Detection and Distribution of Sweetpotato Feathery Mottle Virus in Sweetpotato by In Vitro-Transcribed RNA Probes (Riboprobes), Membrane Immunobinding Assay, and Direct Blotting

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ABSTRACT

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An in vitro-transcribed RNA probe (riboprobe) system was developed to detect sweetpotato feathery mottle virus (SPFMV) in infected plants. The essential components of the system include selection of an SPFMV cDNA clone that reacts with all known strains of SPFMV, optimization of assay procedures, and modified hybridization conditions. Additionally, a direct blotting technique on nitrocellulose membrane was developed to detect SPFMV by either riboprobe or membrane immunobinding assay (MIBA). The riboprobe system provided greater sensitivity of detection of virus in symptomless tissues than MIBA. Reconstitution experiments showed that the limit of detection of SPFMV using the riboprobe was 0.128 pg of RNA per sample, whereas MIBA only detected 170 pg of capsid protein per sample. Experiments that measured the accumulation of viral RNA and capsid protein of SPFMV in naturally infected sweetpotato plants cv. Jewel demonstrated that the riboprobe assay was nearly as effective as grafting to detect the virus, whereas the MIBA was only effective in symptomatic leaves.

Additional keywords: rapid diagnosis, sweetpotato viruses.

Sweetpotato feathery mottle virus (SPFMV), a potyvirus (20), is the most widespread virus known to infect sweetpotato, *Ipomoea batatas* (L.) Lam. (23). Virions of SPFMV are 810–815 nm in length (6,7) with a 38-K capsid protein that encapsidates a positive-sense, polyadenylated single-stranded RNA (ssRNA) of M_r 3.65×10^6 (20). In addition, the SPFMV double-stranded (ds) RNA is purified as one species twice the size of the ssRNA (J. A. Abad, *unpublished results*).

SPFMV-infected sweetpotato plants exhibit a variety of chlorotic patterns on leaves. In most cultivars, the symptoms are relatively mild and transient. Some cultivars also exhibit necrotic symptoms on the surface (russet crack) or interior (internal cork) of roots (6,7,23). Co-infection of sweetpotato by SPFMV and other viruses is also common (14,29). These diseases are frequently more severe than the syndrome resulting from infection by SPFMV alone.

Four strains of SPFMV have been described; the common (C) strain that is limited to convolvulaceae induces only foliar symptoms in sweetpotato and does not infect *Chenopodium amaranticolor* Coste & Reyn. (22). The russet crack (RC) strain induces lesions on roots of some sweetpotato cultivars and produces local lesions in *C. amaranticolor* (6,7). The yellow vein (YV) strain induces severe symptoms in *Ipomoea nil* (L.) Roth, and strain 835 infects *Nicotiana benthamiana* Domin. (18). Strains RC, YV, and 835 are closely related serologically, whereas SPFMV C is distantly related (J. W. Moyer, *unpublished results*). SPFMV is transmitted in a nonpersistent, noncirculative manner by aphids (10), and the probability of seed transmission is very low (33). Cylindrical inclusion proteins ("pinwheels"), typical of potyvirus, are produced in the cytoplasm of SPFMV-infected plants (13).

An improved system is needed for the detection and refined diagnosis of SPFMV. Even though serological procedures have been developed, they are not widely accepted. The low concentration and irregular distribution of SPFMV in sweetpotato are frequently cited as obstacles to reliable use of these tests (5,8,21). Another factor is the presence of high concentrations of phenolics, latex, and inhibitors in sweetpotato tissue that adversely affect reagents used in these tests. A reliable and sensitive detection method for SPFMV that overcomes host factors could serve as a model for other sweetpotato viruses. The recent evidence of worldwide interest in sweetpotato as an important food crop has precipitated a dramatic increase in the international exchange of sweetpotato germ plasm and thus the necessity to improve diagnostic methods for SPFMV as well as other viruses that infect sweetpotatoes.

In this research, a method is described for detection of SPFMV capsid protein and RNA by direct blotting. Development of the system included the identification of a recombinant complementary DNA clone (cDNA) that recognizes all known strains to serve as a template for the synthesis of a riboprobe to detect viral sense, SPFMV RNA. Relative sensitivity of serological and hybridization assays were then compared to the sensitivity of grafting. The probes were also used to determine the distribution of SPFMV in infected sweetpotato tissues.

