Detection of Five Seedborne Legume Viruses in One Sensitive Multiplex Polymerase Chain Reaction Test

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This work was funded by the Wool Research and Development Corporation, Australia.
We thank W. Collins, G. Auricht, and P. Nichols for helpful discussions and P. Keese for valuable discussions and sequence information.
Accepted for publication 10 June 1994.

ABSTRACT

Bariana, H. S., Shannon, A. L., Chu, P. W. G., and Waterhouse, P. M. 1994. Detection of five seedborne legume viruses in one sensitive multiplex polymerase chain reaction test. Phytopathology 84:1201-1205.

A reverse transcription polymerase chain reaction (RT-PCR) assay has been developed that can simultaneously test a sample, in one tube, for the presence of five seedborne legume viruses that are of great concern to legume germ plasm banks: alfalfa mosaic alfamovirus, bean yellow mosaic potyvirus, clover yellow vein potyvirus, cucumber mosaic cucumovirus, and subterranean clover mottle sobemovirus. RT-PCR assays were also developed for the detection of subterranean clover redleaf luteovirus and subterranean clover stunt virus, thus providing a detection system for all well-characterized viruses known to infect subterranean clover. Primers were designed so that the size of the RT-PCR product was indicative of the virus amplified and, where sequences of more than one strain of virus were available, conserved regions were given preference as primer targets. The RT-PCR assay detected all isolates tested for each virus and was up to five orders of magnitude more sensitive than ELISA.

Additional keywords: virus indexing.

It has recently become apparent that a proportion of the seed stored in some legume germ plasm banks harbors a number of seedborne viruses that may be unwittingly distributed along with the seed. The viruses of concern in subterranean clover and medic germ plasm banks are alfalfa mosaic alfamovirus (AMV), bean yellow mosaic potyvirus (BYMV), clover yellow vein potyvirus (CYVV), cucumber mosaic cucumovirus (CMV), and subterranean clover mottle sobemovirus (SCMoV).

Currently the viral disease status of such germ plasm collections is evaluated by visual observation of plants grown for seed propagation. Suspect plants are sometimes tested by ELISA. Screening for all five seedborne viruses by five different virus-specific ELISA tests for each sample is both prohibitively expensive and labor-intensive. Furthermore, antisera to some of these viruses are not readily available. An assay is needed to detect and differentiate all five viruses in a single test that is sufficiently sensitive to be used in assaying large-batch samples. The polymerase chain reaction is a powerful technique used for the amplification of specific DNA sequences and is capable of enrichment by a factor of 10^6 to 10^9, enabling the detection of a few target molecules (6,7). Reverse transcription followed by polymerase chain reaction (RT-PCR) has been used to detect BYMV with a sensitivity in the range of 1-pg quantities of virus (10) and has given sensitive detection of apple scar skin (5) and citrus exocortis and cachexia viroids (13).

This paper describes the development of an RT-PCR assay that can detect and identify five seedborne viruses of subterranean clover in a single, simple, and sensitive RT-PCR assay. It also reports the sensitive detection assay for two aphid-transmitted viruses of subterranean clover, subterranean clover redleaf luteovirus (SCRLV) and subterranean clover stunt virus (SCSV).

MATERIALS AND METHODS

Virus and sample sources. AMV isolates H1, H2, H5, and LA; CMV isolate WC; and BYMV isolates S and G were kindly provided by R. Francki and J. Randles, Waite Institute, Adelaide. R. Jones, W.A. Department of Agriculture, Perth, generously provided CMV isolate SN, AMV isolate EW, SCMoV isolate P23, and BYMV isolates MI, SV, SMB, and LP, as well as CMV-infected seeds. G. Johnstone, Tasmania Department of Agriculture, Hobart, generously provided three isolates of SCRLV.

Four isolates of SCRLV, eight isolates of SCSV, four isolates of SCMoV, one isolate of CYVV, and one isolate of AMV were collected from the field and maintained by our laboratory (Division of Plant Industry, Canberra).

Virus purification. AMV and SCMoV were purified essentially as described by Clark (2) and Francki et al (3), respectively.

