues of the mature leaves. Comparison of the distribution of replicative forms of MSV DNA with the expression of the S-phase specific host gene, H2b, also showed that host DNA synthesis is not a prerequisite for MSV replication.

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RESULTS

Following insect transmission of a severe strain of MSV (MSV-Ns) to maize seedlings, severely chlorotic streak symptoms develop in newly emerged leaves. Leaves showing symptoms covering the complete lamina develop after one or two leaves with incomplete symptoms at the basal region of the leaves. The latter reflect the basipetal transition of leaves from photosynthetic sink to source (Fig. 1A). To investigate the extent and specificity of invasion of various organs and tissues of the plant by MSV, tissues harvested from different areas of the plant (Fig. 1B) were embedded, sectioned, and subjected to immunohistochemistry and in situ hybridization using a variety of MSV-specific probes (Table 1). The probes detected either MSV coat protein or MSV nucleic acids. The use of in situ hybridization probes of two polarities permitted the separate detection of total viral DNA and the free double-stranded form of the viral genome associated with viral DNA replication and expression.

Virus distribution in different tissues.

Uninfected plants and plants that were leafhopper-inoculated with MSV and showing complete systemic symptoms of MSV infection were dissected and various tissues analyzed for virus distribution. In all of the infected tissues there was the same tissue distribution of MSV coat protein or MSV nucleic acids.

Longitudinal sections through the shoot apex identified the apical dome, shoot apical meristem, leaf primordia, and several plastochrons of expanding leaves (Fig. 2A). Use of either anti-coat protein serum (data not shown) or MSV in situ hybridization probes showed that the virus was limited to the differentiated vascular tissues and was therefore not present in the apical meristematic region. A similar distribution was evident when reproductive meristems were examined (data not shown). To gain a more complete picture of the distribution in the apex from such two-dimensional images (Fig. 2A), the staining patterns from a complete series of consecutive sections through a shoot apex were integrated, using a computer, into a single image to show the distribution of virus DNA relative to the complex vascular network (Fig. 2B). From this composite image, we deduced that the virus entered the developing leaves approximately five plastochrons removed from the meristem. There was no evidence from these studies for the infection of cell lineages.

In sections taken through nodal (Fig. 2C and D) and internodal (Fig. 2D) regions in the maize stem, MSV was seen to have a similar vascular restriction to that seen in the shoot-tip. Thus, virus was localized along the vascular routes of this region and was never seen in the surrounding parenchyma tissues (Fig. 2C and D). The specific signal was particularly concentrated within vascular traces around the periphery of the stem, adjacent to the region of primary thickening. This pattern was maintained in developing axillary buds (not shown) and adventitious root primordia emerging from these nodes (Fig. 2E). In the latter, virus remained restricted largely to the vascular traces; MSV was never seen to invade the root meristem.

In transverse sections taken through mature adventitious roots of infected plants, virus DNA could be detected only in pairs of phloem sieve tube-associated pericycle cells sur-

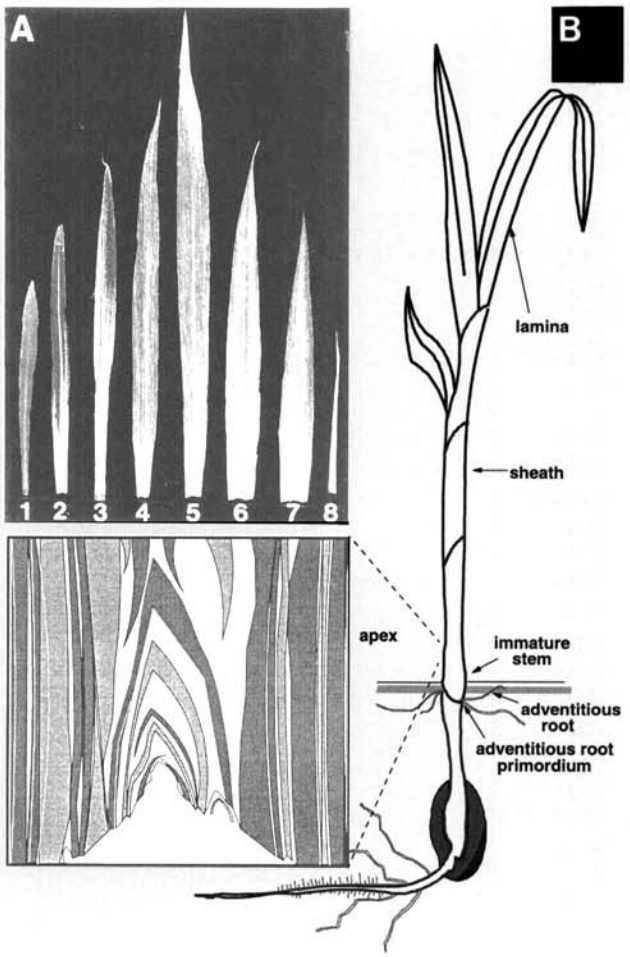


Fig. 1. Analysis of maize streak virus infection of maize. **A,** Streak symptoms on newly formed leaves following insect transmission of the virus to maize seedlings. Leaves 2 and 3 showing symptoms in the basal regions of the leaf were, most likely, in transition from photosynthetic sink to source when the virus arrived in the tissues. Leaf 8 was fully enclosed within the whorl when the infected plant was dissected. **B,** Tissue from the named locations was analyzed by in situ hybridization or immunohistochemistry; inset from the apical region of the plant is included to show general tissue organization in that area.