MATERIALS AND METHODS

Virus strains maintenance and inoculations. The RC (6,7), YV (18), and C (22) strains of SPFMV were isolated in the United States. These isolates were propagated in *I. nil.* An additional isolate from Guatemala (strain 835) (18) was propagated in *N. benthamiana* at North Carolina State University in accordance with an APHIS Departmental permit 59048. The identity of each strain was verified by local lesion production in *C. amaranticolor*, systemic infection of *I. nil,* or *N. benthamiana* and serology. SPFMV-infected plants were mechanically inoculated and grown in a greenhouse for maintenance and purification. Supplemental lighting was provided during the winter months. Mechanical transmission was by homogenizing infected tissue in a chilled mortar containing 0.1 M potassium phosphate buffer, pH 7.5, and rubbed onto leaves previously dusted with Carborundum.

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Sweetpotato plants of cv. Jewel tested free of SPFMV (21), and screened sweetpotato plants naturally infected with SPFMV-RC were also grown in a greenhouse.

Purification of virions and viral RNA. Virions of SPFMV strains RC and C were purified from infected *I. nil* plants according to the method described by Cali and Moyer (6). Viral RNA was isolated from purified virions as described by Moyer and Cali (20). Total RNA from healthy and SPFMV-infected plants were extracted using a method described by Ausubel et al (3), and dsRNA of SPFMV was extracted as described by Morris and Dodds (17).

Cloning and construction of an in vitro transcription system plasmid. Double-stranded cDNA was synthesized from SPFMV-RC RNA and cloned into pBR-322 according to Maniatis et al (15). A 2.0-kb clone was selected after screening the pBR-322 library and subcloned into pGem-4Z (Promega Corp., Madison, WI), an in vitro transcription vector (24,26), generating the plasmid pGFMRC4.20. This plasmid was propagated in competent DH-5α *Escherichia coli* cells. Recombinant clones were selected by ampicillin resistance with negative β-galactosidase activity and northern blot analysis of SPFMV RNA.

Synthesis of riboprobes and hybridization conditions. The in vitro transcription recombinant plasmid, pGFMRC4.20, was purified by CsCl ultracentrifugation and used as a template for riboprobe synthesis. The plasmid was linearized with *Sma*I, and the riboprobe was synthesized by the manufacturer's directions (Fig. 1) (Promega).

Prehybridization was carried out at 42 C for 3 h in 50% formamide, 50 mM $NaPO_4$, pH 6.0, 5× SSC (3 M NaCl and 0.3 M trisodium citrate), 1% dextran sulphate, and 10 μg/ml denatured salmon sperm DNA. Fresh hybridization solution containing the riboprobe (10^3–10^4 cpm/ml of solution) was added to the membranes and incubated at 42 C for 20 h. Membranes were then washed twice in 2× SSC for 5 min at room temperature, twice with 0.1× SSC containing 1% sodium dodecyl sulfate (SDS) at 65 C for 30 min, once with 2× SSC containing RNase A (2 μg/ml) at room temperature for 20 min, twice with 0.1× SSC at room temperature for 20 min, and twice with 0.1× SSC at room temperature for 5 min. Autoradiographic exposure of the membranes with Kodak X-ray film was at −80 C for at least 24 h using intensifying screens.

Sample preparation. Several extraction buffers previously reported for nucleic acid hybridization detection of plant virus or viroids (3,4,12,16,27,31) were previously compared (1), and the best results were consistently obtained with 10× SSC as extraction buffer. Two disks, one from each half of the leaf, with a total weight of about 15 mg were ground in 135 μl of 10× SSC in microfuge tubes and centrifuged at room temperature for 1 min at 10 g. The supernatant (20 μl) was blotted onto nitrocellulose membranes previously soaked in diethyl pyrocarbonate (DEPC)-treated water for 5 min and 10× SSC for 10 min using a Minifold II slot-blotter system (Schleicher & Schuell, Keene, NH) with 10× SSC prewetted Whatman 3 paper as a backing. Nitrocellulose membranes were then dried at room temperature for 20 min, baked for 90 min at 80 C under vacuum, and incubated in sealed plastic bags for prehybridization.

