Development of a strand-specific RT-PCR to detect the positive sense replicative strand of Soybean blotchy mosaic virus

Elrea Strydom[a, b]

[a]Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002, South Africa; [b]Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, 0002, South Africa.

elrea.appelgryn@fabi.up.ac.za

Gerhard Pietersen[a, b, c]

[c]Genetics Department, University of Stellenbosch, Stellenbosch, 7600, South Africa; [a]Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002, South Africa; [b]Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, 0002, South Africa.

Corresponding author at: Genetics Department, University of Stellenbosch, Stellenbosch, 7600, South Africa.

gerhard.pietersen@up.ac.za

Highlights

- A positive-strand specific RT-PCR detecting the intermediate replicative strand of Soybean blotchy mosaic virus (SbBMV) was developed.
- cDNA synthesis using a tagged reverse transcription primer, PCR amplification using a tag-specific primer and removal of residual reverse transcription primer ensures specificity of the assay.
- This assay can be used to study the replication of SbBMV in its insect vector in future.

Abstract

Soybean blotchy mosaic virus (SbBMV), a plant virus of the genus *Cytorhabdovirus* is an economically important virus of soybean reported only from the warmer, lower-lying soybean production areas in South Africa. The virus consistently appears in soybean crops annually in spite of the absence of soybean plants in winter. One possible reason for this may be that the virus replicates and hence persists in the SbBMV vector, a leafhopper, *Peragallia caboverdensis*. RNA viruses with antisense genomes as inferred for SbBMV produce positive sense RNAs as intermediate replicative forms during replication in their hosts, and detection of the positive strand in the plant host or vector is evidence of virus replication. In this study, a positive-strand specific RT-PCR (pss-RT-PCR) was developed to detect the positive RNA strand of SbBMV and validated on nine SbBMV isolates from soybean. The effect of tagged reverse transcription (RT) primers for cDNA synthesis, coupled with PCR using a tag-specific primer, as well as removal of unincorporated RT primers following cDNA synthesis was assessed. The positive RNA strand of SbBMV in infected plants was successfully detected following this protocol. Reverse transcription with forward and unmodified reverse primers confirmed that the assay was not able to detect the genomic sense RNA or self-primed cDNAs, lacking the non-viral tag, respectively. However, Exonuclease I (ExoI) treatment of cDNA was required to eliminate false-positive results during PCR amplification.

Keywords: replication; rhabdovirus; positive strand-specific RT-PCR

1. Introduction

Soybean blotchy mosaic virus (SbBMV) is a single-stranded, negative sense RNA virus of the genus *Cytorhabdovirus* within the family *Rhabdoviridae*. SbBMV was first detected in the North-West, Limpopo and Mpumalanga provinces of South Africa during surveys conducted to identify viruses of soybean in the 1990s (Pietersen and Garnett 1990; Pietersen 1993; Pietersen et al. 1998). Despite the annual nature of soybean production, infected leaves display the characteristic blotchy mosaic symptoms early in the season each year, with symptoms declining with time. Isolation and characterization led to the identification of bacilliform-shaped virions distributed in the cytoplasm of host cells, leading the authors to hypothesizing that the virus was a member of the cytorhabdoviruses (Lamprecht et al. 2010). This was confirmed by phylogenetic analysis when the nucleotide sequence of a 522 nt portion of the RNA-dependent RNA polymerase (L) gene was also determined. The leafhopper *Peragallia caboverdensis* (Lindberg) was identified as the insect vector of SbBMV (Lamprecht et al. 2010).

Four plant-infecting genera, *Cytorhabdovirus*, *Nucleorhabdovirus*, *Dichorhavirus* and *Varicosavirus* are distinguished within the family *Rhabdoviridae* (Amarasinghe et al. 2017). Dichorha- and varicosaviruses have two genome segments, while cyto- and nucleorhabdoviruses have a single genomic RNA and are classified based on their intracellular location and sites of replication (Jackson et al. 2005). The linear genomes of rhabdoviruses are between 12 and 14.5 kb in size, and encode five structural proteins, the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and L protein (Jackson et al. 2005; Dietzgen and Kuzmin 2012). In addition, plant nucleo-and cytorhabdovirus genomes also contain between one and four additional open reading frames at position X between the P and M genes, or position Y between the G and L genes, postulated to be involved in movement from cell to cell and other uncharacterized functions.

Members of the *Cytorhabdovirus* and *Nucleorhabdovirus* genera are transmitted in a persistent, propagative manner by their insect vectors which include leafhoppers (*Cicadellidiae*), planthoppers (*Delphacidae*) and aphids (*Aphidae)* (Hogenhout et al. 2003). Examples include maize mosaic virus (Whitfield et al. 2015; Barandoc-Alviar et al. 2016), barley yellow striate virus, northern cereal mosaic virus and wheat chlorotic streak virus (Conti 1985). Positive sense mRNAs corresponding to each gene and full length complements of the genomic RNA are

produced during the replication of RNA viruses to serve as mRNA and genomic RNAs for packaging into virions respectively (Plaskon et al. 2009). For an antisense RNA virus such as SbBMV, this would implicate the production of positive sense RNAs which function as mRNA and a replicative intermediate for the production of negative sense genomic RNAs. Strand-specific RT-PCR (ss-RT-PCR) and ss-RT-qPCR is used to detect and quantify these replicative forms during studies of RNA virus replication (Craggs et al. 2001; Gu et al. 2007; Gunji et al. 1994; Komurian-Pradel et al. 2004; Lanford et al. 1994; Lin et al. 2002; Plaskon et al. 2009; Purcell et al. 2006).

