Development of Real-Time PCR for the Rapid Detection of Episomal *Banana streak virus* (BSV)

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ABSTRACT

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A real-time assay for the detection of episomal *Banana streak virus* (BSV; strain OL) in banana and plantains that carry integrated BSV sequences is described. Primers specific to the viral DNA were designed using the viral sequence integrated into the cv. Obino l'Ewai genome and the sequence of the genomic DNA of the infecting virus strain OL. They amplify a sequence of 1,336 bp that is detected in real-time by a short fluorogenic 3′ minor groove binder DNA probe. This method enables reproducible and specific detection of episomal BSV from purified DNA as well as from crude extracts from infected plants. The assay is rapid, adaptable for large-scale experiments, and circumvents carryover problems.

Additional keywords: badnavirus, long amplicon, minor groove binder (MGB), *Musa*, 5′ nuclease assay

Banana streak virus (BSV) is the causal agent of viral leaf streak of banana and plantains and is the most widely distributed virus of these crops (5). The virus is a member of the plant pararetroviruses genus *Badnavirus* (16), with nonenveloped bacilliform particles (episomal virus) containing a 7.4-kb circular double-stranded DNA (dsDNA) genome that is replicated by reverse transcription (9). The different virus strains show a high degree of serological and genomic heterogeneity (17).

BSV infection induces yield losses and restricts the international exchange of banana germ plasm. In recent years, the virus has caused increasing concern worldwide as infection of new banana hybrids with many desirable traits frequently occurs, curtailing their exploitation. Recent reports indicate that BSV infection may arise from the activation of viral sequences that are integrated into the *Musa* genome (10,20).

The Onne strain of BSV, now renamed BSV-OL (7), is particularly common in new hybrids developed by the international *Musa* breeding programs in Africa and Central and South America (3). BSV-OL sequences have been found integrated into the nuclear genome of cv. Obino l'Ewai (AAB group), which is used as a parent for several breeding programs (10,20). One of the characterized integrated sequence represents the full genome of the episomal virus BSV-OL, although the sequence is interspersed with a "scrambled" region containing inverted and noncontiguous viral and non-BSV sequences (20). This integrant recently was shown to be present in various BB and AAB cultivars and not in the AAA cultivars tested, suggesting that the integrated BSV-OL sequences are associated with the B genome of *Musa* (7). The sequence similarity between this integrated sequence and the genome of the virus BSV-OL argues against the activation of the integrant by excision of the scrambled region and circularization of the DNA to produce the episomal viral genome (20). Tissue culture and hybridization might be triggers for the activation of the integrant to produce BSV infection. The reasons why this activation occurs only in some plants of a given cultivar are still unknown.

This problem of virus activation suggests that traditional techniques of virus eradication, such as meristem tip culture, are not appropriate because these treatments would merely activate the integrated BSV sequences. Thus, further improvements in the production of BSV-free plants will require a better understanding of the activation process. Such information could be provided by studies on the infection frequency in the progeny of virus-free plants grown or treated under different conditions. These kinds of experiments may require the analysis of a large number of plants and, therefore, require the availability of a high throughput detection tool.

Current methods of detection of BSV include observation of symptoms, which are periodic and may be confused with those of other viruses; immunosorbent electron microscopy (ISEM), which is laborious and requires sophisticated material; enzyme-linked immunosorbent assay (ELISA), which is relatively insensitive (26); and immunocapture-polymerase chain reaction (IC-PCR), which requires pre- and post-PCR steps (4,8).

Real-time PCR, which increasingly is used in molecular diagnostics, eliminates the need for labor-intensive detection steps, prevents carryover contaminations and, therefore, would be particularly suitable for episomal BSV detection in large-scale experiments.

Although various types of fluorogenic probes can be used for real-time monitoring (18,22), the 5′ nuclease assay chemistry (1,12,15) is by far the most commonly used. Nevertheless, the length of the probe (25 to 40 nucleotides [nt]) which is necessary to obtain the required melting temperature (Tm) of 65 to 72°C can be problematic when targeting pathogens with high genomic variability. The conjugation at the 3′ end of the probe of a minor groove binding moiety circumvents this problem by stabilizing the probe-template duplex, increasing the melting temperature of the probe to 65 to 70°C even if shorter probes are used (13,14).

This article describes a 5′ nuclease assay based on a fluorescent 3′ minor groove binder (MGB) probe to rapidly and reproducibly detect episomal BSV. The assay is optimized for BSV strain OL.