Reconstitution experiments. To quantify the relative amount of detectable RNA and capsid protein, the sap of naturally infected sweetpotato leaves (cv. Jewel) was diluted in similarly prepared extracts from healthy plants. Known amounts of purified SPFMV-RC RNA and virus diluted 1:100 in "healthy" sap extracted with 10× SSC and 2× TBS (1× = 0.05 M Tris HCl, 0.5 M NaCl, pH 7.5), respectively, were used as positive controls. Similarly prepared extracts from healthy plants were used as negative controls. Twenty- and 50-μl samples were slot-blotted on nitrocellulose for hybridization with riboprobes and membrane immunobinding assay (MIBA), respectively. The relative amounts of detectable viral RNA or capsid protein were estimated by scanning hybridization signals on X-ray films and the negative films of the MIBA pictures in a soft laser scanning densitometer (Biomed Instruments Inc., Fullerton, CA).

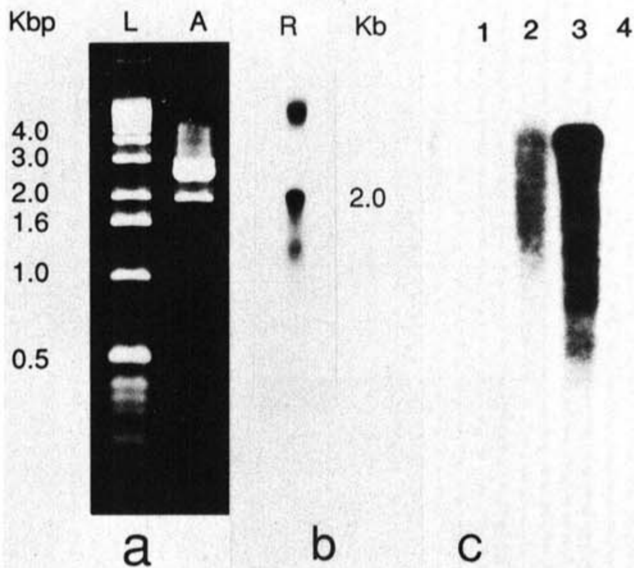


Fig. 1. Characterization of sweetpotato feathery mottle virus (SPFMV) cDNA template, riboprobe, and riboprobe specificity. **A,** agarose electrophoresis of SPFMV strain RC recombinant cDNA. Lane L is ladder of DNA size standards. Lane A is pGFMRC4.20 digested with *Pst*I. **B,** autoradiographic exposure of a previously dried agarose gel containing ^{32}P-labeled riboprobe synthesized from the template in Figure 1A. **C,** northern blot analysis using riboprobe to detect SPFMV RNA in purified virion and total RNA extract. Lane 1 contains total RNA extracted from a "healthy" *I. nil* plant. Lane 2 contains RNA extracted from SPFMV-RC-infected *I. nil*. Purified SPFMV-RC RNA is in lane 3. Lane 4 contains purified potato virus Y (PVY) RNA. The riboprobe (1 × 10^4 cpm/ml) used was obtained from pGFMRC4.20 digested with *Sma*I.

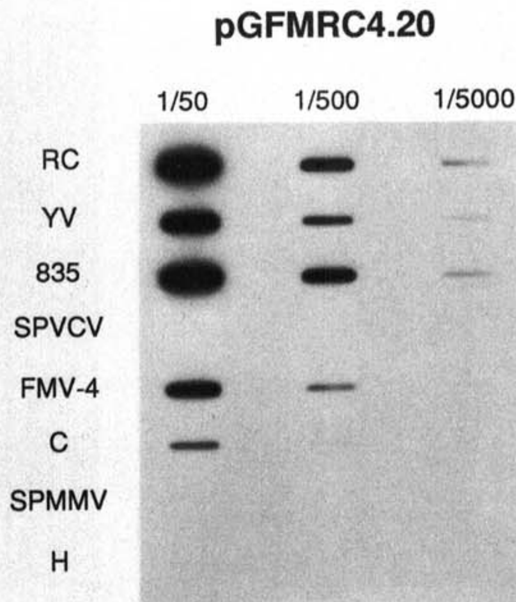


Fig. 2. Detection of different strains of sweetpotato feathery mottle virus (SPFMV) by riboprobe hybridization. Serial dilutions (1:50, 1:500, 1:5,000) of extracts from symptomatic plants infected with SPFMV strains RC, YV, 835, C, and the FMV-4 isolate from Guatemala, and healthy *I. nil* (H) were probed with the riboprobe pGFMRC4.20. Plants infected with sweetpotato vein clearing virus (SPVCV) (11), and sweetpotato mild mottle virus (SPMMV) (23) were used as negative controls.

