Use of Unique RNA Sequence-Specific Oligonucleotide Primers for RT-PCR to Detect and Differentiate Soybean Mosaic Virus Strains

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ABSTRACT

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A reverse transcription–polymerase chain reaction (RT-PCR) assay was developed to detect and discriminate between RNAs of individual soybean mosaic virus strains G2 and G7. This assay utilized oligonucleotides complementary to unique regions in the cylindrical inclusion protein cistron. When G2- and G7-specific primers were used, amplification from total RNAs from trifoliolate soybean leaves infected with either strain or a mixture of strains yielded specific fragments. The specificity of primers was validated further by amplification of only the expected 277-bp fragment characteristic of the G7 strain from infected soybean differential cultivars used to discriminate the strains. Thus, this specifically designed assay allowed discrimination of strains in a mixed infection of a single soybean host plant. This represents a method capable of discriminating between two very closely related pathogens that were previously distinguishable by laborious bioassays.

Additional keyword: potyvirus

Soybean mosaic virus (SMV) is a member of the large and economically important plant virus family, the Potyviridae (29). Several strains of SMV (G1 through G7, G7a, and C14) have been identified on the basis of their pathogenicity to differential soybean lines (2,23) and transmission by aphid species (16). SMV strains have also been divided into three serological groups by means of one-dimensional trypsin peptide maps immunoblotted with 12 monoclonal antibodies (8). Comparison of coat protein tryptic digests from 14 potyvirus isolates from soybean confirmed them to be SMV strains (11). The complete nucleotide sequences of strains G2 and G7 have been determined (12). The genomes of G2 and G7 are 9,588 nucleotides (nt) long, excluding the poly(A) tail, and encode a polyprotein of 3,066 amino acids. Nucleotide sequence identity between the G2 and G7 strains is 94%. Most variation is in the 5′ end of the genome (35K [P1], helper component-protease [HC-Pro], 42K [P3], and cylindrical inclusion protein [CI]). Such variation, an important consideration when identifying strains, may be related to pathogenicity of different strains.

SMV strains G2 and G7, although distinguished by pathogenicity on differential lines, have been difficult to differentiate serologically (8,9). In nature, SMV is spread by aphids (16) and may result in infection of soybeans by mixed strains of the virus. Therefore, the limited sensitivity of serological assays could have practical implications for virus detection of G2 and G7 in doubly infected soybeans. Polymerase chain reaction (PCR), a highly sensitive method for the amplification of genomes, has become widely used as a diagnostic technique for infections by phytoplasmas (formerly called mycoplasma-like organisms) (27), bacteria (20), viroids (21), and plant viruses belonging to several different groups (e.g., the geminivirus, luteovirus, and potyvirus groups) (7,15,24,25). The assay has been applied to enhance detection sensitivity of potyviruses such as plum pox virus (34), sugarcane mosaic virus (30), lettuce mosaic virus (36), zucchini yellow mosaic virus (31), and two sweet potato potyviruses (3). With these viruses, the PCR procedure utilized primers designed to amplify a variable region of the potyvirus genome to distinguish between different viruses within the group rather than between strains of the same virus. Differentiation of strains of the same virus with a reverse-transcription (RT-) PCR–based assay has been reported for cucumber mosaic virus (22), citrus tristeza virus (6), and vertebrate viruses such as equine herpesvirus (EHV) (33). These viruses were first detected with primers derived from variable (cucumber mosaic and citrus tristeza viruses) or conserved (EHV-1 and EHV-4 strains) regions of the viral genome and then differentiated by further analysis of the amplified products by restriction-endonuclease analyses and restriction fragment length polymorphism (6,22, 33) or hybridization with type-specific oligonucleotide probes (28). However, a different approach to PCR, known as nested PCR, allowed detection and differentiation of EHV-1 and EHV-4 without the need for further techniques (1).

More recently, nucleotide-specific PCR was shown to discriminate between two variants of influenza virus C (19). Primers have been shown to discriminate between the P1 and P4 isolates of pea seedborne mosaic virus (13,14). However, they have not been used in reciprocal tests to detect and discriminate between the two isolates inoculated to the same plant.

The objective of this study was to develop a simple and accurate RT-PCR–based method that would detect and discriminate between two SMV strains in the same plant and not require further analysis of amplified products. Here, we describe an RT-PCR assay that uses unique RNA sequences in the CI cistron of SMV to detect and differentiate strains G2 and G7. The method greatly increases the speed and ease of detecting two very closely related plant virus strains.