Table 1. Specificity of the *in situ* hybridization probes

Origin	Clone	Hybridization probe	Specificity
MSV complete genome	pBSMSV10	+ve sense	ds DNA, –ve complementary sense (*rep*) mRNA
		–ve sense	ss DNA, ds DNA, +ve virion sense mRNA
MSV (nt 1-1004)	pBSMSVRH	+ve sense	ds DNA
H2b1 cDNA	pBSH2bs	+ve sense	H2b1 genomic DNA[a]
		–ve sense	H2b1 mRNA H2b1 genomic DNA[a]

[a] Not detectable under the conditions employed.

rounding the vascular cylinder (Fig. 2F). An MSV-specific signal was also detected occasionally in the adjacent endodermal cells.

The progression of MSV into the leaf tissues was followed by examining transverse sections through leaf lamina (Fig. 2G and H) and sheath tissue from mature leaves showing chlorotic symptoms. In these tissues, the MSV DNA was associated with all the classes of vasculature, i.e., midvein (not in the example in Fig. 2G, but frequently observed), large lateral bundles, and the intermediate, small and transverse veins. Within infected vascular bundles, phloem cells (presumably sieve elements) were reproducibly free of virus-specific signal

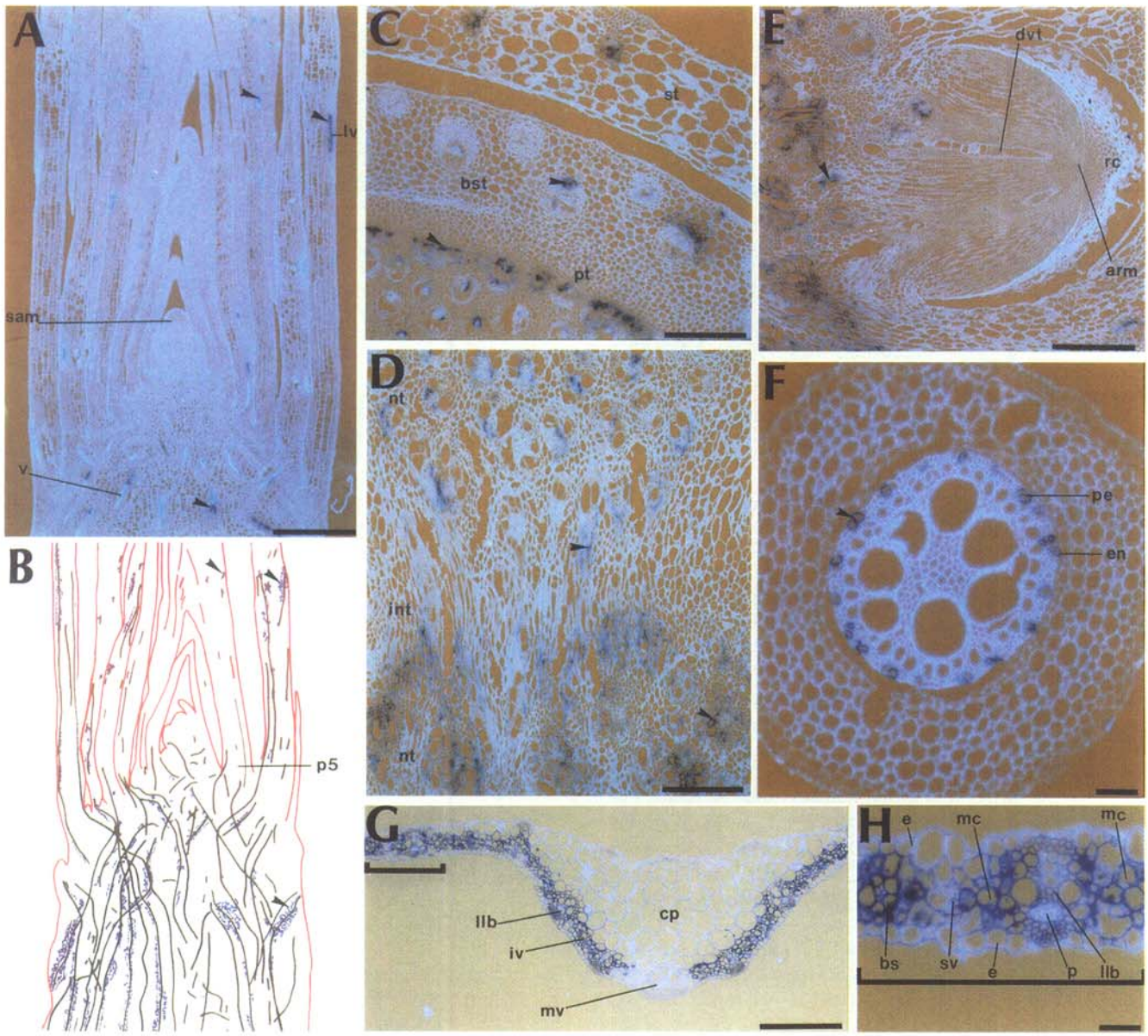


Fig. 2. Localization of maize streak virus (MSV) DNA in different regions of the maize plant. Embedded tissue was sectioned and subjected to in situ hybridization with the full-length –ve sense MSV probe (Table 1). The location of virus infection was shown as dark blue against the light blue fluorescence identifying general tissue organization. In panels **A to F** representative locations of MSV infection are indicated with arrowheads. **A,** Longitudinal median section through the shoot apex showing the shoot apical meristem (sam), surrounding leaf primordia, immature leaves, and the vascular tracery (v) in the developing stem. lv, leaf vasculature. Bar: 500 μm. **B,** A computer-generated integrated composite of the vascular organization (gray) in the shoot apex and the sites of MSV infection (blue and purple spots), in relation to the approximate tissue outline (red lines). This image is the combined data of 17 sections covering approximately 200 μm through the shoot apex. Virus was first detected in leaf tissue at plastochron 5 (p5). **C,** Cross section through the node region of the stem. pt, region of primary thickening; bst, basal sheath tissue; st, sheath tissue. Bar: 500 μm. **D,** Longitudinal section through nodal (nt) and internodal (int) tissues of the stem. Bar: 500 μm. **E,** Longitudinal section through an adventitious root primordium showing the root meristem (arm), root cap (rc), and developing vascular traces (dvt). Bar: 500 μm. **F,** Cross section through an adventitous root showing the virus located in pairs of pericycle cells (pe) and occasionally in the endodermis (en). Bar: 100 μm. **G and H,** Cross section through the midrib and lamina regions of a mature infected maize leaf. **H,** An enlargement of the region in **G** marked with a bracket. MSV can be seen to infect all photosynthetic cell types, including bundle sheath (bs) and mesophyll (mc) cells. The virus appears to be absent from epidermal (e) and colorless parenchyma (cp) cells, and from the phloem elements (p) in infected vascular bundles. mv, midvein; llb, large lateral bundles; iv, intermediate veins; sv, small veins. Bars: **G**, 500 μm; **H**, 100 μm.

(e.g., Fig. 2H) indicating a smaller concentration of translocating virus than in cells in which the virus had multiplied. Surprisingly, and contrary to the suggested vascular limitation of geminiviruses, a wider distribution of virus was seen further into the tissues of the lamina (Fig. 2G and H) and sheath (although the signal was lower in the sheath than in the leaf lamina; see Fig. 3). Indeed, in these regions, and particularly in the lamina, virus was abundant in all photosynthetically active cell types (e.g., bundle sheath and mesophyll cells) but detected rarely in the epidermal cell layers and in the non-photosynthetic packing cells adaxial to the midvein (Fig. 2G). This pattern of detection was maintained in sections taken from all parts of the mature leaf lamina (data not shown). The distribution of the virus was often discontinuous along the cross section of the lamina, reminiscent of the streaked chlorotic symptom seen on intact mature leaves (Fig. 1A).

To assess whether virus accumulation correlated directly with symptom formation, leaf tissue pieces with distinctive symptoms (taken from the region with incomplete symptom formation on an early systemically infected leaf) were photographed and sections of a defined region subjected to in situ hybridization. Comparison of the pattern of virus accumula-