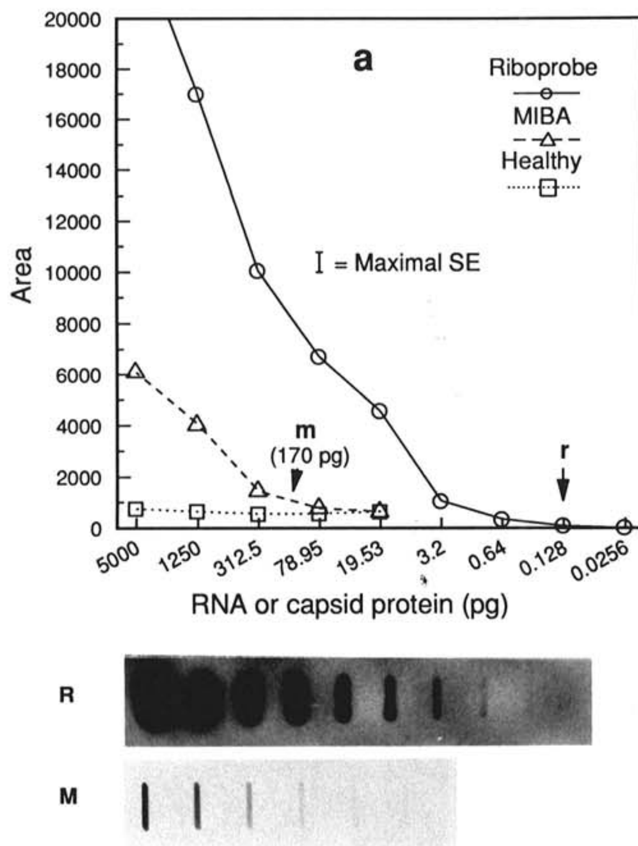
Serology, MIBA. Antisera were prepared against the four strains of SPFMV as previously described (19). Conjugated goat antirabbit alkaline phosphatase was purchased from Bio-Rad (Richmond, CA).

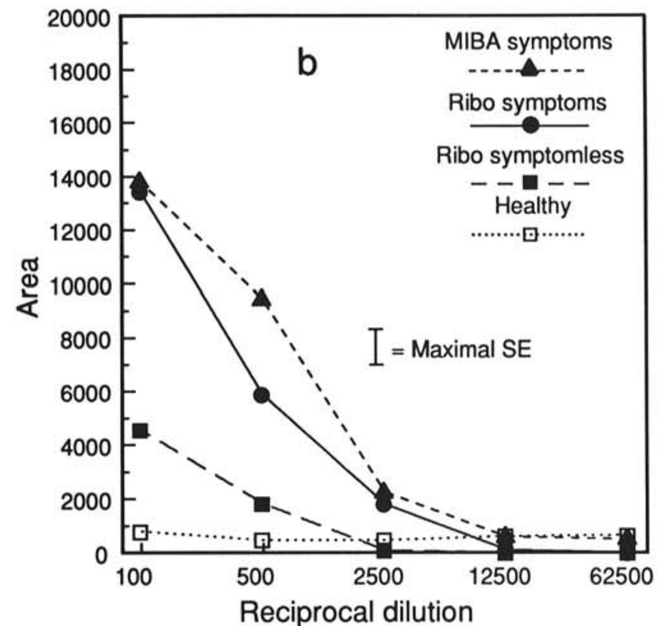
MIBA was performed as a procedure similar to those described before (25,32) with some modifications. Two leaf disks (about 15 mg), 135 μl of TBS buffer, 0.1 M DIECA, and 150 μl of chloroform were homogenized in a tissue grinder (final tissue/buffer; 1:5). After a 2-min centrifugation at 10 g, the supernatant was further diluted 1:10 in TBS buffer. The clarified extract (1:50 final dilution) was blotted, 100 μl per well, onto a buffer-saturated nitrocellulose membrane using a Minifold I microsample filtration manifold (Schleicher & Schuell). Nitrocellulose membranes were subsequently rinsed with deionized water and allowed to dry at room temperature. The membranes were blocked in 2% powdered lowfat milk in TBS for 1 h at room temperature with gentle agitation. The blocking solution was decanted, and the membranes were rinsed with deionized water three times for 15 s each. The first antibody diluted in blocking solution (1:1,000 from 1 mg/ml) was added to the membranes and incubated 2 h at room temperature. The membranes were rinsed with deionized water as before. The second antibody (conjugated goat-antirabbit enzyme) diluted 1:3,000 in fresh blocking solution was added and incubated for 1 h at room temperature. The membranes were rinsed as before, the presence of bound antibody was visualized by the addition of substrate. The substrate consisted of 5 mg of 5-bromo-3-chloro indolyl phosphatase dissolved in 100 μl of dimethyl formamide diluted in 30 ml of substrate buffer (0.1 M Tris HCl, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5) containing 10 mg of nitrobenzene-tetrazolium previously warmed at 37 C. Reactions were terminated after 10 min by washing the membranes with tap water. Then, residual green color was bleached by soaking the membranes in 10% chlorox for about 1 min followed by a tap water rinse.