Previous studies have reported that standard, unmodified virus-specific primers are not able to differentiate between strand-specific cDNAs transcribed from virus-specific primers or those generated through false priming, especially when the complementary strand is present at high levels (Plaskon et al. 2009; Purcell et al. 2006; Komurian-Pradel et al. 2004; Craggs et al. 2001). cDNA synthesis using strand-specific primers with a non-viral tag sequence at the 5' end incorporates the unique tag into the cDNA. Subsequent PCR with a tag-specific primer results in the specific amplification of cDNAs with the tag sequence, which eliminates amplification and thus detection of cDNAs that are the result of false priming. The use of thermostable reverse transcriptases, such as rTth which allow for reverse transcription at temperatures as high as 70 °C, increasing specificity, has also been reported (Craggs et al. 2001; Lanford et al. 1994). Despite the use of tagged primers in reverse transcription and tag-specific primers during PCR, factors such as reverse transcriptase activity or unincorporated tagged reverse primer in the PCR reaction can lead to false positive results (Craggs et al. 2001).

The replication of SbBMV within its vector may represent one of the mechanisms by which the virus persists between growing seasons. In the past, traditional and time intensive techniques such as electron microscopy and serial dilution passages were used to investigate whether viruses were transmitted by their insect vectors in a propagative manner (Nault and Rodriguez 1985; Jackson et al. 2005). The development of ss-RT-PCR directed at the replicative strands of SbBMV thus offers a fast, efficient tool to study the replication of SbBMV in *P. caboverdensis*.

In this study, the effect of a tagged RT primer and tag-specific PCR primer combination, as well as removal of ssDNA (primers) from cDNA prior to PCR amplification on the specificity of a positive strand-specific RT-PCR (pss-RT-PCR) for SbBMV was assessed. We report the

development of the first pss-RT-PCR for SbBMV. The assay relies on the use of a 5'-tagged antisense primer, of which the 3' end is complementary to the SbBMV L gene for reverse transcription, and Exonuclease I (Exo I) digestion of cDNA to remove primers before PCR. PCR amplification is performed using a sense primer specific to the SbBMV L gene and a tag-specific antisense primer. This protocol was then tested on nine SbBMV isolates from different temporal and geographical origins in South Africa. An internal control gene was first amplified to confirm RNA quality, and the presence of SbBMV in field samples confirmed using a diagnostic Soyblotch RT-PCR. This was followed by detection of replication using the newly established Soyblotch pss-RT-PCR.

2. Materials and methods

To determine the specificity of the Soyblotch pss-RT-PCR for the positive strand of SbBMV, different primers were used for reverse transcription to produce cDNAs of specific polarities to serve as positive and negative controls for the ss-RT-PCR. The Soyblotch F primer was used to prime reverse transcription in order to generate cDNAs of negative polarity, while the Soyblotch R and Soyblotch R-Tag primers were used to produce cDNAs corresponding to the positive sense strand, which is the replicative intermediate of SbBMV. The cDNAs transcribed from the Soyblotch F and Soyblotch R primers served as negative controls for the Soyblotch pss-RT-PCR, as the ss-RT-PCR should be specific for the positive sense strand and positive sense cDNAs containing the non-viral tag sequence. Similarly, reverse transcription in the absence of primer was performed to confirm that it is not possible to amplify cDNAs lacking the non-viral tag sequence using the Soyblotch pss-RT-PCR. cDNAs transcribed using the Soyblotch R-Tag primer served as positive control for the Soyblotch pss-RT-PCR as a result of a positive polarity and the presence of the non-viral tag sequence.

2.1 Primer design

Soyblotch F and Soyblotch R primers (Table 1) were designed to amplify a 354 bp portion of the SbBMV L gene sequence (EU 877231.1) available on the National Centre for Biotechnology Information (NCBI) database by RT-PCR, and serves as diagnostic Soyblotch RT-PCR for detection of SbBMV in plants and *P. caboverdensis* (Strydom and Pietersen 2017, 2018). In the Soyblotch pss-RT-PCR a modified Soyblotch R primer (Table 1) with a 5' tag was used for

reverse transcription. The non-viral sequence used as tag on the Soyblotch R-Tag primer and the tag-specific primer was obtained from literature (Lin et al. 2002; Komurian-Pradel et al. 2004). The Soyblotch F and tag-specific reverse primer (Table 1) were used for PCR amplification of cDNA transcribed using the Soyblotch R-Tag primer in the Soyblotch pss-RT-PCR. PCR products of the Soyblotch pss-RT-PCR were 387 bp in size.

Table 1. Nucleotide sequences (5'-3') of oligonucleotide primers used in this study.

Primer	Sequence
rbcLa F	ATGTCACCACAAACAGAGACTAAAGC
rbcLa R	GTAAAATCAAGTCCACCRCC
Soyblotch F	CTTTGCCCAACTGGACTCCC
Soyblotch R	TCCAAACAGTCTTCCCAGGC
Soyblotch R-Tag	GGCCGTCATGGTGGCGAATAATCCAAACAGTCTTCCCAGGC
Tag-specific primer	AATAAATCATAAGGCCGTCATGGTGGCGAATAA

The non-viral tag sequence on the Soyblotch R-Tag primer is underlined.