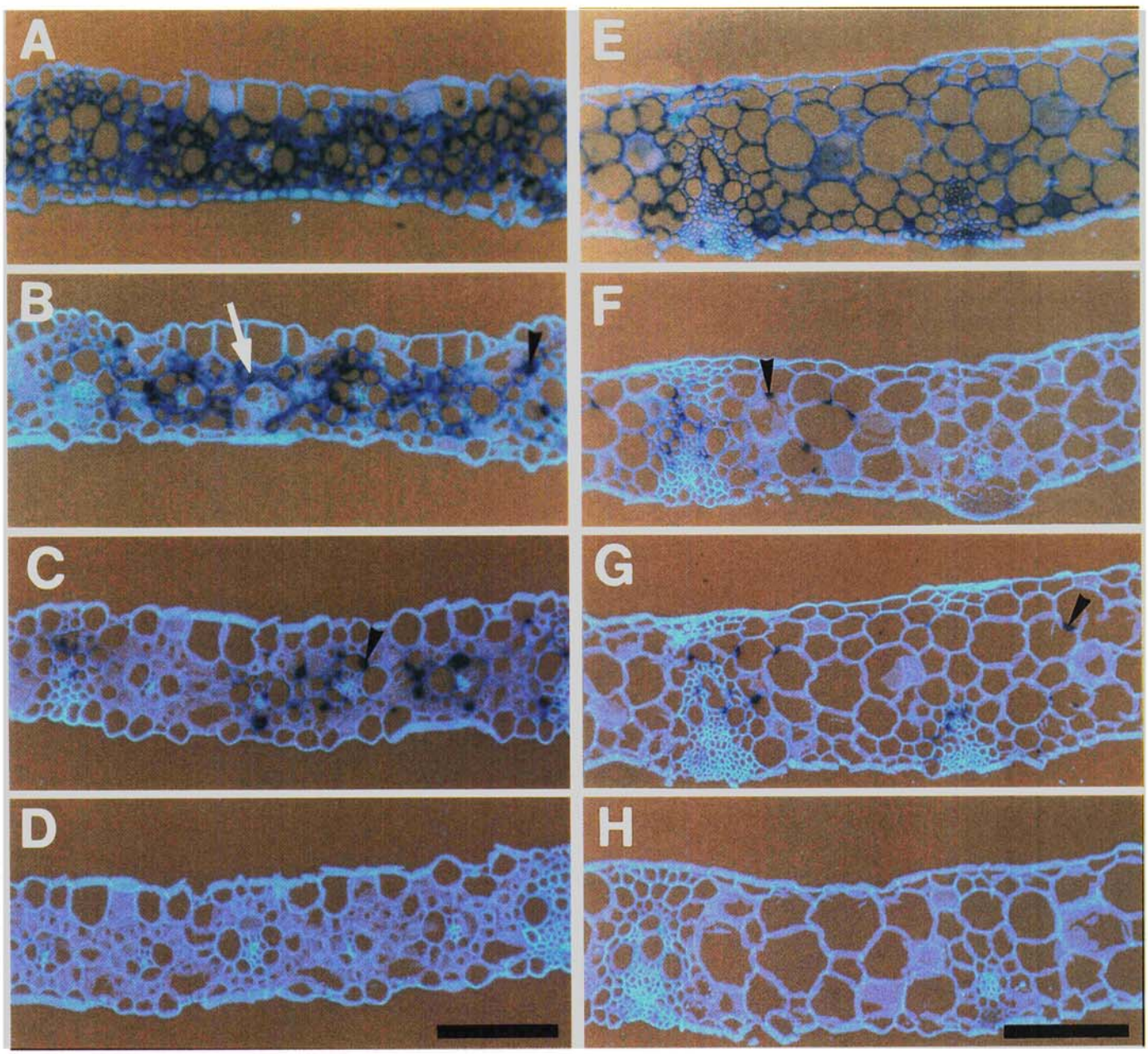


Fig. 3. An assessment of maize streak virus (MSV) replication in leaf and sheath tissue. Consecutive sections of leaf lamina (**A to C**) and sheath (**E to G**) showing nearly complete chlorotic symptoms, and sections of control, uninfected lamina (**D**) and sheath (**H**), were subjected to in situ hybridization with the full-length –ve sense MSV probe (**A, D, E, H**), the full-length +ve sense MSV probe (**B, F**) or the right hand (RH +ve) MSV probe (**C, G**). Accumulation of MSV –ve sense nucleic acids in nuclei (arrowheads) was seen with each +ve sense probe and in both tissue types. In contrast, the accumulation of MSV –ve sense nucleic acids in the cytoplasm (white arrow) was only seen in leaf tissue when using the full-length +ve sense MSV probe. Sections from uninfected tissue showed no hybridization of the probe (**D,H**). Bars: 200 μm.

tion in the section with the chlorotic symptoms on the leaf showed that the two were precisely correlated (Fig. 4).

The distinct difference in the tropism of MSV in the shoot apex (including the young leaves; Fig. 2A) and the mature leaf lamina (Fig. 2G and H) suggested that there was a loss of vascular limitation of MSV during the development of the leaf. To determine the stage at which this occurred, the leaves of a plant showing complete symptoms on all newly emerging leaves were analyzed separately for viral coat protein distribution using immunohistochemistry. All leaves that had emerged from the whorl showed virus accumulation in a broad range of photosynthetic cell types. The first enclosed leaf (e.g.. leaf 8, Fig. 1A) showed the broader distribution only in the distal half, and all smaller leaves showed increasing vascular limitation (data not shown). Leaves showing the broader virus distribution contained thin-walled and thick-walled metaphloem elements in vascular bundles (data not shown) providing an indication of their developmental age.

MSV replication in laminal tissues.

The presence of viral DNA in the mesophyll of mature leaves where cell division would no longer be expected to occur is at odds with the proposed requirement for cell division to be associated with MSV replication. It could be argued that the location of virus-specific protein or nucleic acid outside of the vasculature in the leaf and sheath was a consequence of virus movement without replication. We used three approaches to investigate whether MSV replication