SCMoV cloning and sequencing. RNA was extracted from a satellite-free strain of SCMoV essentially as described by Waterhouse et al (11), and cDNA was synthesized as described by Gubler and Hoffman (4). The cDNA was digested with restriction endonucleases *Hae*III and *Rsa*I, and the fragments were fractionated by PAGE. The bands were eluted and cloned into the *Sma*I site of pGem3Zf- (Promega). Nucleotide sequences of the inserts were obtained by the dideoxynucleotide chain termination method (8). Primers were designed using these sequences.

Tissue and nucleic acid extraction. Samples of 200 mg of leaves or seeds (about 30 seeds) were ground in liquid nitrogen, then in 300 µl of TE3D buffer (2 M Tris-HCl, 200 mM EDTA, 15% lithium dodecyl sulfate, 10% sodium deoxycholate, 10% Nonidet-P40) in microcentrifuge tubes using a tight-fitting metal rod and a little washed sand. Then, 250 µl of 3 M ammonium acetate and 400 µl of chloroform:isoamyl alcohol (24:1) were added to each tube and the emulsion was shaken for 1 min before being centrifuged at 13,000 *g* for 5 min at 4 C. The supernatants (200 µl) were recovered and transferred to new tubes containing 600 µl of cold 3.6 M lithium chloride and 1 mM MEDTA (pH 8.0) and left on ice for 15 min. The mixtures were centrifuged at 13,000 *g* for 5 min, and the supernatants were discarded. The pellets were resuspended in 100 µl of TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA) and vortexed with 100 µl of a 1:1 mixture of phenol:chloroform. The emulsion was separated by centrifugation at 13,000 *g* for 5 min. The supernatant was recovered and

added to 10 μl of 3 M sodium acetate and 100 μl of cold isopropanol, left on ice for 1 h, and then centrifuged at 13,000 *g* for 5 min. The pellet was retained, washed with 1 ml of 70% ethanol, and then resuspended in 30 μl of distilled water or TE.

Viral cDNA synthesis and amplification. One-third (5–10 μg) of the total RNA extracted from plant material was reverse transcribed into cDNA using Superscript RNase H-reverse transcriptase (GIBCO BRL) in reaction conditions of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1.5 mM dNTPs, and 10 mM dithiothreitol. Because SCSV has a single-stranded DNA genome, there was no need for the reverse transcription step.

PCR amplification. A reaction volume of 50 μl was used for individual virus PCR assays. The reaction mixture comprised: 16.6 mM $(NH_4)2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.45% Triton X-100, 200 μg/ml of gelatin, 4 mM $MgCl_2$, 800 μM dNTPs, 10 pmol of each primer, 1 μl of reverse transcription product, and 1 unit of *Taq* polymerase (Biotech International, Perth, Australia). The thermal cycling was done either with a multiblock PCR machine (Ericomp, USA) or in a fast thermal sequencer (Corbett Research, Australia); a 10-μl aliquot of the reaction mixture was used in the latter machine.

For the simultaneous assay of all the seedborne viruses, RNA was extracted from individually infected plants, then the extracts were pooled and used in the reverse transcriptase reaction. A 10-μl aliquot of the cDNA product was used for the PCR step.

The regime for amplification of either single or multiplex assays was 30 cycles of 45 s of denaturation at 94 C, 45 s of primer annealing at 50 C, and 2 min of primer extension at 72 C. The reaction was completed by a final period of 5 min at 72 C.

Primers. The primers were designed from sequence information obtained from EMBL and Genbank databases and from colleagues within our laboratory (SCSV sequences, P. Keese, P. Boevink, and P. Chu; SCRLV sequences, N. Habili, R. Symons, and P. Waterhouse; and SCMoV sequences, A. Shannon, H. Bariana, and P. Waterhouse). The primer sequences were derived from the coat protein genes of all the viruses except SCSV and SCMoV.

Sequence information is available for more than one strain of each of CYVV, BYMV, AMV, and CMV. The regions of the coat protein genes of these viruses showing less variability were chosen as targets for the primers. There is no sequence information about different strains of SCRLV, so primers were designed to hybridize with regions expected to show sequence conservation by comparison with other luteovirus genomes.

Although there is no sequence information about different strains of SCSV, its genome consists of at least seven different segments and five of the genome segments appear to share a conserved region (P. Keese, *personal communication*). Primers for the detection of SCSV were designed to hybridize to this region.

The sequences of all the primers and the expected size of the PCR products when used with the appropriate virus are shown in Table 1.