2.2 RNA extraction

Infected soybean leaf material in which SbBMV replicates has RNA of both polarities present, and two isolates, 16/4265 and 16/4266 (Table 2) were used as positive controls to optimize and test a new pss-RT-PCR for SbBMV. Once established, the protocol was tested on nine other soybean field samples collected from different years and geographic origins in South Africa. Symptomatic leaf material was collected in the field, assigned a unique accession number and stored in plastic bags until processed. Dried leaf material was also obtained from the Virus, Antiserum and Seroreagent (PVAS) collection at the Agricultural Research Council-Plant Protection Research Institute (ARC-PPRI) in Pretoria, South Africa. Leaf material was homogenized in liquid nitrogen, and total RNA extracted according to the method of White et al. (2008) with modifications as described by Strydom and Pietersen (2017, 2018). Shortly, 1.8 ml cetyltrimethylammonium bromide (CTAB) buffer (2% CTAB; 2% polyvinylpyrrolidone (PVP) K-40; 25 mM ethylenediaminetetraacetic acid (EDTA); 100 mM Tris-Hydrochloride (Tris-HCL) pH 8; 2 M sodium chloride (NaCl) and 3% β-mercaptoethanol), heated to 65°C was used per sample. Homogenized leaf material was added to buffer and incubated at 65°C for 30 min with vortexing every 5 min. Supernatant was collected by centrifugation, followed by two sequential

chloroform:isoamylalcohol (C:I) (24:1) extractions through the addition of an equal volume C:I and centrifugation. All centrifugation steps on the first day were performed at 11 337 x*g* for 15 min. Lithium chloride (LiCl) was added to a final concentration of 2M, and incubated at 4°C overnight. The following day, RNA was precipitated by centrifugation for 60 min, the supernatant discarded and the pellet washed with 500 µl 70% ethanol. Ethanol was removed, and pellet resuspended in 50 µl molecular grade H_2O after air drying.

Table 2. Host, geographical origin, year of collection and accession information of SbBMV isolates used in this study.

Accession	Host	Origin	Year
15/3079	*Glycine max*	Loskop Irrigation Scheme, Mpumalanga, South Africa	2015
15/3080	*Glycine max*	Loskop Irrigation Scheme, Mpumalanga, South Africa	2015
15/3083	*Glycine max*	Loskop Irrigation Scheme, Mpumalanga, South Africa	2015
16/4265	*Glycine max*	Brits, North West, South Africa	2016
16/4266	*Glycine max*	Brits, North West, South Africa	2016
17/5002	*Glycine max*	Brits, North West, South Africa	2017
95/0015	*Glycine max*	Loskop Irrigation Scheme, Mpumalanga, South Africa	1995
95/0038	*Glycine max*	Thabazimbi, Limpopo, South Africa	1995
95/0073	*Glycine max*	Pretoria, Gauteng, South Africa	1995
03/4025	*Glycine max*	Lusikisiki, Eastern Cape, South Africa	2003
03/4033	*Glycine max*	Lusikisiki, Eastern Cape, South Africa	2003

2.3 Reverse transcription

Complementary strand synthesis followed a standard protocol with only different primers utilized. The Soyblotch F, Soyblotch R or Soybotch R-Tag primers were used to initiate reverse transcription in order to test the specificity of the pss-RT-PCR assay. Five µl primer (4 µM primer in final reaction) was allowed to anneal to 2 µl RNA by heating to 70 °C for 5 min, and cooling for 5 min at 4 °C. 2.5 µl M-MLV Reverse transcriptase 5x Reaction buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT in final working solution) (Promega, Madison, USA), 1.25 µL dNTPs (10 mM each dATP, dCTP, dGTP and dTTP) (Kapa Biosystems, Wilmington, USA), 0.125 µl (25 U) M-MLV Reverse Transcriptase (Promega) and 1.7 µl molecular grade H_2O to a final volume of 12.5 µl was added to the annealed primer and template. The reaction was incubated at 42 °C for 60 min. Reactions were performed in

duplicate, with one reaction subjected to ExoI digestion following reverse transcription to remove any remaining RT primer to evaluate the effect of unincorporated primers on the specificity of the Soyblotch pss-RT-PCR assay. This involved cDNA (12.5 µl) being incubated with 1 µl Exo I (20 U) (Thermo Scientific, Waltham, USA) at 37 °C for 15 min, followed by incubation at 85 °C for 15 min.

2.3.1 Reverse transcription of the genomic (negative) strand of SbBMV

The Soyblotch F primer was used in cDNA synthesis for the diagnostic Soyblotch RT- PCR to confirm the presence of SbBMV in soybean plant material. cDNAs corresponding to the genomic sense RNA of 16/4265 and 16/4266 served as negative control in Soyblotch pss-RT-PCR optimization.

2.3.2 Reverse transcription of the antigenomic (positive) strand of SbBMV

The Soyblotch R and Soyblotch R-Tag primers were used to transcribe the positive (replicative) strand of the SbBMV L gene. The unmodified and tagged Soyblotch R primers were compared for use in detecting virus-specific RNAs.

2.3.3 Reverse transcription of falsely primed cDNAs

To assess the effect of non-specific priming during reverse transcription and the specificity of the Soyblotch pss-RT-PCR for cDNAs with the non-viral tag, cDNA was produced as described above with primer replaced with water.

2.3.4 Reverse transcription of internal control gene

As internal control for RNA quality, the plant gene ribulose 1,5-bisphosphate carboxylase (RuBisCo) was amplified from RNA of all isolates using the rbcLa primer pair (Levin et al. 2007; Kress and Erickson 2007) following cDNA synthesis using random hexamer primers.

2.4 PCR amplification and Sanger sequencing

Each 25 µl PCR amplification reaction consisted of 2 µl cDNA, 5 µl MyTaq Reaction buffer (1 mM dNTPs, 3 mM $MgCl_2$) (Bioline, London, UK), 0.5 µl (0.2 µM) forward primer, 0.5 µl (0.2 µM) reverse primer, 0.25 µl (1.25 U) MyTaq DNA polymerase (Bioline) and 16.8 µl molecular

grade H_2O. PCR cycling conditions for the Soyblotch RT-PCR and pss-RT-PCR consisted of an initial denaturation step at 95 °C for 1 min, followed by 35 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 10 s. Final extension of PCR products took place at 72 °C for 5 min. PCR products (6 µl) were separated by agarose gel electrophoresis on ethidium bromide-stained 2% agarose gels, and viewed under UV light. PCR products were purified for sequencing through the addition of 0.5 µl (10 U) Exo I (Thermo Scientific) and 2 µl (2 U) FastAP Alkaline Phosphatase (Thermo Scientific) to each 19 µl PCR product. This was followed by two 15 min incubation steps, first at 37 °C, followed by incubation at 85 °C. Each 10 µl sequencing reaction consisted of 1 µl template DNA, 1 µl of BigDye Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Foster City, USA), 2.25 µl 5x Sequencing buffer (Applied Biosystems) and 2 µM of each primer. Sequences obtained were used in Basic Local Alignment Search Tool (BLASTn) searches on the NCBI database.