occurs in mesophyll cells in the leaf. First, sections of leaf lamina were treated with a full length MSV (+) sense in situ hybridization probe (Table 1) to detect jointly double-stranded (ds) MSV DNA, and complementary sense transcripts specific for the *rep* gene products (Fig. 3B). Comparison of the signal from full-length negative (Fig. 3A) and positive (Fig. 3B) sense probes on consecutive sections showed a similar tissue distribution but a different overall signal intensity and a different subcellular distribution. The (+) sense probe showed a lower signal intensity, present as spots of hybridization (presumably reflecting the presence of ds DNA in nuclei) and diffuse staining in the cytoplasm (Fig. 3B).

In the second approach, we attempted to distinguish between MSV ds DNA and *rep* gene transcripts by nuclease digestion of the sections before hybridization with the full length (+) sense probe. Treatment with either DNase or RNase at high concentration reduced the intensity of the signal after hybridization (data not shown). However, sections subjected to treatment with both enzymes did not reproducibly lose all of the hybridization signal. Such resistance to nuclease digestion, probably attributable to the tissue fixation and embedding procedures, left us unable to interpret the nuclease treatment data meaningfully.

In the third approach, the full-length (+) sense probe was similarly compared with a (+) sense probe corresponding to the virion-sense genes (nt 1 to 1004; Table 1) on the MSV genome. These probes identified, respectively, ds MSV DNA and complementary sense (*rep*) transcripts, and ds MSV DNA alone (Table 1). Hence, the difference between the signals obtained with the two probes should be attributable to the *rep* gene transcripts. When consecutive sections from infected leaf lamina and sheath tissue were analyzed, alternative differential patterns of staining were observed. In sheath tissue, there was little difference between the two (+) sense probes (Fig. 3F and G) suggesting that virus replication was no longer active in these tissues. In contrast, in the laminal tissue, the smaller (+) sense probe identified DNA in nuclei, whereas the full-length (+) sense probe identified a nuclear and cytoplasmic location for MSV nucleic acids (Fig. 3, compare panels B and C). This difference provides strong evidence for the existence of *rep* gene transcripts, and therefore viral DNA replication, in these tissues.