Analysis of RT-PCR amplified products. The RT-PCR reactions were electrophoresed in 1.5% agarose gels with a loading of 10 μl per well. *Spp*I DNA restricted with *Eco*RI endonuclease and pUC19 DNA restricted with *Hpa*II endonuclease were used as dsDNA size makers. The gels were stained with ethidium bromide, and the presence of a single band of the expected size specific for a given virus was considered diagnostic for that virus.

ELISA. Sap was extracted from leaf tissue and tested by ELISA essentially as described by Xin et al (12) using an AMV-specific antiserum kindly provided by R. Francki. All tests were done in duplicate wells, using six uninoculated and four infected plant samples as controls in each plate. Samples were considered positive when both duplicate values exceeded the total value of the mean background value plus five times its standard deviation.

RESULTS

Partial SCMoV sequence. There was no information about the sequence of the SCMoV genome, so random segments of the genome were cloned and sequenced. From these sequences two primers were designed: SCMoV5′ (CCCTGCACTTATCA-ATGTTCCGTC) and SCMoV3′ (GATTGGTCAGTCCA-ATCATGG). These primers amplify a 160-bp fragment from the SCMoV genome.

PCR assay conditions. Different buffers, nucleotide concentrations, cycle temperatures, and durations were tested to find the optimum conditions for both single virus and multiplex assays. In our hands, the conditions previously described gave the optimum results for both types of assay. The multiplex assay was less robust and required very specific conditions. For this assay, we found that it was essential to have a high dNTP concentration, to use the $(NH_4)2SO_4$–Tris-HCl buffer and to have a 2-min extension time. With these conditions the assay worked well for all seven different viruses, either singly or as a multiplex. Surprisingly, the extraction procedure designed for the RNA viruses also worked efficiently for extraction of SCSV DNA.

Detection of different strains by RT-PCR. Six isolates of AMV (Fig. 1); six isolates of BYMV (Fig. 1); eight isolates of SCSV (Fig. 2); seven isolates of SCRLV (Fig. 2); three isolates of CMV, including a sample from a CMV-infected seed lot (Fig. 3); five isolates of SCMoV (Fig. 3); and one isolate of CYVV (Fig. 3) were assayed by RT-PCR. In all cases the expected amplification

TABLE 1. Primer sequences and expected size of PCR product for each primer pair when used to amplify the appropriate virus template

Primer name	Primer sequence[a]	Target	Expected size (bp)
SCMoV5′ SCMoV3′	CCCTGCACTTATCAATGTTCCGTC GATTGGTCAGTCCAATCATGGG	SCMoV	160
BYMV5′ BYCY3′	GGTTTGGCYAGRTATGCTTTTG GAGAATTTAAAGACGGATA	BYMV	240
AMV5′ AMV3′	CGTCAGCTTTCGTCGAACA GCCGTCGCGCATGGTAAT	AMV	288
CMV5′ CMV3′	TATGATAAGAAGCTTGTTTCGCGCA TTTTAGCCGTAAGCTGGATGGACAACCC	CMV	500
SCRLV5′ SCRLV3′	CGAGTATGGTCGCGGTTAG CCCGGTTCTCCGTCTACCTATTT	SCRLV	650
CYVV5′ BYCY3′	CATTCCAGACAGAGACATCAATGCAG GAGAATTTAAAGACGGATA	CYVV	750
SCSV5′ SCSV3′	CAGATCCTGAGCGTCC TGATGCTAGTGAAGCG	SCSV	1,000
S18S5′ S18S3′	TCATTAAATCAGTTATAGTTTGTTTG GAAATACGAATGCCCCCGACTGTCC	Positive control (ribosomal 18S RNA)	800

[a]Y = C or T, R = A or G.

product was observed. Unexpectedly, the amplification of SCRLV also gave an additional band of about 190 bp for all five isolates tested. This was presumably due to priming at similar nontarget sequences in the genome.