2.4.1 Diagnostic Soyblotch RT-PCR

The Soyblotch F and Soyblotch R primers were used in the diagnostic Soyblotch RT-PCR to confirm the presence of SbBMV in field samples. To compare the specificity in detection between the diagnostic Soyblotch RT-PCR and the Soyblotch pss-RT-PCR cDNAs transcribed in the absence of primer was also subjected to the Soyblotch RT-PCR.

2.4.2 Soyblotch pss-RT-PCR

To investigate the possible increased specificity in detection conferred by the Soyblotch R-Tag RT primer cDNA transcribed from both the standard and modified Soyblotch R primer were subjected to PCR amplification using the Soyblotch F and tag-specific primer. cDNAs transcribed from the Soyblotch F primer and in the absence of RT primer were also subjected to the Soyblotch pss-RT-PCR to confirm the specificity of the Soyblotch pss-RT-PCR for the positive strand and to compare differences in the specificity of detection between the diagnostic Soyblotch RT-PCR and the Soyblotch pss-RT-PCR respectively. PCR amplification of cDNAs transcribed in the absence of primer, together with the corresponding positive controls was performed at 66°C in order to eliminate low-level amplification in the Soyblotch pss-RT-PCR.

2.4.3 PCR amplification of the RuBisCo gene region

The rbcLa F and rbcLa R primer pair were used for amplification of the RuBisCo gene. The PCR programme used was identical to what is described above, with the exception that an annealing temperature of 55 °C was used.

2.5 Sensitivity of Soyblotch pss-RT-PCR

To assess the sensitivity of the pss-RT-PCR, 10-fold serial dilutions of Soyblotch RT- PCR products were prepared. The initial starting concentration was determined with a Qubit 2.0 fluoremeter (Life Technologies, Carlsbad, California), according to the manufacturer's instructions. Second strand synthesis using the Soyblotch R-Tag primer, Exo I treatment and PCR amplification was performed as described above using the Soyblotch F and tag-specific primer pair (Soyblotch pss-RT-PCR), with the exception that primer and template were heated to 95 °C and cooled to 4 °C to denature double stranded template DNA before second strand synthesis.

3. Results

3.1 Specificity of Soyblotch pss-RT-PCR

The specificity of the assay was tested on RNA extracts of two plant isolates, 16/4265 and 16/4266, which had tested positive for SbBMV using the diagnostic Soyblotch RT-PCR. RNA of both the genomic and anti-genomic strand should be present in infected plant material in which the virus is replicating. Reverse transcription of the positive sense RNA strand was primed by the Soyblotch R-Tag and the Soyblotch R primer, and cDNA transcribed from the Soyblotch F primer was used to confirm that PCR amplification of the antisense RNA strand did not occur.

3.1.1 Absence of detection of genomic RNA and misprimed cDNAs using the Soyblotch pss-RT-PCR

cDNA synthesis using the Soyblotch F, unmodified Soyblotch R and no RT primer was performed to assess whether the assay non-specifically detects the negative RNA strand, and its ability to detect falsely-primed cDNAs. PCR amplification of cDNA not digested with ExoI transcribed from the Soyblotch F primer resulted in multiple bands corresponding to non-specific

PCR amplification (Fig 1). A band of the size of the PCR product of the unmodified Soyblotch primer pair (354 bp) was also present, but following Exo I digestion of unincorporated Soyblotch F primers, a single band of approximately 200 bp was obtained. BLASTn analysis of the sequence of the 200 bp PCR product indicated cross reaction of primers with multiple plant chloroplast genomes (E-value 0.0) in the NCBI database. The Soyblotch pss-RT-PCR thus failed to amplify negative strand RNA when the Soyblotch pss-RT-PCR included an Exo I digestion following reverse transcription.