MATERIAL AND METHODS

Plant material. Banana cvs. FHIA 21 (AAAB, ITC 1332), Pisang Lawadin (AAB, ITC 0449), and Obino l'Ewai (AAB, ITC 0109) were provided by the International Network for the Improvement of Banana and Plantain (INIBAP) Transit Center (ITC, Leuven, Belgium). Healthy cvs. Pisang Ceylan (AAB), Williams (AAA), and Cavendish (AAA) were kindly provided by Jean-Jacques Etame (Agricultural University of Gembloux, Belgium); and cv. Butuhan (BB) was provided by Glyn Harper (John Innes Center, Norwich, UK).

Nucleic acid extraction. The extraction protocol for total nucleic acids was adapted

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from the one described by Spiegel et al. (25) for *Prunus* spp. Fresh material (500 mg) was ground in five volumes of extraction buffer (200 mM Tris-HCl, pH 8.5, 1.5% sodium dodecyl sulfate, 300 mM LiCl, 10 mM EDTA, 1% Na deoxycholate, 1% Nonidet P40, 0.5% β-mercaptoethanol). Leaf extracts were transferred to microtubes and clarified by centrifugation for 5 min at 7,000 × *g* followed by a second centrifugation of the supernatant mixed with a half-volume of potassium acetate (6 M, pH 6.5) for 10 min at 12,000 × *g*. Nucleic acids contained in the supernatant were precipitated by 10 min of incubation at –20°C with one volume of cold isopropanol followed by centrifugation for 10 min at 12,000 × *g*. The pellet was rinsed with cold ethanol (70°C) and resuspended in 50 µl of sterile water after air drying. Extraction purity and nucleic acid concentration were determined by measurement of the absorbance at 260 and 280 nm in a spectrophotometer.

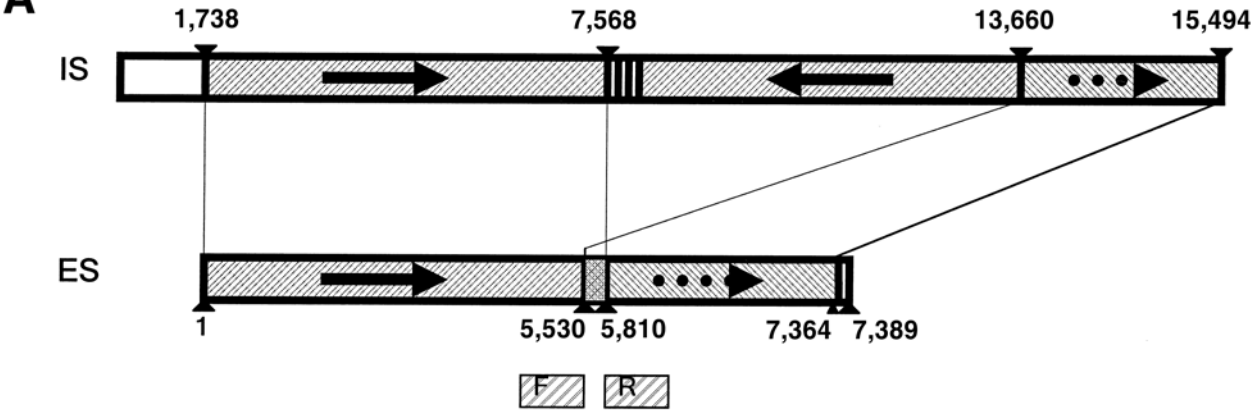
Crude extract preparation for real-time PCR. Crude extracts were prepared from *Musa* leaves by grinding leaf tissue in 10 volumes of 137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 3 mM NaN_3, 0.05% Tween 20, and 80 mM Na_2SO_3, pH 7.2 to 7.4, followed by centrifugation at 7,000 × *g* for 5 min. The supernatant was diluted 10 times in sterile water and kept on ice prior to PCR.

Primer and probe design. Primers and probe for episomal BSV were selected from sequence alignments using PILEUP (Wisconsin Package version 10.1; Genetics Computer Group GCG, Madison, WI) on sequences available in EMBL and Genbank databases.