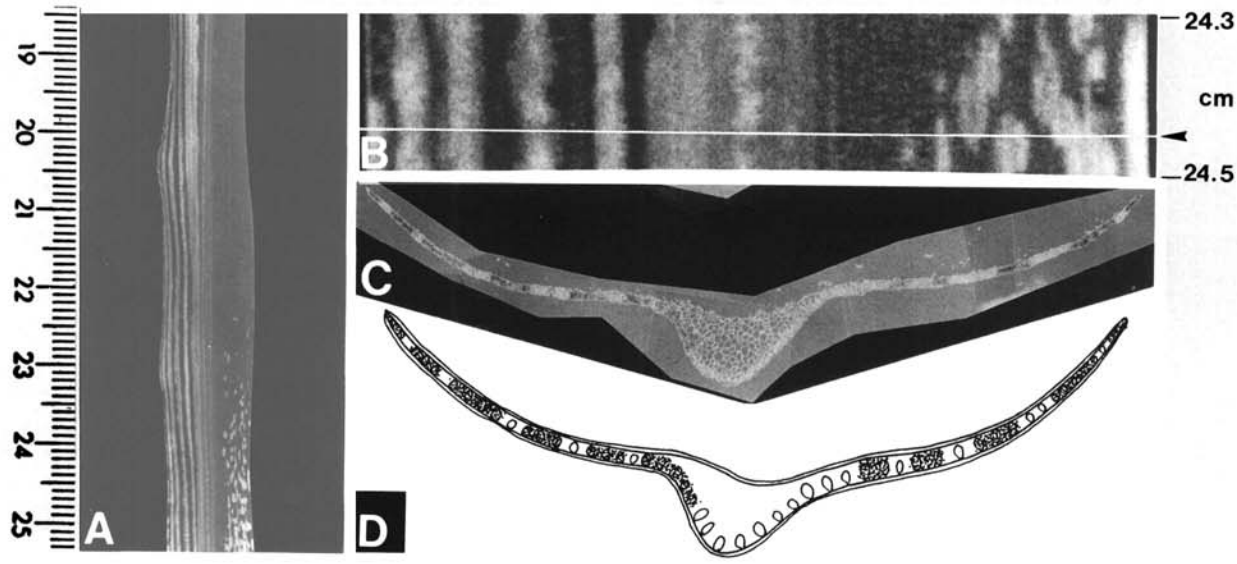


Fig. 4. Correlation between the distribution of maize streak virus (MSV) infection and the chlorotic symptoms on the infected maize leaf. A region of an infected leaf showing intermediate symptoms (**A**) was embedded and sectioned at a specific location (**B**; arrow; leaf photographed from the adaxial surface). The section was subjected to in situ hybridization with the full length –ve sense MSV probe (Table 1) and the complete section photographed (**C**). The location of virus-specific reactions is shown as stippled regions on the adjacent sketch (**D**). The pale region over the midrib in **B** resulted from the non-chlorophyllous cells adaxial to the midvein rather than virus symptoms.

Is the replication of host DNA and MSV DNA spatially correlated?

To investigate the requirement for host cell mitotic activity to be associated with MSV DNA replication, sets of consecutive sections from several different tissues from infected plants were hybridized with virus-specific probes and a probe to the maize histone gene, H2b (Joanin et al. 1992). In common with other histone genes, the expression of this gene is tightly linked with the S-phase of mitosis (Heintz et al. 1983).

The expression of H2b was examined in the tissues of the shoot apex, stem node, adventitious root apex, and the leaf lamina. In all cases, there was no difference in the pattern of H2b expression between healthy and infected tissues (data not shown). Consecutive sections of shoot apical tissues were analyzed for the accumulation of MSV (–) sense nucleic acids (Fig. 5A) and H2b transcripts (Fig. 5B). As before (Fig. 2A), MSV-specific molecules were seen only in vascular-associated cells. H2b expression was observed in cells close to the apical meristem, in leaf primordia and the young expanding leaves until approximately plastochron 8 to 10 (Fig. 5B). Expression was also detected in the developing vascular traces. In the stem nodal tissue, H2b expression was seen in cells (judged to be procambial cells) of the vasculature and in cells of the epidermis (presumably representing periclinal divisions; Fig. 5C). MSV accumulation in this region was again limited to the vasculature (Fig. 2C and D). In an adventitious root primordium (Fig. 5D), H2b expression was evident in the root cap, and all young developing tissues of the root, but particularly the provascular traces. None of these tissues was invaded by MSV (Fig. 2E). H2b expression was not observed in the mature vasculature of the adventitious root where MSV accumulation had been observed (Fig. 2E) previously.

Therefore, for some cell types (e.g., those of the vasculature) there appeared to be a positive correlation between H2b transcript and MSV accumulation while elsewhere MSV was unable to invade tissues most active in cell division. For tissues showing such a positive correlation we did not know whether viral DNA replication was active or had been completed sometime previously. For developing leaves, however, we had shown that in young leaves MSV was invading new sets of cells and that this invasion was associated with the accumulation of replication-specific nucleic acids (Fig. 4). When consecutive sections of such leaf tissues (Fig. 5E to G) were analyzed for H2b expression and viral (+) sense or MSV (–) sense nucleic acids, no H2b transcripts were detected anywhere in the tissue while, as before, (–) sense viral nucleic acids were readily detected. Hence, a correlation between H2b expression and MSV DNA replication could not be established.