Direct blotting. Each of the previous methods involved sample preparation protocols specifically designed for serological or hybridization assays. Direct blotting of tissue sap has been used for detection of other antigens (14). For the sweetpotato assays, petioles or rolled leaves were cut with a razor blade. The freshly cut tissue surfaces were immediately pressed directly onto dried and previously buffer-saturated nitrocellulose membranes. The presence of viral protein or RNA was determined by the previously described procedures for either MIBA or hybridizations.

RESULTS

Clone analysis. The viral complementary RNA probe was synthesized from pGFMRC4.20 linearized with *Sma*I. Synthesis was initiated at the T-7 promoter. Probes with specific radioactivity of 4×10^8 cpm/μg were usually obtained. In preliminary experiments, probes generated from the opposite strand of pGFMRC4.20 only hybridized with SPFMV dsRNA and not with purified SPFMV RNA. The viral complementary riboprobe





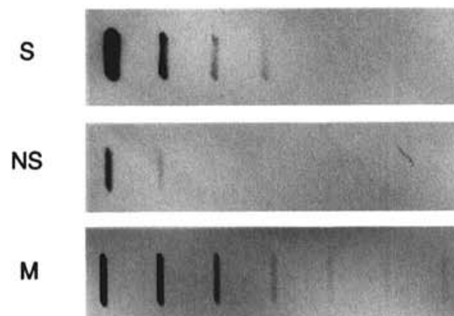


Fig. 3. The relative sensitivity of riboprobe hybridization and membrane immunobinding assays (MIBA) for detecting sweetpotato feathery mottle virus (SPFMV) RNA and capsid protein in sweetpotato tissues. **A,** the limits of detection of SPFMV RNA and capsid protein were determined by scanning densitometry of exposed X-ray film (R = riboprobe ○) and photographic images of immunoblots (M = MIBA △), respectively. Healthy sweetpotato tissue assayed by MIBA (□) is presented, but when assayed by riboprobe no signal was detected. **B,** the relative concentrations of detectable SPFMV capsid protein and RNA in naturally infected sweetpotato plants. Leaves on healthy plants and symptomless leaves on infected plants gave similar responses by MIBA (□). SPFMV was detected in symptomless (■) and symptomatic (●) leaves by riboprobe, and in symptomatic tissue (△) by MIBA. Characteristic riboprobe blots of symptomatic (S) and symptomless (NS) leaves and a MIBA blot of a symptomatic (M) leaf are shown below the Figure. Dilutions on the abscissa axis are dilutions (weight/volume) of the sweetpotato tissue.

synthesized from pGFMRC4.20 detected all five SPFMV isolates (RC, YV, 835, C, and FMV-4) from extracts of symptomatic leaves of *I. nil* by slot-blot hybridization (Fig. 2). The signals from RC, YV, 835, and FMV-4 (an isolate of SPFMV from Guatemala) probed with the riboprobe were of similar intensity, whereas C consistently gave a much weaker hybridization signal suggesting a lower homology in nucleotide sequence. The riboprobe did not hybridize with the other viruses or with healthy tissue.