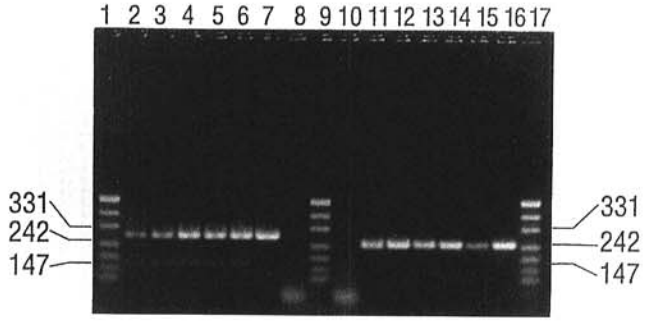

Fig. 1. PCR products amplified from total nucleic acids purified from plants infected with either alfalfa mosaic virus (AMV) (six isolates, lanes 2–7) or bean yellow mosaic virus (BYMV) (six isolates, lanes 11–16) electrophoresed in 1.5% agarose/TBE and stained with ethidium bromide. Reaction mixture contained primers specific to each virus. Lanes 8 and 10 are PCR products from nucleic acids from uninoculated plants amplified with AMV and BYMV primers, respectively. Lanes 1, 9, and 17 contain size markers of *Hpa*II digested pUC19 DNA.

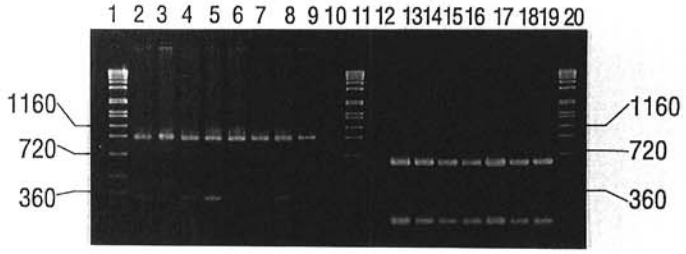

Fig. 2. PCR products amplified from total nucleic acids purified from plants infected with either subterranean clover stunt virus (SCSV) (eight isolates, lanes 2–9) or subterranean clover redleaf virus (SCRLV) (seven isolates, lanes 13–19) electrophoresed in 1.5% agarose/TBE and stained with ethidium bromide. Reaction mixture contained primers specific to each virus. Lanes 10 and 12 are PCR products from nucleic acids from uninoculated plants amplified with SCSV and SCRLV primers, respectively. Lanes 1, 11, and 20 contain size markers of *Hpa*II digested pUC19 DNA.

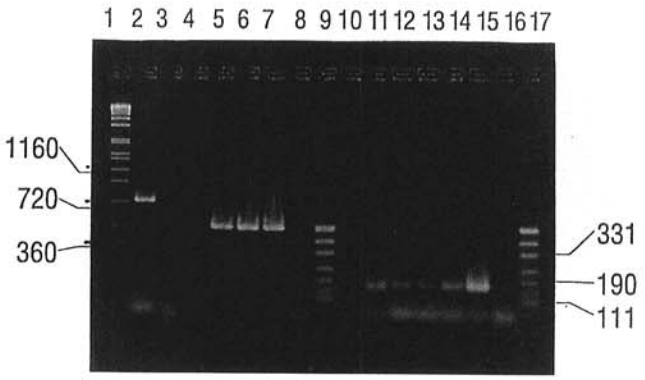

Fig. 3. PCR products amplified from total nucleic acids purified from plants infected with either clover yellow vein virus (CYVV) (lane 2), cucumber mosaic virus (CMV) (three isolates, lanes 5–7), or subterranean clover mottle virus (SCMoV) (five isolates, lanes 11–15) electrophoresed in 1.5% agarose/TBE and stained with ethidium bromide. Reaction mixture contained primers specific to each virus. Lanes 3, 8, and 10 are PCR products from nucleic acids from uninoculated plants amplified with CYVV, CMV, and SCMoV primers, respectively. Lane 1 contains size markers of *Eco*RI digested *Spp*I DNA, and lanes 9 and 17 contain size markers of *Hpa*II digested pUC19 DNA.

Sensitivity of RT-PCR and comparison with ELISA. The sensitivity of RT-PCR detection was compared with that of ELISA for the detection of AMV (isolate H2). A purified preparation of AMV particles was serially diluted in extract from uninfected plants to give a range of concentration from 10 mg/ml to 1 pg/ml; 200 μl of each dilution was used for ELISA and RT-PCR. The detection end point by ELISA was between 2 and 20 ng/ml, whereas RT-PCR detected AMV in all the dilutions (Table 2). Fourteen samples of white clover from the field suspected of being infected by AMV and six glasshouse samples were tested by bioassay, ELISA, and RT-PCR, with five, five, and six positive results, respectively (Table 3).