DISCUSSION

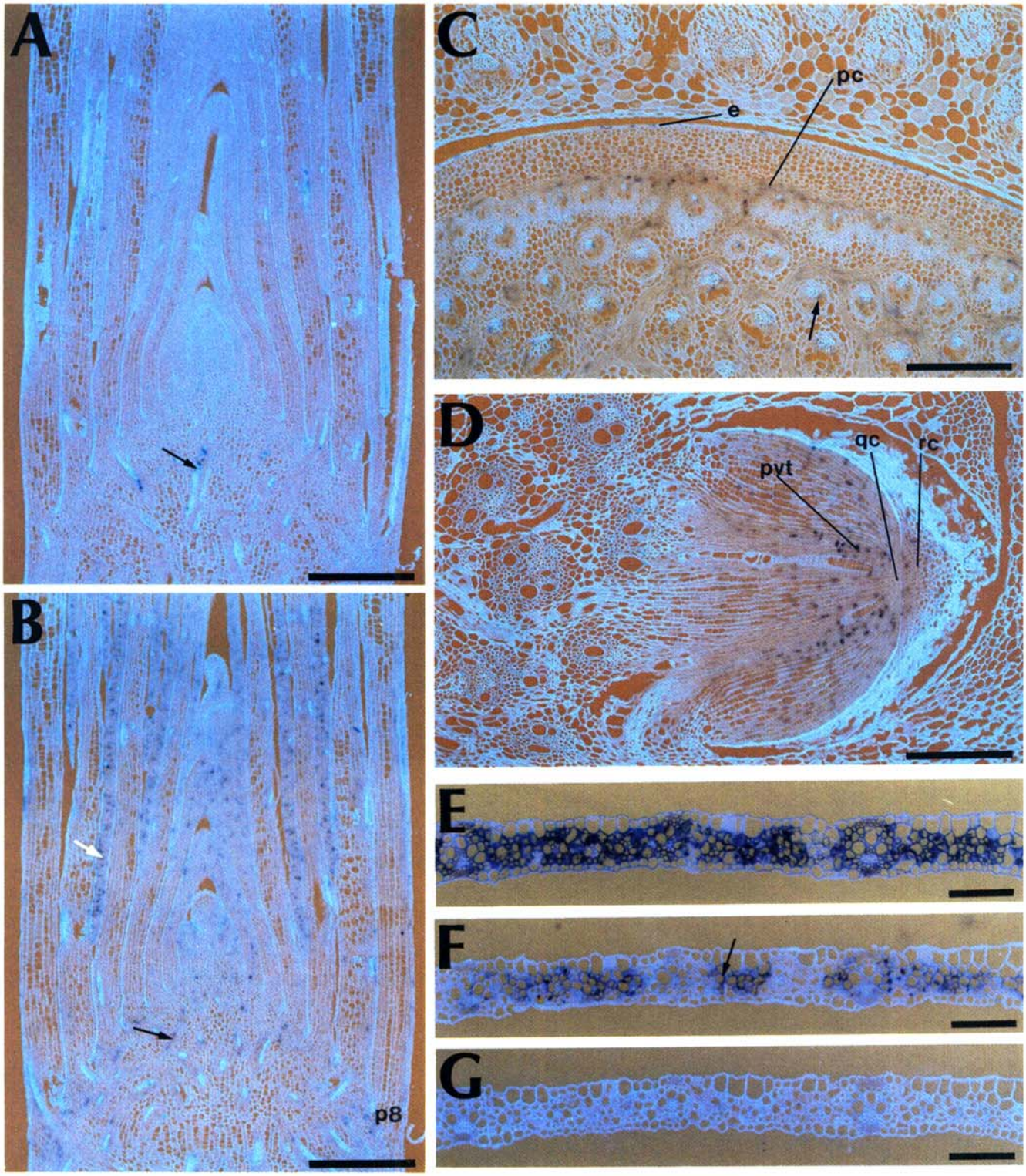
The pattern of accumulation of a virus in a plant host will be the consequence of a range of factors, including the genetic and physiological suitability of particular cell types to support virus replication, the capacity of particular tissues or cell types to mount a defense response, and the existence and nature of translocation pathways between tissues and of symplastic pathways between cells. Our evidence suggests that the first and last of these factors have strong influences in the infection of maize by MSV.

The requirement for host DNA replication to be associated with the replication of MSV DNA was not supported by our experiments using expression of the histone (H2b) gene as an S-phase specific marker, although the pattern of H2b expression in the shoot and root apical regions conformed with that reported for similar markers in other systems (Fobert et al. 1994). The lack of correlation between H2b and MSV accumulation was most notable in the developmentally mature leaf laminal tissues where viral nucleic acids associated with MSV replication could be detected. The difference between this conclusion and previously published reports (Boulton et al. 1993; Accotto et al. 1993) could reflect the difference in experimental systems and that a factor(s) associated with dividing cells, rather than cell division itself, was required for virus replication. Recently, colocalization of tomato golden mosaic geminivirus *rep* protein (TGMV AL1) and proliferating cell nuclear antigen (PCNA) in host cells was reported (Nagar et al. 1995). PCNA is an auxillary factor for DNA polymerase Δ functioning during mitosis but is also a factor required for DNA excision repair (Shivji et al. 1992). Certainly, it is the current popular hypothesis that geminivirus replication takes advantage of host DNA polymerases but the precise factors involved have yet to be characterized. Whether these factors are limiting for the initiation of infection or can be induced following infection, is also not yet known. The latter may apply for PCNA since it is induced in terminally differentiated cells expressing TGMV AL1 (Nagar et al. 1995).