MATERIALS AND METHODS

Plants and viruses. SMV strains G2 (ATCC PV-723) and G7 (ATCC PV-722) have been previously described (17). Soybean cvs. Williams '82 (susceptible to all SMV strains) and PI 96983 and L 78379 (susceptible to G7, immune to G2) (23,35) were used for all experimental tests; cv. Williams was also used as the maintenance host for the viruses. Primary leaves of 12-day-old soybean seedlings of these cultivars were mechanically inoculated separately with strain G2 or G7. Test plants were maintained in growth chambers operating at a constant temperature of 20°C with a 16-h photoperiod and an irradiance of 50 W/m^2. Approximately 3 weeks after inoculation, infected trifoliolate leaf tissue from plants inoculated with either strain was combined (1:1 wt/wt) to obtain 300 mg of leaf sample for total RNA extraction and cDNA synthesis. Alternatively, total RNA was extracted from plants infected separately with either G2 or G7, cDNA synthesized by reverse transcription from the first PCR primer, and the resulting cDNAs combined (1:1 vol/vol) before PCR amplification.

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In a separate experiment, 12-day-old cv. Williams seedlings were inoculated with both strains G2 and G7 by means of one strain per leaf or half leaf. The first treatment consisted of inoculating plants with both strains at the same time. In the second treatment, plants were inoculated with the G2 strain 24 h after plants had been inoculated with G7. Confirmation of infection was done first by enzyme linked immunosorbent assay (ELISA) and then by RT-PCR amplification of cDNA synthesized from total RNA from plants of PI 96983 and L 78379 inoculated with the two strains. As controls for all experiments, uninoculated plants maintained in the same growth chamber as the inoculated plants were used for parallel assays.

ELISA. Non-strain-specific monoclonal antibody-based biotin-avidin ELISA (MAb ELISA) was performed as described previously (4,10) with some modifications. All steps involving incubation of reactants were performed at 26°C in polystyrene flat-bottomed ELISA plates (Dynatech Laboratories, Chantilly, VA). Antibodies used were MAb S7 (100 µl/well, 5.0 µg/ml) (8) as a coating antibody and biotinylated MAb S2-G12 (100 µl/well, 2.5 µg/ml) as a second antibody.

RT-PCR. Four 20-nt SMV strain-specific primers, designated G2-5′CI (cylindrical inclusion protein), G2-3′CI, G7-5′CI, and G7-3′CI were synthesized by means of an Applied Biosystems DNA Synthesizer 394 (Perkin Elmer, Norwalk, CT) by the Iowa State University Nucleic Acids facility. The oligonucleotides were designed with the PRIMER DESIGNER program version 2.0 (Scientific & Educational Software, State Line, PA) based on sequence divergence between SMV strains G2 and G7 (12) at positions 4937 to 4956 in the CI region (Fig 1). The downstream primers G2-3′CI (5′-GCAGTCTTGTGTC AATCACG-3′) and G7-3′CI (5′-GCAATC TGTGGATCTCTGGG-3′) were complementary to nt 4937 to 4956 of SMV strains G2 and G7 (common nucleotides of G7 and G2 are underlined). The upstream primers G2-5′CI (5′-CCACACTTCATAG TCGCAAC-3′) and G7-5′CI (5′-CTTGGC AGAGTTGGTCGTTG-3′) were homologous to RNA nt 4518 to 4537 of SMV G2 and 4680 to 4699 of SMV G7, respectively. An additional upstream primer G2-5′CI-0 (5′-CTTGGCAGAGTTGGTCGTT G-3′) homologous to RNA nt 4680 to 4699 of G2 was also tested.

Total RNA was extracted from trifoliolate leaves by the method of Wadsworth et al. (32). Single-stranded cDNA was synthesized from approximately 80 ng of total RNA by using a 3′ RACE (rapid amplification of cDNA ends) system for first-strand cDNA synthesis (GIBCO BRL, Gaithersburg, MD), according to the manufacturer's instructions except that we utilized the downstream primers G2-3′CI and G7-3′CI (Fig. 1) instead of those primers supplied by the manufacturer. Ten picomoles of the specific primer was used in a final volume of 21 µl.