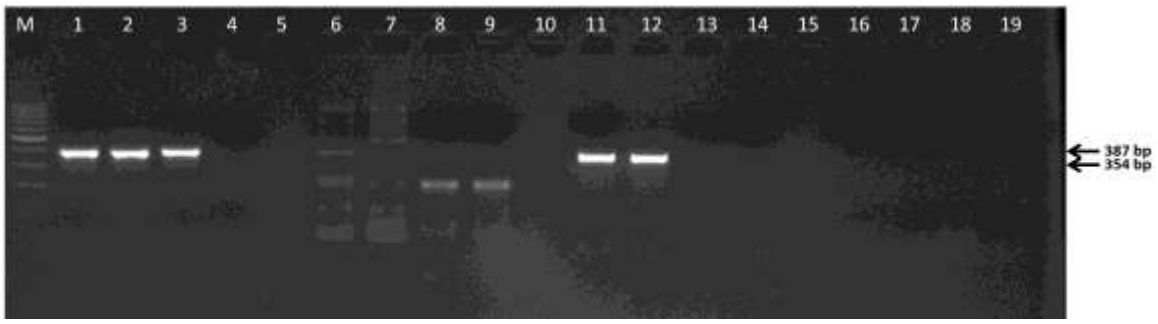

Fig 1. **Impact of the primer used for cDNA transcription and Exonuclease I (Exo-I) treatment on the specificity of the Soyblotch pss-RT-PCR assay'.**. M, 100 bp DNA ladder; 1, 16/4265 Soyblotch R-Tag; 2, 16/4266 Soyblotch R-Tag; 3, 16/4265 Soyblotch R-Tag digested with ExoI following cDNA synthesis; 4, 16/4266 Soyblotch R-Tag digested with ExoI following cDNA synthesis; 5, unused lane; 6, 16/4265 Soyblotch F; 7, 16/4266 Soyblotch F; 8, 16/4265 Soyblotch F digested with ExoI following cDNA synthesis; 9, 16/4266 Soyblotch F digested with ExoI following cDNA synthesis; 10, unused lane; 11, 16/4265 Soyblotch R; 12, 16/4266 Soyblotch R; 13, 16/4265 Soyblotch R digested with ExoI following cDNA synthesis; 14, 16/4266 Soyblotch R digested with ExoI following cDNA synthesis; 15, unused lane; 16, Soyblotch R-Tag no template cDNA control; 17, Soyblotch F no template cDNA control; 18, Soyblotch R no template cDNA control, 19, no template PCR control. The bigger band present in lanes 1-3 corresponds

PCR products from amplification of cDNA produced from the Soyblotch R primer were present in the absence of Exo I treatment of cDNA (Fig 1). Unincorporated Soyblotch R primers from the reverse transcription reaction enabled amplification of a portion of the SbBMV polymerase gene as a result of the presence of both the Soyblotch F and Soyblotch R primer. This PCR product corresponds to the PCR product usually obtained from the unmodified Soyblotch primer pair. Sequence analysis of the PCR product also showed identity to a portion of the L gene of SbBMV (BLASTn E-values of 0.0 to EU 877231.1). However, following Exo I digestion of cDNA, this non-specific amplification was eliminated.

PCR amplification of cDNA transcribed in the absence of a primer using the Soyblotch RT-PCR yielded a band of 354 bp (Fig 2). Amplification using the Soyblotch F and tag-specific primers of the Soyblotch pss-RT-PCR was however better able to discriminate between falsely-primed cDNAs and cDNAs transcribed from the positive sense RNA strand, as no amplification occurred in the latter case. The low-level false-positive results observed at lower annealing temperatures in this experiment may possibly be the result of contamination of reagents or the environment with Soyblotch R-Tag primer or PCR products of the Soyblotch pss-RT-PCR. The increased annealing temperature used in this experiment was not used in the standard Soyblotch pss-RT-PCR as this was balanced with an increased probability of obtaining false negative results.

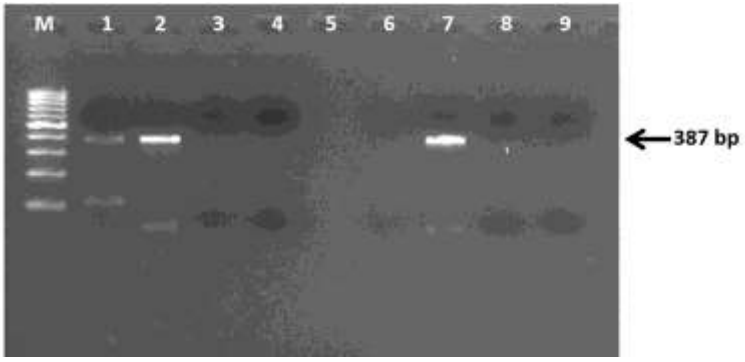

Fig 2. Specificity analysis of pss-RT-PCR using a tag-specific primer for PCR amplification. Lanes 1-4 represent PCR amplification using the Soyblotch F and Soyblotch R primer pair, and lanes 6-9 PCR amplification using the Soyblotch F and tag-specific primer. M, 100 bp DNA ladder; 1, 16/4265 reverse transcription in absence of primer; 2, 16/4265 reverse transcription with Soyblotch R-Tag primer; 3, no template cDNA synthesis control; 4 no template PCR control; 5, unused lane; 6, 16/4265 reverse transcription in absence of primer; 7, 16/4265 reverse transcription with Soyblotch R-Tag primer; 8, no template cDNA synthesis control; 9, no template PCR control.

3.1.2 Specificity of Soyblotch pss-RT-PCR for positive sense strand

PCR amplification of cDNA not subjected to ExoI digestion transcribed from the Soyblotch R-Tag primer yielded amplification in both 16/4265 and 16/4266 (Fig 1). In contrast, amplification was only observed for 16/4265 after unincorporated Soyblotch R-Tag primer from cDNA synthesis was removed by Exo I digestion. The sequence of the PCR product amplified from cDNA synthesis using the Soyblotch R-Tag primer showed significant homology (BLASTn E-value of 0.0) to the SbBMV polymerase sequence (EU 877231.1) in the NCBI database

(http://www.ncbi.nlm.nih.gov/). Specific detection of the positive strand RNA of SbBMV thus relies on Exo I digestion of unincorporated primers to avoid false positive results.

3.2 Sensitivity of Soyblotch pss-RT-PCR

The sensitivity of the pss-RT-PCR was evaluated by performing cDNA synthesis using a dilution series of PCR products, ranging between 196 fg and 19.6 ag. The pss-RT-PCR was able to amplify template across the first five dilutions, and was thus able to detect the tagged, positive RNA strand of SbBMV across five orders of magnitude (Fig 3).