Comparison of the published sequences of the virus BSV-OL (AJ002234) (9) and the sequence integrated in the cv. Obino l'Ewai (AF106946) (20) allowed the selection of primers that only amplified sequences from episomal BSV using standard conditions. They were designed from the most conserved sequences obtained by the alignment of the sequences BSV-OL (AJ002234) (9), BSV-Mysore (AF214005), BSV-Goldfinger (AF215814), and BSV-Red Dacca (AF215816) (6). The software Primer Express 5.1 (Applied Biosystems, Foster City, CA) was used for Tm calculations and primer-dimer evaluation.

The 3′ MGB DNA probe was selected by following the general rules outlined by Livak et al. (15) and Kutyavin et al. (13) using the software Primer Express 5.1 for accurate Tm calculations. Sequences of other badnaviruses were used for the alignments: *Cacao swollen shoot virus* (CSSV, L14546), *Sugarcane bacilliform virus* (SCBV, M89923), and *Rice tungro bacilliform virus* (RTBV, D10774).

A



B

Couple of primer	Forward primer		Reverse primer		Product length (bp)
	Position	Sequence (5′-3′)	Position	Sequence (5′-3′)	
5477F-5897R	5477-5496	CATCAAGTAGCAATGG ACCC	5876-5897	CCAGCCATTTCCTTA ATCCTTT	420
5345F-5897R	5345-5370	TTGGTGTTTAACTATAA GAGGCTGAA	5876-5897	CCAGCCATTTCCTTA ATCCTTT	552
5318F-5897R	5318-5339	GGAAAGGAAAGAAGA GGAAAGG	5876-5897	CCAGCCATTTCCTTA ATCCTTT	579
5477F-6765R	5477-5496	CATCAAGTAGCAATGG ACCC	6765-6783	GGGTCTGGAAAGAC CAAAT	1288
5477F-6105R	5477-5496	CATCAAGTAGCAATGG ACCC	6085-6105	ATACAGCCATCCGTT TCAATT	628
5477F-6119R	5477-5496	CATCAAGTAGCAATGG ACCC	6101-6119	CTCCCCAGCCTTCCA TACA	642
5477F-6681R	5477-5496	CATCAAGTAGCAATGG ACCC	6659-6681	TTATTGCATCCACATT TGAAAAC	1204
5345F-6681R	5345-5370	TTGGTGTTTAACTATAA GAGGCTGAA	6659-6681	TTATTGCATCCACATT TGAAAAC	1336

Fig. 1. Selection of primers that specifically detect episomal *Banana streak virus* (BSV). **A,** Schematic representation of the genomic regions selected (F: forward primers, R: reverse primers) by comparison of the sequences of episomal BSV-OL (ES; AJ002234, 7,389 bp) and the sequence integrated in the cv. Obino l'Ewai (IS; AF106946, 15,494 bp). Regions of homologous sequence are represented by similar arrows, for which the left orientation report an inverted sequence. The vertically chopped sequence in the IS represents noncontiguous viral and non-BSV sequences. **B,** Sequence and position (referring to the BSV-OL sequence, AJ002234) of the selected couples of primers.

Primers and probe were optimized for the BSV-OL strain. Primer synthesis was performed by Invitrogen (Paisley, Scotland). The MGB probe was supplied by Applied Biosystems with 6-carboxyfluorescein (FAM) as the reporter dye at the 5′ terminus, a nonfluorescent quencher, and the MGB moiety at the 3′ end.

5′ nuclease assay. PCR was performed in a reaction volume of 50 µl containing 0.2 mM of each dNTP, 0.28 µM of each primer, 0.1 µM MGB probe, 1.4× PCR buffer, 2 mM final $MgCl_2$ concentration, one unit of Taq DNA polymerase (Roche Diagnostics, Belgium), and 1 µl of sample. Thermal cycling conditions were 95°C for 1 min and 40 cycles of 95°C for 30 s, 53°C for 20 s, and 60°C for 60 s. To amplify from crude extracts, the number of cycles was increased to 50. The GeneAmp 5700 Sequence Detection System (Applied Biosystems) was used for thermal cycling and fluorescence recording. The threshold cycles (Ct) were calculated by plotting normalized fluorescence (ΔRn) in relation to cycle number, and correspond to the cycle number at which the fluorescence passes the threshold, from insignificant to detectable fluorescence levels (18). In all experiments, 10 µl of amplified products was analyzed by gel electrophoresis for validation.