Optimization of PCR buffer conditions for amplification of SMV strains employed a PCR Optimizer kit (Invitrogen, San Diego, CA). Briefly, reactions were performed in 50-µl volumes that contained buffers (300 mM Tris-HCl, 75 mM $(NH_4)_2SO_4$) ranging from pH 8.5 to 9.5 and with $MgCl_2$ concentrations from 1.5 to 3.5 mM; 2.5 mM of each dNTP; 30 to 40 pmol each of downstream and upstream primers; 1 to 2 µl of cDNA (100 to 300 ng) and 1 unit of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). The thermocycler (Ericomp TwinBlock System, San Diego, CA) was programmed for template denaturation at 94°C for 1 min (2 min first cycle), primer annealing at 55°C for 2 min, and DNA synthesis at 72°C for 3 min. A final 7-min extension step at 72°C was performed at the end of 25 cycles. In subsequent amplifications of the first-strand cDNA, samples were held at 80°C, prior to amplification according to the Invitrogen's Optimizer kit instructions.

Other analyses. Nucleic acids were separated on 6.5 cm by 9.0 cm, 2% (wt/vol) agarose gels in TBE buffer (0.09 M-Tris-borate, 0.09 M-boric acid, 0.02 M-EDTA, pH 8.0) at 80 V for 30 to 40 min. Gels were stained with ethidium bromide and photographed with a UVP GDS 5000 Imaging System (San Gabriel, CA).

*Hae*III restriction digests were performed as recommended by the manufacturer (Promega, Madison, WI) and purified by standard methods (26). Direct sequencing of PCR products was performed with the Applied Biosystems DNA Synthesizer 373 (Perkin Elmer Cetus, Norwalk, CT) by the Iowa State University Nucleic Acid facility with either downstream or upstream primers, or both. The DNA was

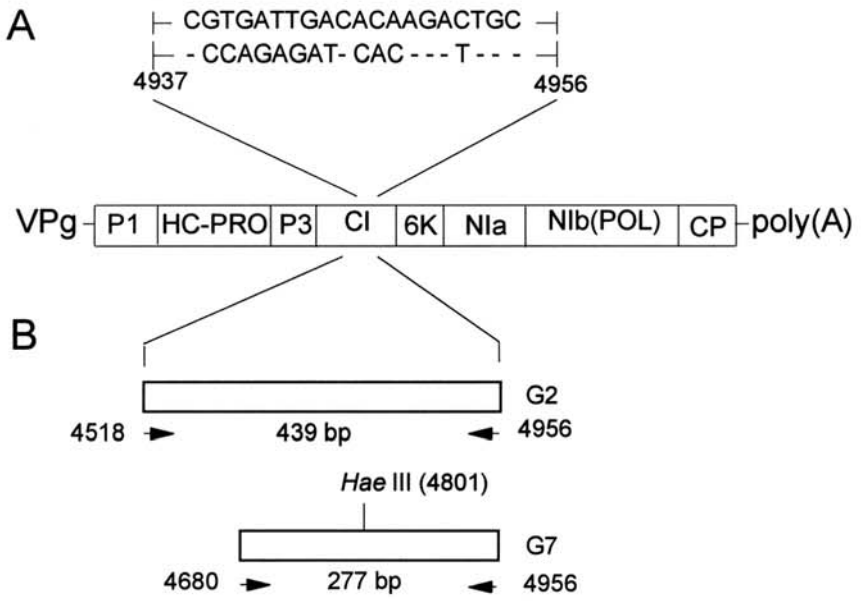

Fig. 1. (A) Location of nucleotide sequence divergence between soybean mosaic virus (SMV) strains G2 and G7 from positions 4937 to 4956 within the cylindrical inclusion (CI) coding region. Bases of G7 sequence are shown below G2 sequence where they differ from G2. Positions of other proposed SMV coding regions are shown. Abbreviations: P1, first protein (35K); HC-Pro, helper component-protease; P3, third protein (42K); CI, cylindrical inclusion protein; 6K putative peptide; NIa, nuclear inclusion 'a' protein (21K and 27K peptides, VPg and protease, respectively); NIb (POL), putative RNA polymerase; and CP, coat protein. **(B)** RNA sequence positions of primers used for reverse transcription–polymerase chain reaction amplification of SMV G2 and G7 RNAs are shown with expected amplification products. Location of specific *Hae*III restriction site is shown for G7 DNA.