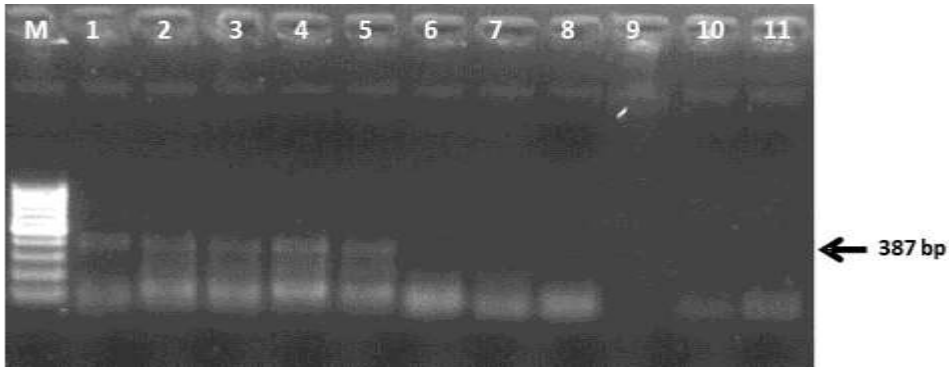

Fig 3. Sensitivity analysis of pss-RT-PCR for SbBMV. M, 100 bp DNA ladder; 1, 196 pg; 2, 19.6 pg; 3, 1.96 pg; 4, 196 fg; 5, 19.6 fg; 6, 1.96 fg; 7, 196 ag; 8, 19.6 ag; 9, unused lane; 10, no template cDNA synthesis control; 11, no template PCR control.

3.3 Screening of SbBMV isolates using Soyblotch pss-RT-PCR

PCR amplification using the RuBisCo-RT-PCR and Soyblotch RT-PCR was successful in all nine isolates tested, confirming RNA quality for RT-PCR analysis and presence of SbBMV in soybean plant material respectively (Fig 4). For all isolates except 95/0015 and 03/4033 the positive strand was successfully detected using the Soyblotch pss-RT-PCR, confirming the replication of the specific accessions in soybean plant material.

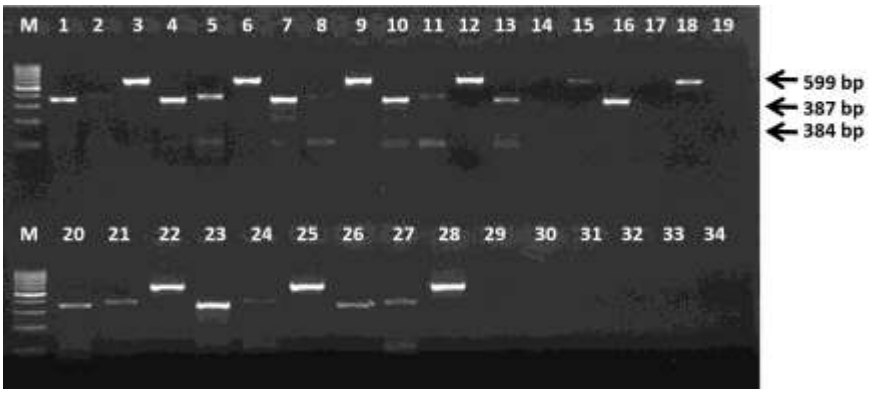

Fig 4. Analysis of field samples using the pss-RT-PCR. M, 100 bp DNA ladder; 1, 15/3083 Soyblotch RT-PCR product; 2, 15/3083 pss-RT-PCR product; 3, 15/3083 *rbcLa* PCR product; 4, 15/3080 Soyblotch RT-PCR product; 5, 15/3080 pss-RT-PCR product; 6, 15/3080 *rbcLa* PCR product; 7, 95/0038 Soyblotch RT-PCR product; 8, 95/0038 pss-RT-PCR product; 9, 95/0038 *rbcLa* PCR product; 10, 17/5002 Soyblotch RT-PCR product; 11, 17/5002 pss-RT-PCR product; 12, 17/5002 *rbcLa* PCR product; 13, 95/0015 Soyblotch RT-PCR product; 14, 95/0015 pss-RT-PCR product; 15, 95/0015 *rbcLa* PCR product; 16, 03/4033 Soyblotch RT-PCR product; 17, 03/4033 pss-RT-PCR product; 18, 03/4033 *rbcLa* PCR product; 19, unused lane; 20, 15/3079 Soyblotch RT-PCR product; 21, 15/3079 pss-RT-PCR product; 22, 15/3079 *rbcLa* PCR product; 23, 95/0073 Soyblotch RT-PCR product; 24, 95/0073 pss-RT-PCR product; 25, 95/0073 *rbcLa* PCR product; 26, 03/4025 Soyblotch RT-PCR product; 27, 03/4025 pss-RT-PCR product; 28, 03/4025 *rbcLa* PCR product; 29, Soyblotch RT-PCR no template cDNA synthesis control; 30, pss-RT-PCR no template cDNA synthesis control; 31, *rbcLa* RT-PCR no template cDNA synthesis control, 32, Soyblotch RT-PCR no template PCR control; 33, pss-RT-PCR no template PCR control; 34, *rbcLa* no template PCR control.

4. Discussion

In this study, a pss-RT-PCR, enabling the detection of the positive sense, anti-genomic RNA strand of SbBMV was developed. The use of a reverse primer tagged with a non-viral sequence during reverse transcription, combined with a tag-specific primer during PCR amplification allowed for the specific detection of the SbBMV replicative strand, eliminating false positives as a result of mis-priming. Exo I treatment of cDNA before PCR amplification further enhanced the specificity of the assay, preventing amplification as a result of false-priming and contaminating Soyblotch R-Tag RT primer in the PCR reaction.

Reports in literature of reverse transcription of cDNAs in the absence of primers illustrates how false-priming can occur (Timofeeva and Skrypina 2001; Peyrefitte et al. 2003). Priming of reverse transcription by the reverse transcriptase through secondary structures present in RNA or

other short endogenous or exogenous sequences have been postulated as possible mechanisms facilitating false priming of cDNAs. Thus when unmodified or no reverse primers are used for cDNA synthesis, both specific and falsely-primed cDNAs can be amplified by virus specific primers in a PCR reaction.