Sensitivity of the riboprobe versus MIBA. The sensitivity of the two assay systems was compared by adding known amounts of virus or viral RNA to extracts of healthy sweetpotato tissue. The limits of sensitivity were based on comparisons of densitometric scans (expressed as dimensionless units of area) of a range of known amounts of virus or RNA in healthy sweetpotato extracts to blots of healthy tissue. Concentrations were adjusted using healthy sweetpotato extracts. Examples of the riboprobe and MIBA blots are at the bottom of Figure 3. The density readings of the healthy tissues were zero for the riboprobe and between 402 and 975 for the MIBA. Using two standard deviations greater than the mean for healthy tissue as statistical criteria, the MIBA could detect as little as 170 pg of capsid protein (area = 1,220) per sample, and the riboprobe could detect as little as 0.128 pg of RNA (area = 185) (Fig. 3A). This estimate corresponds to 179 pg and 2.56 pg of whole virus for the MIBA and riboprobe, respectively, when the values are corrected to reflect the relative amounts of virus and RNA per virion. This represents a 60-fold increase in sensitivity of the riboprobe over the MIBA.

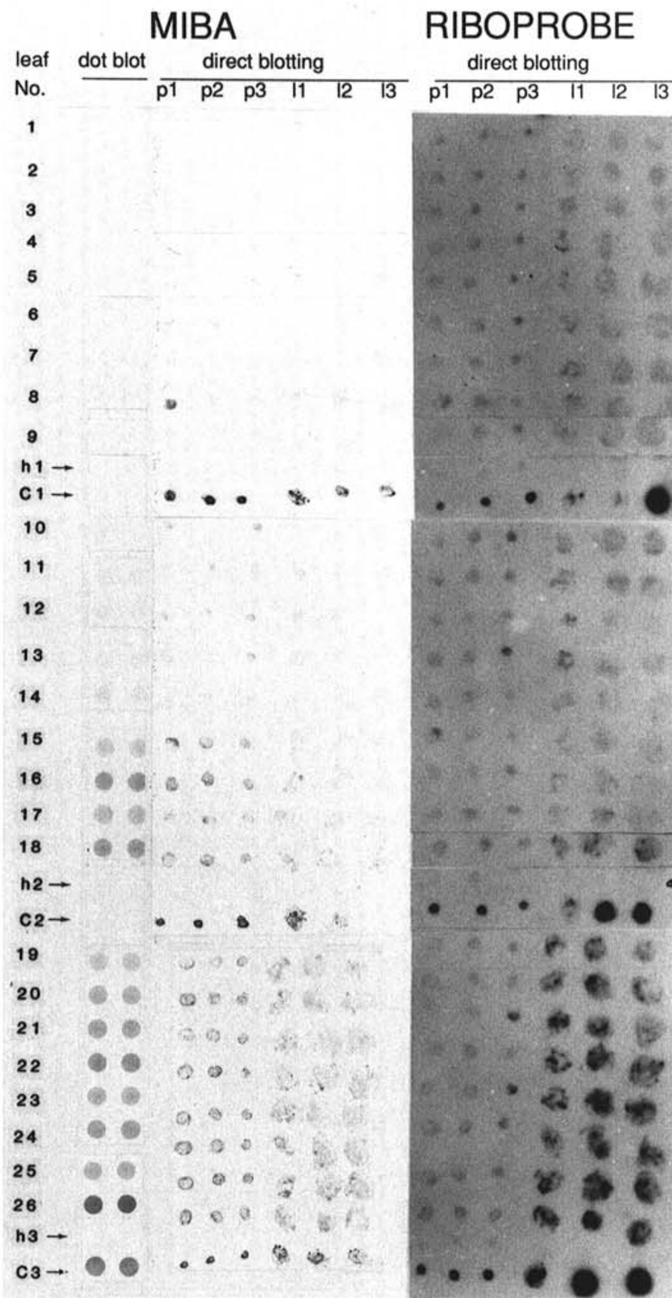


Fig. 4. Comparison of membrane immunobinding assay (MIBA) versus riboprobe for detection of sweetpotato feathery mottle virus (SPFMV)-RC naturally infected sweetpotato vines (cv. Jewel). Leaf number is the position of leaf on the vine from youngest (1) to oldest (26). P1, P2, and P3 are relative positions of petioles, and l1, l2, and l3 are relative leaf positions from proximal to distal, respectively. H1–H3 are healthy sweetpotato controls (cv. Jewel) and C1–C3 are SPFMV-RC-infected *Ipomoea nil* controls.