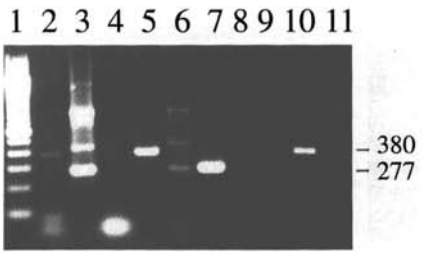

Fig. 2. Ethidium bromide–stained agarose gel of samples amplified from soybean cv. Williams plants separately infected with soybean mosaic virus strains G2 or G7. Lane 1, DNA markers, 100 to 2072 bps (100 ng total DNA [GIBCO BRL, Gaithersburg, MD]); lanes 2 and 3, samples amplified from a mixture of RNA from G2- and G7-infected plants; lane 2, primers G2-3′CI and G2-5′CI; lane 3, primers G7-3′CI and G7-5′CI; lanes 4 and 5, G2 primer pair; lane 4, no RNA; lane 5, RNA amplified from G2-infected plants; lanes 6 and 7, G7 samples amplified from G7-infected plants by means of G7 primer pair; lane 7, hot-start polymerase chain reaction; lanes 8 and 9, uninoculated controls amplified with G2 and G7 primer pairs, respectively. Samples in lanes 10 and 11 resulted from amplification of a mixture of cDNAs mixed after synthesis of RNAs of strains G2 and G7 by G2 and G7 primer pairs, respectively. Band in lane 11 was faint and did not reproduce clearly in the image.

prepared for sequencing with centricom 30 (Amicon, Beverly, MA) and or gel purification in low-melting Nusieve GTG agarose (FMC BioProducts, Rockland, ME).

RESULTS

Primers G2-3′CI, G2-5′CI, G2-5′CI-0, G7-3′CI, and G7-5′CI for PCR amplification of SMV G2 and G7 cDNA were designed to anneal to the region of the SMV genome that showed the highest level of local divergence between the strains (Fig. 1) (12). The RT-PCR amplification of SMV-specific RNA from total nucleic acid extracts yielded products that migrated at about 380 bps and 280 bps for strains G2 and G7, respectively (Fig. 2). The G2 product migrated faster than the expected size of 439 bps, whereas the G7 product migrated exactly as expected (277 bps) from the position of the known SMV sequence (Fig. 1). The G2 product indicated anomaly, probably due to secondary structure, but it still allowed specific detection.

Successful amplification worked over a range of magnesium concentrations and pH levels. Optimal buffer conditions consisted of 3.5 mM $MgCl_2$ and pH 8.5 for the G2 strain and 2.0 mM $MgCl_2$ and pH 9.0 to 10.0 for the G7 strain. Buffer containing 2.0 or 3.5 mM $MgCl_2$ and pH 9.0 or 9.5 gave the highest yields of amplified products and therefore was used for subsequent amplifications. Primer annealing at 55°C for 2 min and amplification for 25 cycles worked well for both strains. The G2-3′CI and G2-5′CI-0 pair inconsistently amplified a 277-bp fragment for G2 and also amplified the characteristic G7 fragment under all conditions. Thus, we did not pursue use of G2-5′CI-0 further.

We next tested whether the assay could specifically detect G2 and G7 in a mixture of the two. Total RNA was extracted from a mixture of trifoliolate leaves from a soybean plant infected with the G2 strain and leaves from a G7-infected plant. RT-PCR amplification with the primer pairs G2-3′CI and G2-5′CI or G7-3′CI and G7-5′CI of this mixture yielded products of 380 bps and 277 bps, respectively (Fig. 2). G2 RNA gave stronger signals when amplified alone than in the presence of G7 RNA. In the latter case, the G2-specific primers gave unusual primer-dimers. No fragments were amplified from RNA extracted from uninoculated soybean plants. Strains were further differentiated by amplification of the expected products on mixed samples of cDNAs prepared from total RNAs of separately inoculated plants and showed distinguishable signals for both G2 and G7 (Fig. 2). The occasional formation of some nonspecific bands with the G7-3′CI and G7-5′CI primer pair is not due to loss of discrimination ability because the 380-bp fragment primed by the G2-3′CI and G2-5′CI pair is absent. The nonspecific bands could be eliminated by taking care to ensure thorough mixing of the extracted tissue with phenol-chloroform during isolation of the RNA or by hot-start PCR (compare Figure 2, lanes 6 and 7).