This was observed when RNA of 16/4265 was subjected to reverse transcription in the absence of any primer. Subsequent PCR amplification of cDNA using the Soyblotch RT-PCR led to the amplification of the 354 bp portion of the SbBMV L gene against which the Soyblotch RT-PCR primer pair is directed. Due to the lack of primers in the reaction, the strand from which false-priming occurred cannot be determined, but it is likely that the genomic strand, which is more abundant, served as template (Plaskon et al. 2009). False-priming of cDNA synthesis in the absence of primer in the reaction has also been reported in o' nyong-nyong (ONNV), dengue and hepatitis viruses (Plaskon et al. 2009; Peyrefitte et al. 2003; Gunji et al. 1994; Lanford et al. 1994).

By using a non-viral tagged RT primer coupled with a tag-specific primer during PCR amplification, only virus-specific cDNAs in which the non-viral tag sequence was incorporated during reverse transcription should be detected, eliminating amplification of falsely-primed cDNAs. No amplification occurred when PCR of falsely-primed cDNAs (reverse transcription without primer) was performed with the Soyblotch F and tag-specific primers (Soyblotch pss-RT-PCR). This specificity was also illustrated by failure to amplify positive RNA strand-specific products from cDNA transcribed from the Soyblotch F and unmodified Soyblotch R products. Reverse transcription using the Soyblotch F primer detects the genomic sense RNA of SbBMV, and is used in the diagnostic Soyblotch RT-PCR, and thus served as negative control for the Soyblotch pss-RT-PCR. Failure to amplify the 387 bp PCR product of the Soyblotch pss-RT-PCR thus confirmed the specificity of the assay for the positive strand RNA.

However, failure to remove unincorporated RT primer can also lead to false positive results. Despite the improvements in the specificity of ss-RT-PCR as a result of the use of tagged primers and tag-specific primers, a number of other factors can still contribute towards non-specific amplification. This includes contamination of synthetic RNA transcripts with plasmid DNA, and contamination of PCR reactions with the primer used in reverse transcription (Craggs et al. 2001). The lack of strand-specificity observed after reverse transcription of 16/4266 with

the Soyblotch R-Tag primer as illustrated by the absence of amplification following Exo I treatment was attributed to self-priming and contamination of the PCR reaction with RT primer (Purcell et al. 2006). The presence of the Soyblotch R-Tag primer in the PCR reaction containing Soyblotch F and tag-specific primers allowed amplification of mis-primed cDNA.

The importance of the removal of all free, unincorporated RT primer was also observed in the PCR amplification of cDNA transcribed from the Soyblotch R primer. In the absence of Exo I digestion of cDNA, contaminating Soyblotch R primer in the PCR reaction allowed amplification of the Soyblotch RT-PCR PCR product without the tag. Exo I digestion of unincorporated primers, however, eliminated this false positive result of the slightly smaller PCR product. These results highlights the importance of combining Exo I digestion of cDNA before PCR amplification with tag-specific PCR primers to eliminate detection of false positive results.

After establishment of this protocol, nine isolates of SbBMV from five different locations in South Africa sampled over 12 years were selected for screening using the Soyblotch pss-RT-PCR. An internal plant barcoding gene was first amplified to ensure RNA integrity and the presence of SbBMV confirmed before performing the Soyblotch pss-RT-PCR. In seven out of the nine isolates, the positive strand of SbBMV was detected. The negative results in two isolates were unexpected, and might be due to a recovery phenotype and plant defence responses or simply tissue selection during the RNA extraction. The recovery of tobacco plants from symptoms induced by tobacco ringspot virus was the first report of a recovery phenotype in plants infected by plant viruses (Wingard 1928). Symptoms decline due to a decrease in virus titre, but the virus can still be present in recovered leaves, which are also often resistant to subsequent infections (Ghoshal and Sanfaçon 2015). This, however, further illustrates the specificity of the assay, as the positive sense RNA was not detected in all isolates positive for the presence of the virus.

In this study we described the development of a pss-RT-PCR to detect the replicative intermediate of SbBMV. The assay is capable of detecting the positive RNA strand of SbBMV specifically over five orders of magnitude, and will be used in future to detect the intermediate replicative RNA strand of SbBMV in its insect vector *P. carboverdensis*. An enhanced understanding of the replication dynamics of SbBMV in its vector will improve control strategies against the virus in future.

5. Acknowledgements

Financial support was provided by the Association of African Universities (AAU) and through the National Research Foundation Incentive Grant for Rated Scientists.

6. References

Amarasinghe, G. K., Bào, Y., Basler, C. F., Bavari, S., Beer, M., Bejerman, N., et al. (2017). Taxonomy of the order *Mononegavirales*: update 2017. *Archives of Virology, 162*(8), 2493-2504, doi:10.1007/s00705-017-3311-7.

Barandoc-Alviar, K., Ramirez, G. M., Rotenberg, D., & Whitfield, A. E. (2016). Analysis of acquisition and titer of Maize mosaic rhabdovirus in its vector, *Peregrinus maidis* (*Hemiptera: Delphacidae*). *Journal of Insect Science, 16*(1), doi:10.1093/jisesa/iev154.

Conti, M. (1985). Transmission of plant viruses by leafhoppers and planthoppers. In L. R. Nault, & J. G. Rodriguez (Eds.), *The leafhoppers and planthoppers* (pp. 289-307). New York: Wiley.

Craggs, J. K., Ball, J. K., Thomson, B. J., Irving, W. L., & Grabowska, A. M. (2001). Development of a strand-specific RT-PCR based assay to detect the replicative form of hepatitis C virus RNA. *Journal of Virological Methods, 94*, 111-120.

Dietzgen, R. G., & Kuzmin, I. V. (Eds.). (2012). *Rhabdoviruses: Molecular taxonomy, evolution, genomics, ecology, host-vector interactions, cytopathology and control.* Norfolk: Caister Academic Press.

Ghoshal, B., & Sanfaçon, H. (2015). Symptom recovery in virus-infected plants: Revisiting the role of RNA silencing mechanisms. *Virology, 479-480*, 167-179, doi:https://doi.org/10.1016/j.virol.2015.01.008.