Distribution of detectable SPFMV in sweetpotato plants by MIBA and riboprobe. It is widely accepted that SPFMV is most reliably detected by ELISA and mechanically transmitted from symptomatic leaves on the older half of the vine, while being graft-transmitted from all regions of an infected vine (5,8). We were able to detect SPFMV equally well by both the riboprobe and MIBA assays in symptomatic leaves (Fig. 3B). MIBA did not detect SPFMV in symptomless leaves. Although the riboprobe signal in symptomless leaves was significantly less than the signal obtained from symptomatic leaves, it was significantly above background. Hybridization conditions were adjusted until the healthy control did not give a detectable signal by the scanning densitometer.

We further examined the distribution of detectable SPFMV by graft, MIBA, riboprobe assays, and by visual symptoms in 15 sweetpotato vines (Figs. 4 and 5). In this experiment, we used the direct blotting procedure rather than spotting tissue extracts, as the two procedures gave similar results (Fig. 4). Mild chlorotic spotting typical of SPFMV-infected sweetpotato was evident on leaves on the older part of the vine (leaves 11–26, Fig. 4). As expected, the MIBA only detected SPFMV in symptomatic leaves and their respective petioles; 1,275 out of 1,440 samples from leaves showing symptoms and only 17 out of 900 symptomless leaves (leaves 1–10) reacted positively. The riboprobe consistently detected SPFMV in nearly all leaves and petioles throughout the infected vines; 2,235 positive samples were detected from 2,340 tested. There was no consistent trend in intensity of reactions with respect to proximal (p1, L1) or distal (p3, L3) orientation of the source of blotted tissue on individual petioles or leaves. The presence of SPFMV in the plant was confirmed by grafting individual nodes from assayed leaves of two vines into *I. setosa* (Fig. 5). All 52 grafts were positive.

We have further tested the riboprobe procedure on symptomless and symptomatic field-grown sweetpotato plants. SPFMV was detected in 42 out of 44 samples assayed. The results of these tests were consistent with the extracts and tissue blots of greenhouse-grown plants.

DISCUSSION

The goal of this research was to develop a system for the detection of viruses in sweetpotato. In these investigations, SPFMV was used as the representative virus as it is the most thoroughly characterized (23), and patterns of symptom expression are similar to other viruses such as sweetpotato latent virus, sweetpotato mild mottle, sweetpotato yellow dwarf, and an ilarlike virus. The components of the detection system have overcome several of the frequently cited limitations of virus detection in sweetpotato. These investigations have revealed the basis for host-related problems affecting virus distribution as well as assay factors related to increased sensitivity and probe specificity.

Our findings of increased sensitivity of the riboprobe over serological assays (Fig. 3) and labeled cDNAs (data not shown)