The specificity of PCR was validated by detecting RNA of each strain from individual plants infected with both strains. As a control, we used ELISA to ensure that plants were infected, but this assay does not distinguish between strains. Positive signals in PCR correlated with ELISA readings. The primer pair G7-3′CI and G7-5′CI amplified only the 277-bp fragment of strain G7; no such band occurred with the G2-3′CI and G2-5′CI pair that primed amplification of the 380-bp fragment of G2 (Fig. 3). As observed with the mixed RNAs from separately infected plants, the G2-3′CI and G2-5′CI pair amplified the target less well when used to amplify G2 RNA from mixed infection by strains G2 and G7 than from plants inoculated with G2 alone (Fig. 3).

To provide further confirmation of primer specificity, PCR amplifications were performed on total RNA extracted from the differential soybean lines PI 96983 or L 78379, each separately inoculated with strain G7 or G2. These soybean lines are immune to G2 but not G7, and thus were used to correlate the differential host reactions with presence of specific viral RNA. The expected fragments (277 bps) were obtained with RNAs extracted from G7-inoculated plants by using the primer pair G7-3′CI and G7-5′CI, whereas no fragments resulted from G2-inoculated PI 96983 or L 78379 (Fig. 4A). The primer pair G2-3′CI and G2-5′CI yielded negative results (data not shown). Digestion of the amplified SMV G7 CI product with *Hae*III (Fig. 1) produced the expected restriction fragments of 155 and 122 bps (Fig. 4B), and the nucleotide sequence of this DNA product was as predicted (data not shown).

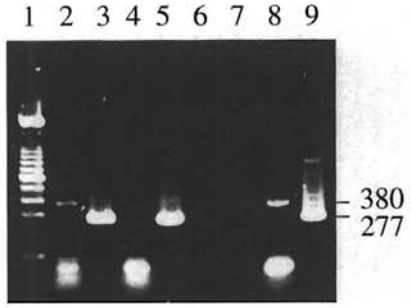

Fig. 3. Reverse transcription–polymerase chain reaction (RT-PCR) of soybean mosaic virus (SMV) RNA from plants inoculated with SMV strains G2 and G7. Analysis of PCR products amplified with RNA sequence-specific primer pairs, from soybean cv. Williams. All samples from trifoliolate leaves after inoculation of primary leaves. Lanes 2 and 3, opposite leaves inoculated with SMV strains G2 and G7 simultaneously; lanes 4 and 5, opposite leaves inoculated with G2, 24 h after G7; lanes 6 and 7, uninoculated controls; and lanes 8 and 9, leaves inoculated with G2 or G7, respectively. Primers used for PCR amplification were G2-3′CI and G2-5′CI (lanes 2, 4, 6, and 8), and G7-3′CI and G7-5′CI (lanes 3, 5, 7, and 9). Lane 1 DNA markers, 100 to 2072 bps (100 ng total DNA [GIBCO BRL, Gaithersburg, MD]).

DISCUSSION

Results of this study demonstrated the differentiation of SMV strains G2 and G7 by RT-PCR based on unique RNA-specific oligonucleotide fragments. This differs from previous approaches employed with other viruses that required further analyses of amplified products (6,22,28,33) or use of nucleic acid hybridizations based on radiolabeled PCR-amplified 3′ noncoding sequences as probes (5). Although the ob-