Gu, C., Zheng, C., Shi, L., Zhang, Q., Li, Y., Lu, B., et al. (2007). Plus- and minus-stranded foot-and-mouth disease virus RNA quantified simultaneously using a novel real-time RT-PCR. *Virus Genes, 34*(3), 289-298, doi:10.1007/s11262-006-0019-2.

Gunji, T., Hijikata, M., Hayashi, K., & Saitoh, S. (1994). Specific detection of positive and negative stranded hepatitis C viral RNA using chemical RNA modification. *Archives of Virology, 134*, 293-302.

Hogenhout, S. A., Redinbaugh, M. G., & Ammar, E.-D. (2003). Plant and animal rhabdovirus host range: a bug's view. *Trends in Microbiology, 11*(6), 264-271, doi:http://dx.doi.org/10.1016/S0966-842X(03)00120-3.

Jackson, A. O., Dietzgen, R. G., Goodin, M. M., Bragg, J. N., & Deng, M. (2005). Biology of plant rhabdoviruses. *Annual Review of Phytopathology, 43*, 623-660.

Komurian-Pradel, F., Perret, M., Deiman, B., Sodoyer, M., & Lotteau, V. (2004). Strand specific quantitative real-time PCR to study replication of hepatitis C viral genome. *Journal of Virological Methods, 116*, 103-106.

Kress, W. J., & Erickson, D. L. (2007). A two-locus global DNA barcode for land plants: The coding *rbcL* gene complements the non-coding trnH-psbA spacer region. [doi:10.1371/journal.pone.0000508]. *PLOS ONE, 2*(6), e508.

Lamprecht, R. L., Kasdorf, G. G. F., Stiller, M., Staples, S. M., Nel, L. H., & Pietersen, G. (2010). Soybean blotchy mosaic virus, a new cytorhabdovirus found in South Africa. *Plant Disease, 94*(11), 1348-1354, doi:10.1094/pdis-09-09-0598.

Lanford, R. E., Surau, C., Jacob, J. R., White, R., & Fuerst, T. R. (1994). Demonstration of *in vitro* infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT-PCR. *Virology, 202*, 606-614.

Levin, R. A., Wagner, W. L., Hoch, P. C., Nepokroeff, M., Pires, J. C., Zimmer, E. A., et al. (2007). Family-level relationship of *Onagraceae* based on chloroplast *rbcL* and *ndhF* data. *American Journal of Botany, 90*(1), 107-115.

Lin, L., Fevery, J., & Hiem Yap, S. (2002). A novel strand-specific RT-PCR for detection of hepatitis C virus negative-strand RNA (replicative intermediate): evidence of absence or very low level of HCV replication in peripheral blood mononuclear cells. *Journal of Virological Methods, 100*(1–2), 97-105, doi:http://dx.doi.org/10.1016/S0166-0934(01)00399-8.

Nault, L. R., & Rodriguez, J. G. (Eds.). (1985). *The leafhoppers and planthoppers*. New York: John Wiley & Sons.

Peyrefitte, C. N., Pastorino, B., Bessaud, M., Tolou, H. J., & Couissinier-Paris, P. (2003). Evidence for *in vitro* falsely-primed cDNAs that prevent specific detection of virus negative strand RNAs in dengue-infected cells: improvement by tagged RT-PCR. *Journal of Virological Methods, 113*, 19-28.

Pietersen, G. Importance of a rhabdovirus-associated disease of soybeans in South Africa. In *6th International Congress of Plant Pathology, Montreal, Canada, 1993*

Pietersen, G., & Garnett, H. M. (1990). A survey for the viruses of soybeans (*Glycine max*) in the Transvaal, South Africa. *Phytophylactica, 22*(1), 35-40.

Pietersen, G., Staples, S. M., Kasdorf, G. G. F., & Jooste, A. E. C. (1998). Relative abundance of soybean viruses in South Africa. *African Plant Protection, 4*(2), 65-70.

Plaskon, N. E., Adelman, Z. N., & Myles, K. M. (2009). Accurate strand-specific quantification of viral RNA. *PLOS ONE, 4*(10).

Purcell, M. K., Hart, S. A., Kurath, G., & Winton, J. R. (2006). Strand-specific, real-time RT-PCR assays for quantification of genomic and positive-sense RNAs of the fish rhabdovirus, Infectious hematopoietic necrosis virus. *Journal of Virological Methods, 132*(1–2), 18-24, doi:http://dx.doi.org/10.1016/j.jviromet.2005.08.017.

Strydom, E., & Pietersen, G. (2017). Alternative hosts and seed transmissibility of soybean blotchy mosaic virus. *European Journal of Plant Pathology*, doi:10.1007/s10658-017-1361-z.

Strydom, E., & Pietersen, G. (2018). Diversity of partial RNA-dependent RNA polymerase gene sequences of soybean blotchy mosaic virus isolates from different host-, geographical- and temporal origins. *Archives of Virology*, doi:https://doi.org/10.1007/s00705-018-3722-0.

Timofeeva, A. V., & Skrypina, N. A. (2001). Background activity of reverse transcriptases. *Biotechniques, 30*, 22-24, 26, 28.

White, E. J., Venter, M., Hiten, N. F., & Burger, J. T. (2008). Modified cetyltrimethylammonium bromide method improves robustness and versatility: The benchmark for plant RNA extraction. *Biotechnology Journal, 3*, 1424–1428.

Whitfield, A. E., Falk, B. W., & Rotenberg, D. (2015). Insect vector-mediated transmission of plant viruses. *Virology, 479–480*, 278-289, doi:http://dx.doi.org/10.1016/j.virol.2015.03.026.

Wingard, S. A. (1928). Hosts and symptoms of ring spot, a virus disease of plants. *Journal of Agricultural Research, 37*, 127-153.