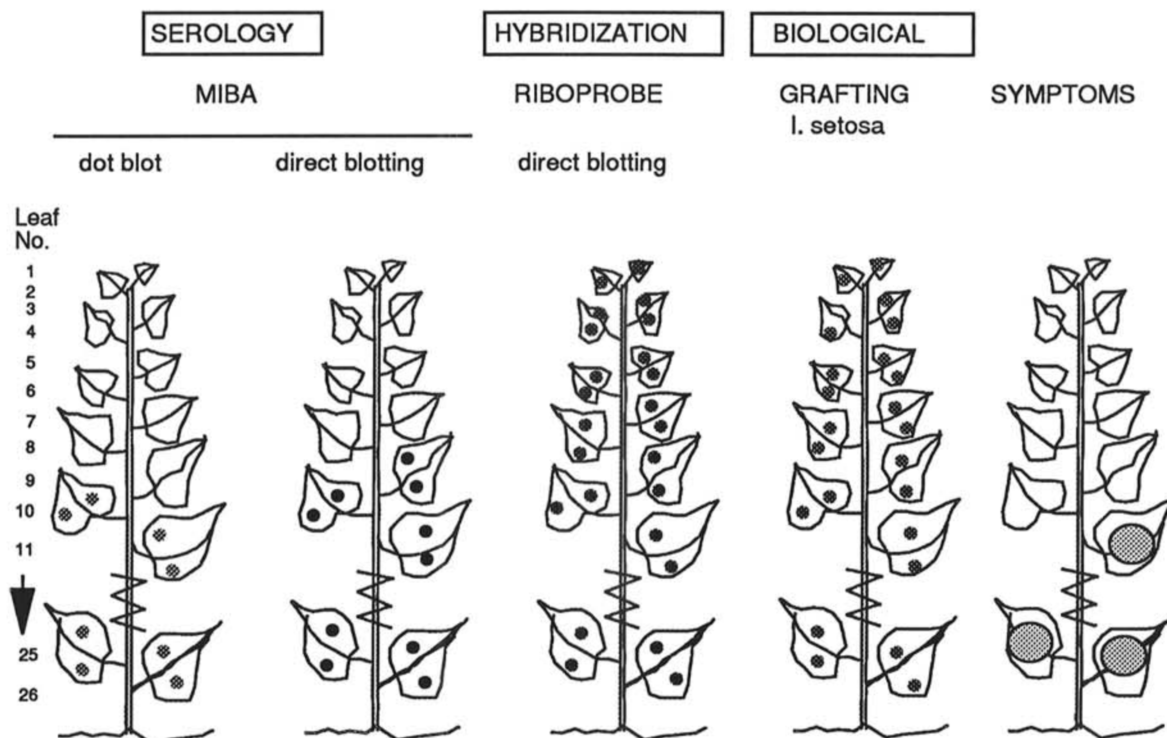


Fig. 5. Detection of the sweetpotato feathery mottle virus (SPFMV)-RC viral components in infected sweetpotato leaves monitored by membrane immunobinding assay (MIBA), riboprobe, and grafting. Leaf no. = position of the sample leaves (1 is the youngest, 26 the oldest). Shadowed dots indicate leaves testing positive for the viral component. MIBA was used in dot blot and direct blotting to monitor capsid protein. Riboprobe direct blotting detected RNA, and grafting monitored virions. This Figure is representative of 15 different vines assayed. Symptoms were recorded the day of sampling.

is consistent with previous studies (9,12,27,31). Our experience with sweetpotato indicated that the increased sensitivity was due not only to the high specific activity of the riboprobe but also the removal of a nonhybridized probe with RNase to minimize nonspecific background. The limiting factor in the latter step is that the concentration of RNase must be carefully titrated. The specificity of the probe is another consideration. Wide spectrum probes are preferable for use in indexing and quarantine programs. cDNA templates for riboprobe synthesis with a high probability of having a broad spectrum of activity for strains of potyviruses should contain the 3′ region of the capsid gene that shows a high degree of homology in the potyvirus group (2,30). In an investigation of the sequence of the SPFMV capsid gene to be published elsewhere, the SPFMV clone pGFMRC4.20 contained the entire capsid gene and a portion of the nontranslated region. The clone did not contain any of the 3′ terminal polyadenylation tract that could result in hybridization with host mRNA.

Direct comparison of the riboprobe and MIBA assays demonstrated that the riboprobe had greater sensitivity to detect SPFMV infection (Fig. 3A). However, there was no difference in the ability of the two probes to detect SPFMV in symptomatic sweetpotato tissue (Fig. 3B). The ability of the riboprobe to detect SPFMV RNA in the symptomless tissue, which was confirmed by grafting, suggests that differences in the nature of the virus may exist between symptomless and symptomatic tissue. Further, it suggests that the basis of these differences are a reduction in virus concentration that may be associated with modification of the virion. Although one possible explanation may be the proteolytic modification of virions recently proposed to account for recovery from infection (28), our experiments did not address the nature of the differences.

Preparation of tissues for assays is frequently the limiting factor in evaluating large numbers of plants. We found that the direct blotting of freshly cut petioles and leaves was at least as reliable as using tissue extracts. Direct blotting has been used for detection of many plant viruses; however, it was ineffective when combined with immunodetection of SPFMV in symptomless leaves and petioles of sweetpotato and *I. setosa* (14). Not only does direct blotting permit processing of large numbers of samples, but it also facilitates cooperative research between investigators in various countries. Diagnostic surveys can be performed with minimal training, and blots can be shipped to a central facility for developing (14). Thus, the procedures described are well suited for sweetpotato that is primarily grown in developing countries and is currently receiving increased attention because of its nutritional advantages.

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