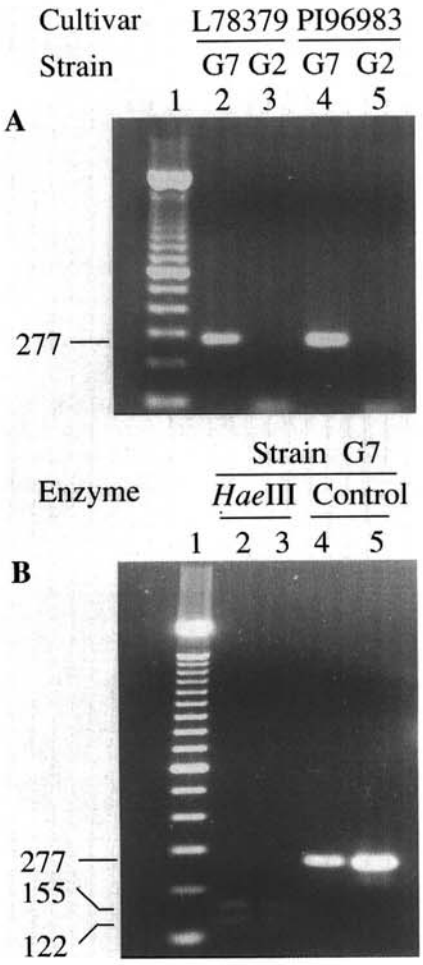

Fig. 4. Electrophoretic analysis of reverse transcription–polymerase chain reaction (RT-PCR) amplified products of soybean mosaic virus RNAs and *Hae*III restriction enzyme digests. **(A)** Products of amplification with primer pair G7-3′CI and G7-5′CI of RNA extracts from differential soybean lines inoculated with strains G2 and G7. Lane 1, DNA marker, 100 to 2072 bps (100-bp ladder [GIBCO BRL, Gaithersburg, MD]); lanes 2 and 3, products from L 78379 inoculated with G7 and G2, respectively; lanes 4 and 5, products from PI 96983 inoculated with G7 and G2, respectively. **(B)** *Hae*III restriction enzyme digests of G7 amplification products. Lane 1, DNA marker 100 to 2072 bps (100-bps ladder [GIBCO BRL]); lanes 2 and 3, *Hae*III digests; lanes 4 and 5, undigested controls.

jective of this study was not to differentiate all SMV strains, the assay would also differentiate other strains such as G1 (ATCC PV-716), G3 (ATCC PV-718), G4 (ATCC PV-719), G5 (ATCC PV-720), and G7a (ATCC PV-724) from G2 (data not shown). Strain G1 could be differentiated from strains G3, G4, G5, and G7a by further digestion of the G7-3′CI to G7-5′CI amplified product with *Hae*III. Restriction fragments of 155 and 122 bps were produced for all of these strains except G1 (data not shown).

For this study, primers were selected from the CI region because it showed one of the highest levels of variation when sequences of the G2 and G7 genomes were compared (12). Within this CI region, design of primers complementary to strains G2 and G7 allowed mismatch of 12 bases, and the homologous (upstream) primers mapped to different positions to ensure a size difference of 162 bases between amplification products. Initially, we had designed upstream primers that mapped to the same position for both G2 and G7. This approach presented some difficulties in finding primers and conditions to discriminate between G2 and G7. Consequently, we used the alternative approach involving primers that gave different sizes of products. Under optimized conditions, amplification of total RNA extracted from soybean plants separately infected with strains G2 and G7 yielded 380- and 277-bp fragments, respectively, indicating that RT-PCR can be used to detect and differentiate between SMV strains.

Amplification on total RNA isolated from plants doubly infected with both strains G2 and G7 confirmed and validated the specificity of the oligonucleotide-primer pairs. The procedure allows the use of primers that ensure differences in size of amplified products and provides unqualified discrimination between strains. Signals were readily reproducible. The apparent reduced amplification of G2 in the presence of G7 suggests that G2 may replicate more slowly, relative to G7, in Williams infected with both strains. This observation would not have been possible with primers that give the same size products from G2 and G7. Such information may be valuable for future studies of mixed infection.

Further validation of the reliability and specificity of the RT-PCR assay was established by using soybean differential lines that contain the *Rsv* resistance gene (35) and used to group SMV strains (2). Although the G7 strain is able to induce disease in plants containing the *Rsv* gene, the plants remain immune to G2. The combined infectivity and RT-PCR assays demonstrated that only strain G7 and not the G2 strain could be identified in virus-inoculated plants containing the *Rsv* gene.

Virus strain differentiation becomes important for viruses such as SMV, which exists as a complex of several very closely related strains that can occur as a mixture in infected soybean host plants. Highly sensitive and rapid techniques for detection and differentiation of SMV strains are important for identification of resistance genes in breeding lines, establishment of naturally occurring variation within strain groups, monitoring spatial and temporal spread of strains, and molecular studies to discern the potential for complementation among SMV strains in mixed infection (18). This RT-PCR assay has shown that unique RNA sequence-specific primers can be employed to detect and discriminate between RNAs of very similar strains.

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