Simultaneous detection of seedborne viruses by RT-PCR. A cocktail of all nine primers to the five seedborne viruses—CYVV, CMV, AMV, BYMV, and SCMoV—was used to determine whether a single reaction mixture could be used to detect one or more of the five viruses in a single sample. The results showed that such an assay detected all the viruses both individually and collectively (Fig. 4). However, we found that when both CYVV and BYMV were present in the same sample, the amplification of both bands was reduced, particularly that of CYVV (data not shown).

Internal control using ribosomal sequences. It would be desirable to have an internal positive control for our RT-PCR assay that would give a band irrespective of the presence of virus. We

TABLE 2. Comparison of the sensitivity of detection of alfalfa mosaic virus by ELISA and reverse transcription-polymerase chain reaction (RT-PCR)

Virus (μg/ml)	ELISA	RT-PCR
2,000	+[a]	+
200	+	+
20	+	+
2	+	+
0.2	+	+
0.02	+	+
0.002	−	+
0.0002	−	+
0.00002	−	+
0.000002	−	+
0.0000002	−	+
Virus-free sap	−	−

[a] + = Positive assay, − = negative assay.

TABLE 3. Comparison of the results obtained with ELISA, reverse transcription-polymerase chain reaction (RT-PCR), and bioassay for detection of alfalfa mosaic virus

Source of material	ELISA	RT-PCR	Bioassay
Glasshouse			
Virus-free control	−[a]	−	−
Glasshouse	+	+	+
Glasshouse	+	+	+
Glasshouse	−	−	−
Glasshouse	+	+	+
Glasshouse	+	+	+
Field	−	+	−
Field	−	−	−
Field	−	−	−
Field	−	−	−
Field	−	−	−
Field	−	−	−
Field	+	+	+
Field	−	−	−
Field	−	−	−
Field	−	−	−
Field	−	−	−
Field	−	−	−
Field	−	−	−
Field	−	−	−

[a] + = Positive assay, − = negative assay.

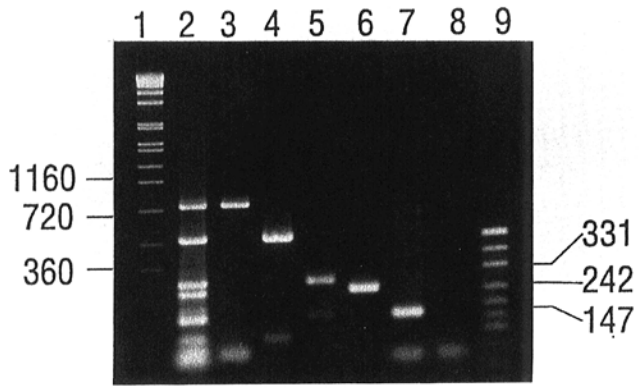

Fig. 4. PCR products amplified from total nucleic acids purified from plants infected with clover yellow vein virus (CYVV) (lane 3), cucumber mosaic virus (CMV) (lane 4), alfalfa mosaic virus (AMV) (lane 5), bean yellow mosaic virus (BYMV) (lane 6), subterranean clover mottle virus (SCMoV) (lane 7), or a pooled sample of all five extracts (lane 2) electrophoresed in 1.5% agarose/TBE and stained with ethidium bromide. Reaction mixture contained a cocktail of primers, each specific to one of the five viruses. Lane 8 contains PCR products from nucleic acids from uninoculated plants amplified with the same primer cocktail. Lane 1 contains size markers of *Eco*RI digested *Spp*I DNA, and lane 9 contains size markers of *Hpa*II digested pUC19 DNA.