Virus purification. Purifications of BSV were done using a modified protocol from T. Ndowora (*unpublished*). Samples of laminae tissue (50 to 100 g) powdered in liquid nitrogen were extracted with two volumes of 0.2 M potassium phosphate, pH 6, with 0.25% sodium sulfite (wt/vol). The extract was filtered through cheesecloth, stirred for 2 h with 2.5% (vol/vol) Triton X-100, and centrifuged at 16,270 × *g* for 15 min (4°C). The supernatant was stirred for 4 h with 5% (wt/vol) polyethylene glycol (PEG) 8000 and then centrifuged at 23,430 × *g* for 20 min. The pellet was resuspended in 1/10 of the original volume in 10 mM phosphate buffer (pH 7) containing 0.85% NaCl and conserved overnight at 4°C. After centrifugation at 12,000 × *g* for 10 min, the supernatant was layered over a 3.1-ml pad of 30% (wt/vol) sucrose in 10 mM phosphate buffer, and centrifuged at 112,000 × *g* for 1 h (4°C). The pellet was rinsed with 120 µl of 10 mM phosphate buffer through three centrifugations at 1,200 × *g* for 5 min. The supernatants were pooled and concentration and purity were assessed by spectrophotometric measures at 260 and 280 nm (A_{260} and A_{280}, respectively). The viral concentration *c* is calculated as follows: $c = A_{260}/7$ (µg/ml) (B. E. L. Lockhart, *personal communication*).

ELISA. The triple antibody sandwich (TAS)-ELISA protocol developed by Ndowora (19) was used with BSV antisera kindly provided by Lockhart (University of Minnesota, St. Paul). Microtiter plates were incubated (4 h, 37°C) with a rabbit polyclonal antiserum to BSV (2 µg/ml) in coating buffer (35 mM $NaHCO_3$, 14.4 mM Na_2CO_3, 3 mM NaN_3, pH 9.6). Leaf extracts were prepared by grinding in buffer (80 mM Na_2SO_3 in phosphate-buffered saline [PBS]-T) at 1:4 dilution and centrifugation at 7,000 × *g* for 5 min. Then, supernatant was added to duplicate wells and incubated for one night at 4°C. Chicken polyclonal antibody at 1:15000 dilution in ECI buffer (0.2% bovine serum albumin, 2% polyvinyl pyrrolidone-40, 3 mM NaN_3 in PBS-T, pH 7.4) was added as second antibody and incubated for 4 h at 37°C. Anti-chicken immunoglobulin G-alkaline phosphatase conjugate (Sigma-Aldrich, Bornem, Belgium) at 1:35000 dilution in ECI buffer was used as the third antibody and revealed after 2 h of incubation at 37°C with p-nitrophenyl phosphate substrate diluted 1:50 in diethanolamine at 97 ml/liter, 1 mM $MgCl_2 \cdot 6H_2O$, and 3 mM NaN_3. Absorbance readings were recorded at 405 nm (A_{405}) on a spectrophotometer after one night incubation at 4°C. Absorbance values greater than twice the mean of negative controls and greater than 0.200 were considered as positive. All washes between incubation steps were done three times with PBS-T (137 mM NaCl, 8 mM $Na_2HPO_4 \cdot 12H_2O$, 1.5 mM KH_2PO_4, 2.7 mM KCl, 3 mM NaN_3, 0.05% Tween 20, 80 mM Na_2SO_3, pH 7.2 to 7.4) for 3 min. All volumes were 100 µl.

RESULTS

Primers and probe design. Although the test was developed using BSV strain OL, primers and probes always were selected in the most conserved sequences among the BSV isolates.

The comparison of the organization of the episomal BSV-OL genome with the integrated sequence in cv. Obino l'Ewai led to the selection of eight pairs of primers with forward primers located upstream of position 5,530 (BSV-OL genome) and reverse primers downstream of position 5,810 (Fig. 1). Such primers amplify a sequence from 420 to 1,336 nt from the episomal virus. As the primers flank the "scrambled region" of the integrated sequence (minimum 6,093 bp), they will not give rise to amplification from the integrated sequence in standard conditions.

All pairs of primers were tested on plants either infected with BSV-OL (cv.