The highly defined pattern of chlorotic streak symptoms seen on infected mature leaves is reminiscent of clonal sectoring suggesting that the virus may be propagated at or very close to the meristematic apex. Our sections of the shoot apex show that this is not true as the virus is restricted to the developed vasculature, and there was no evidence for the infection of cell lineages (unpublished observations). Presumably, the absence of vascular routes and high division rate of cells around the meristem serve to exclude virus from that area. The streak symptoms on leaves are also suggestive of a vascular association of the virus. In situ hybridization was used to show that a bipartite geminivirus, abutilon mosaic virus, was limited to vascular tissues in infected leaves of *Abutilon* even though virus-induced symptoms were seen all over the leaf lamina (Horns and Jeske 1991). In contrast, immunofluorescence staining showed that another bipartite geminivirus, African cassava mosaic virus, was present beyond the vasculature, in epidermal and cortical cells of stems of *Nicotiana benthamiana* (Sequeira and Harrison 1982). Similarly, MSV showed extensive spread beyond the bundle sheath cells in the maize lamina. Indeed, the chlorotic symptom of broad and narrow streaks across the leaf lamina was a precise reflection of the location of the virus. A similar broad distribution of infection has also been seen for another monopartite geminivirus (tomato yellow leaf curl virus) in a dicot host (I. Michelson and H. Czosnek, personal communication).

To understand the overall pattern of MSV accumulation it is necessary to appreciate aspects of maize development (Sharman 1942; Esau 1943; Kumazawa 1961; Freeling 1992), and the importance of plasmodesmal connections for virus movement (Maule 1991; Lucas and Gilbertson 1994). During maize leaf development there is continuous acropetal development of the midvein and large lateral bundles, maintaining

vascular continuity with the nodal network of the stem and providing routes for virus entry into the leaf at, or around, plastochron five. Early in leaf development there is a parallel but opposite basipetal development of an alternative network of intermediate and small veins developing in partial continuity with the former network and interconnected by small transverse veins (Evert et al. 1977; Evert et al. 1978; Fritz et al. 1983). Initially, the large veins use protophloem for systemic translocation but these structures are destroyed during subsequent development (Esau 1943). The protophloem is replaced by thin-walled metaphloem elements, developing basipetally, which are believed to be important in long-distance translocation (Fritz et al. 1983). These latter structures are also present in the second network of veins and are



intimately associated with their adjacent companion cells. These two-cell complexes have few plasmodesmal connections with any surrounding cell types (Fritz et al. 1983), and probably load photoassimilate apoplastically (Evert et al. 1977, 1978). Additionally, the second network of veins contains thick-walled metaphloem complexes with abundant plasmodesmal connections to facilitate symplastic loading of photoassimilate. The development of metaphloem elements only occurs after the leaf has completed its elongation by cell division (Esau 1943), presumably after plastochron 12 (Fig. 5) and this complete pathway for phloem loading must be in place by the time the leaf makes the transition from photosynthetic sink to source, providing maximal opportunity for the export of photosynthate. Metaphloem elements are visible in leaves within the whorl in which virus escape from the vasculature can first be observed and hence, the virus may be able to exploit the abundance of symplastic connections associated with these cells. However, MSV was also seen frequently in the midvein and large lateral bundles and, hence, we must assume that there are sufficient plasmodesmata in the thin-walled phloem complexes to allow virus to escape into the surrounding parenchyma and further.

This distinction in metaphloem function is not apparent in root tissue, all phloem/companion cell complexes being abundantly symplastically connected to surrounding cell types (Warmbrodt 1985) and, yet, MSV shows a tighter tissue limitation here than in leaves. In leaf tissues, MSV was preferentially located in photosynthetic cells, rarely invading epidermal cells or the parenchymatous tissue adjacent to the midvein even though there is symplastic continuity between these cell types (Esau 1965; Evert et al. 1977). The correlation with photosynthesis in cells outside of the vasculature indicates a physiological requirement unable to be supplied by the cortical cells of the root and stem. Clearly, this requires further investigation.

Generally, these observations of MSV invasion of host tissue emphasize the importance of considering host physiology and patterns of host development when attempting to understand the progression of disease.

MATERIALS AND METHODS

Growth and inoculation of plants.

Zea mays (cv. Golden Bantam × Yellow Hybrid) was grown and maintained as described by Boulton et al. (1989b). Maize seedlings with two emergent leaves (visible to the ligule) were infected by insect transmission (Pinner et al. 1988) with the severe Nigerian strain of MSV (MSV-Ns; Boulton et al. 1991) or left uninfected for parallel processing as healthy, control plants. The virus was transmitted from the symptomatic leaves of agroinoculated maize seedlings (Boulton et al. 1989b). Infected and control plants were maintained until symptoms had appeared on all newly emerging leaves (approximately 2 to 3 weeks).

Tissue preparation.

Tissues for both immunocytochemistry and in situ hybridization were fixed, embedded (Paraplast X-tra, Sigma) and used essentially as described by Jackson (1991), with the following modifications: (i) Tissues were fixed in FAA (5% formalin, 5% glacial acetic acid, 45% methanol, (ii) post-hybridization washes were buffered with saline sodium phosphate (1× SSPE: 15 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), (iii) digoxigenin-labeled probes were used at a concentration of 0.2 ng μl^{-1} per kilobase of target sequence. Hybridized probes were detected as described by Coen et al. (1990), again with some modifications: (i) 0.3% Triton X-100 was replaced with 1% polyoxyethylene sorbitan monolaurate (Tween-20; Sigma), (ii) two additional washes in 50 mM Tris-Cl, 75 mM NaCl, 1% Tween-20 (pH 7.5) were used to remove unbound anti-digoxigenin antibody, (iii) color development was conducted overnight at +4°C in a solution containing 10% polyvinyl alcohol as described by Block and Debrouwer (1993) and stopped by sequential washes in 1× PBSE (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM EDTA; pH 7.0). Sections were dehydrated then rehydrated through an ascending and descending ethanol series, and counterstained in 0.1% Calcofluor (Sigma). The slides were finally rinsed in distilled water, mounted in Entellan (Merck) and photographed using combined epifluorescence (UV; Calcofluor staining of cell walls) and transmitted light microscopy.