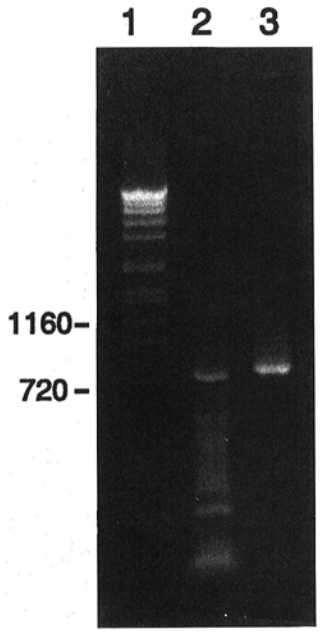

Fig. 5. PCR products amplified from total nucleic acids purified from uninoculated plants. Reaction mixture in lane 2 contained a cocktail of primers, each specific to one of the five viruses and primers specific to ribosomal 18S sequence. Lane 3 contains PCR products from nucleic acids from uninoculated plants amplified with the 18S-specific primers. Lane 1 contains size markers of *Eco*RI digested *Spp*I DNA.

investigated the use of conserved ribosomal sequences for this purpose. As there is no sequence available for the ribosomal RNA genes of subterranean clover but there is a conservation of these genes among all organisms, the 18S ribosomal RNA sequences (from Genbank) of radish, mung bean, soybean, tomato, strawberry, mustard, and *Arabidopsis* were aligned. Two conserved regions 800 bases apart were selected as primer targets. Performing PCR with these primers and extracts from uninfected subterranean clover cvs. Mount Barker, Karridale, and Woogenellup gave a single band of the expected size for each cultivar. However, when the nine-primer cocktail for the seedborne viruses was added to the 18S primers and used to amplify extracts from uninfected material, a number of smaller bands (Fig. 5) were produced in addition to the expected 800-bp band. Further investigation showed that most of the virus-specific primers in the cocktail were involved with the production of these spurious bands. We therefore did not include the 18S primers in subsequent assays.

DISCUSSION

With increasing distribution of germ plasm between researchers and countries, it is important that germ plasm banks do not harbor and distribute seedborne viruses. There are five seedborne viruses known to be potential threats to the integrity of subterranean clover and medic seed banks in Australia. Currently, seed banks monitor for viral infection largely on the basis of symptoms in the plants when grown to propagate the seed stocks. A number of methods could be used for detecting these viruses, including ELISA and radioactive or nonradioactive nucleic acid probes (1). However, these techniques are expensive and labor-intensive. We have defined the primer sequences, extraction procedure, and reaction conditions for an RT-PCR assay for these viruses that is likely to be an attractive alternative method for screening such germ plasm banks.

Our RT-PCR assay is in excess of 10^5 times more sensitive than ELISA and can detect and identify all five seedborne viruses in one assay in a single tube reaction. In fact, this sensitivity necessitates the use of great care to avoid contamination between samples. The assay was as effective as bioassay or ELISA for the detection of AMV in glasshouse and field samples and was also able to detect CMV in an infected seed lot. This has also been true in two further experiments comparing ELISA and PCR for the detection of SCSV in 55 field samples (data not shown). Furthermore, in our experiments with a range of different strains of the viruses we were able to readily detect all strains. Viruses have regions within their genomes that are more conserved than others (9), and where possible, our primers were designed to hybridize to these sequences. However, as more virus sequence information becomes available, the conserved regions of a particular virus should be more easily identified and thus lead to the design of even better primers.

It would be desirable to have an internal positive control in our assay to confirm that a negative virus result was not due to failure of the PCR reaction such as may be caused by inhibitors in the sample extract. We explored this possibility by including in the primer cocktail a pair of primers that would amplify a plant RNA irrespective of the presence of virus. This primer pair was targeted at the ribosomal 18S RNA. However, using these primers in conjunction with the virus-specific primers resulted in numerous spurious bands. This is probably due to the relative abundance of ribosomal RNA and the viral primers acting as random primers on this RNA. A better internal control would be using primers targeted at the mRNA of a constitutively expressed single gene that is conserved in legumes.

When both CYVV and BYMV were present in the sample and amplification of their appropriate regions was dependent on a common 3′ primer, we observed a marked reduction in the amplification of the larger band diagnostic of CYVV. This is presumably due to competition for the common primer by the two different templates. However, this should not be a problem, as plants are unlikely to be coinfected with both viruses.

We have also designed primers and found RT-PCR conditions for the detection of SCSV and SCRLV, which are the two other viruses known to infect subterranean clover. The capacity to readily detect all seven viruses that infect subterranean clover should not only aid germ plasm bank curators maintain a virus-free hygiene but should also provide a tool for research into the incidence, ecology, and epidemiology of these viruses in subterranean clover pastures.

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