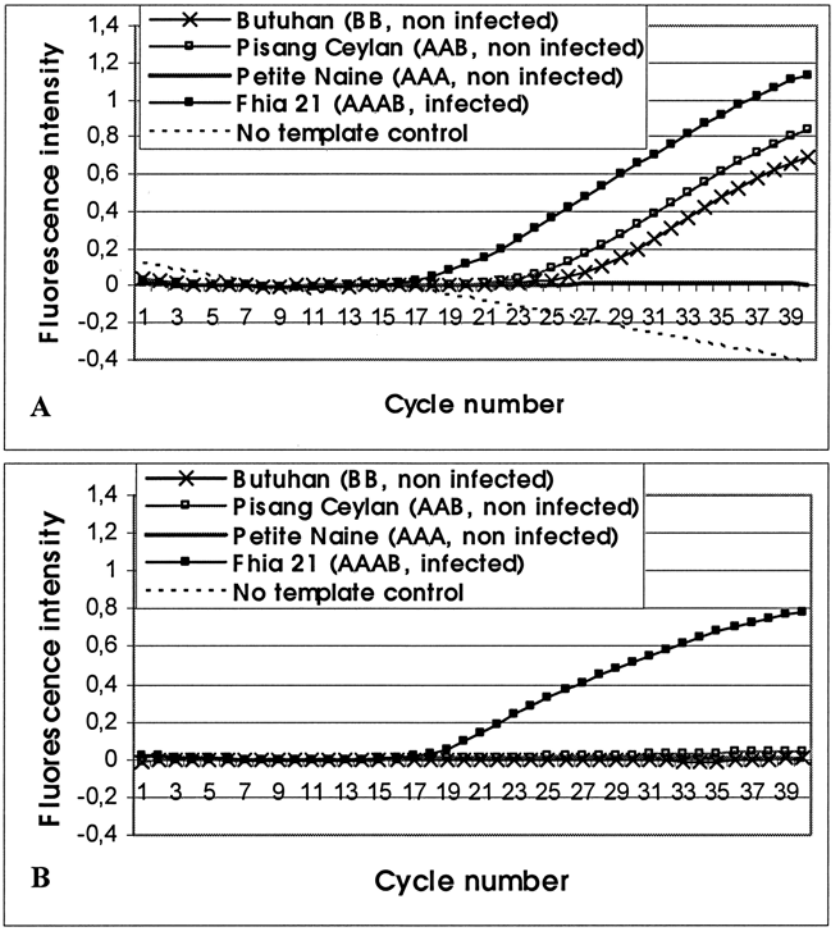


Fig. 2. Identification of primers that specifically amplify episomal viral DNA. **A,** Primers 5345F-5897R, nonspecific; **B,** primers 5345F-6681R, specific. Amplifications were realized on total nucleic acids extracts from plants infected with *Banana streak virus* (BSV)-OL (cv. Fhia 21, AAAB group), plants noninfected and carrying (cv. Pisang Ceylan, AAB group and cv. Butuhan, BB group), or not (cv. Petite Naine, AAA group) integrated sequences related to BSV-OL.

FHIA 21, group AAAB) or noninfected but carrying integrated sequences related to the Obino l'Ewai's integrant (cv. Pisang Ceylan, group AAB and cv. Butuhan, group BB). Five primer pairs gave amplification products from both episomal and integrated BSV and were not used further (Fig. 2). These unforeseen amplifications were not explained by the sequence of the Obino l'Ewai's integrant and could possibly arise from unknown rearrangements or undescribed viral integrated sequences. One pair generated a nonspecific band and two pairs produced, as expected, a unique and specific band for episomal BSV. Therefore we selected the forward primer 5345F (5′TTGGTGTTTAACTATAAGAGGCTGAA) and the reverse primer 6681R (5′TTATTGCATCCACATTTGAAAAC). They amplified a 1,336-bp sequence across the consensus sequences of the reverse transcriptase and RNaseH genes.

Therefore, the MGB probe (5′CAGCCTCATATTAT) was selected between primers 5345F and 6681R. It matched perfectly with both BSV-OL and BSV-Mysore strains. Probe Tm was estimated at 64.2°C.