For the nuclease digestion of tissue sections prior to hybridization, pronase-digested sections (Jackson 1991) were washed extensively in PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.0) and treated with either DNase-free RNase at 20 $\mu g\ ml^{-1}$ in PBSE or RNase-free DNase at 25 $\mu g\ ml^{-1}$ in a buffer containing 50 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$ and 20 U ml^{-1} RNA-guard (Pharmacia). Control sections were treated with both enzymes in 50 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$. Sections were incubated with the nucleases at 37°C for 90 min before being treated again with pronase and further processed for in situ hybridization.

Immunocytochemistry.

Antibodies were raised in rabbits against whole MSV virions, and cross-absorbed against sap extracts from healthy maize (Boulton et al. 1989a).

Fig. 5. S-Phase specific accumulation of maize H2b transcripts in relation to the sites of maize streak virus (MSV) replication. Sections from the shoot apex (**A, B**), stem (**C**), adventitious root primordium (**D**) and leaf lamina (**E to G**) were subjected to in situ hybridization with a probe to H2b (**B to D, G**), the full-length –ve MSV probe (**E**) or the full-length +ve MSV probe (**A, F**). **A,** Longitudinal section through the shoot apex. MSV –ve sense nucleic acids were restricted to the developing vasculature (arrow) of the shoot apex. Bar: 500 μm. **B,** Longitudinal section through the shoot apex (**A, B** and Fig. 2A are near-consecutive median sections). H2b expression was similarly detected in cells of the vasculature (arrow) of the immature stem and leaves but was also seen in the nonvascular cells (white arrow) of young leaves up to plastochron 8 (p8) to 10. Bar: 500 μm. **C,** Cross section of stem nodal tissue. H2b expression was observed in procambial cells (pc; indistinguishable in these sections from the location of MSV in Fig. 2C) and in the expanding stem epidermis (e). H2b expression was also seen in the vascular bundles (arrow). Bar: 500 μm. **D,** Longitudinal section through an adventitious root primordium (near-consecutive section with Fig. 2E). H2b expression was most prevalent in the root cap (rc) and the developing provascular traces (pvt). qc, quiescent center. Bar: 500 μm. **E to F,** Near-consecutive sections of young leaf tissue (equivalent to leaf 7 in Fig. 1A) showing full chlorotic symptoms. MSV +ve sense nucleic acids (**E**) were present in all photosynthetic cell types. MSV DNA replication, as revealed by the presence of extranuclear location for MSV –ve sense nucleic acids (**F**, arrow; see Fig. 4), appeared to be active in some of these cells. H2b expression (**G**) could not be detected at all in leaves at this stage in development. Bars: 200 μm.

Sections were rehydrated in 1× PBSE before being transferred to a blocking solution (A) consisting of 3% dried milk powder, 2% bovine serum albumen (BSA), 1% Tween-20 in 1× PBSE. Following overnight incubation at +4°C, sections were treated for 3 h at room temperature with the cross-absorbed antiserum at a dilution of 1:500 in 2% BSA and 1% Tween-20 in 1×PBSE (Buffer B). Excess antibody was removed by washing in buffer A, three times in 1× PBSE, 1% Tween-20, and twice in 1/2× PBSE, 1% Tween-20. For colorimetric detection the sections were incubated for 2 h at room temperature with a protein-A-alkaline phosphatase conjugate (Sigma) diluted 1:1,000 in buffer B, washed as before and immediately assayed for bound alkaline-phosphatase activity according to standard protocols.

Generation of probes for *in situ* hybridization.

Standard molecular biology procedures (Sambrook et al. 1989) were used throughout. A clone of MSV DNA (pBSMSV10) contained a full-length *Bam*HI insert of the MSV-Ns sequence flanked by T3 and T7 transcriptional promoter sequences in pBS- (Stratagene). To construct the clone pBSMSVRH containing MSV DNA nucleotides 1 to 1004 (i.e., right-hand half of the circular genome) approximately corresponding to the virion-sense genes of the virus, pBSMSV10 DNA was cut with *Bam*HI and *Nco*I and the approximately 1-kb fragment inserted into similarly cut pBS-. A 459-bp *Bam*HI to *Eco*RI fragment from a cDNA clone of the maize histone (H2b1) mRNA (Joanin et al. 1992) was subcloned into similarly cut pBS- to give pBSH2b.

For the transcription reactions, cloned DNA was linearized downstream of the inserted sequence at a site chosen to leave a 5′ overhang, thus avoiding the transcription of undesired plasmid sequences. Using these templates, positive and negative sense probes for in situ hybridization were labeled with digoxigenin-11-rUTP using standard in vitro transcription conditions (Jackson 1991). Carbonate-hydrolyzed transcript probes were stored in small aliquots in 1× TE in the presence of 200 U ml^{-1} RNA-guard (Pharmacia) at –20°C.

The specificity of the probes is listed in Table 1. Positive sense (control) probes for H2b1 sequences never gave a positive hybridization.

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