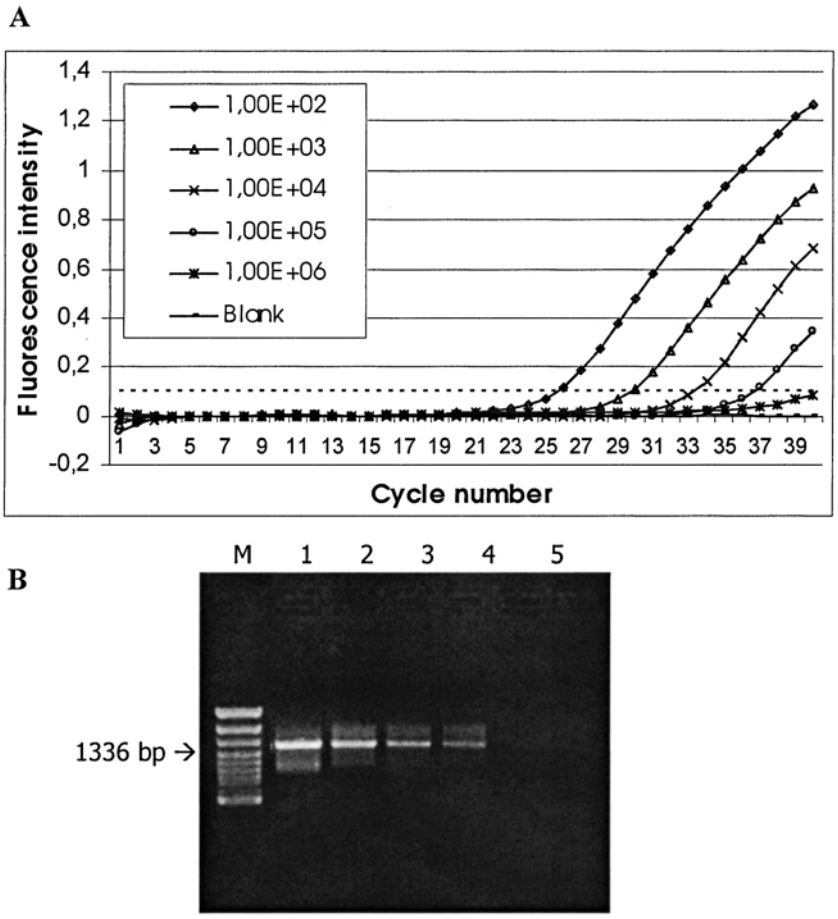


Fig. 3. Detection of episomal *Banana streak virus* (BSV) in total nucleic acids extracts by real-time polymerase chain reaction (PCR) using a minor groove binder DNA probe. Ten-fold dilution series (starting from dilution 1:100) of total nucleic acids extracted from BSV-infected cv. FHIA 21 (377 ng/µl). **A,** Real-time amplification plot. **B,** Detection of PCR products by electrophoresis in 1% agarose gel. M: 100-bp DNA ladder (GibcoBRL); lanes 1 to 5: dilutions 10^2, 10^3, 10^4, 10^5, and 10^6, respectively; lane 7: blank (sterile water). The horizontal dotted line indicates the threshold.

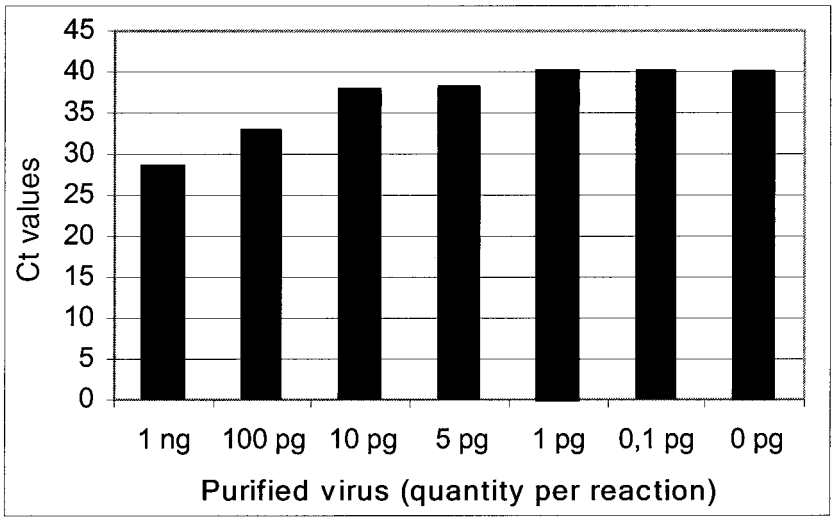


Fig. 4. Sensitivity of the real-time assay evaluated using purified virus. Virus was purified from cv. Pisang Lawadin (absorbance at 260 nm/absorbance at 280 nm = 1.48) and added to the polymerase chain reaction in the amounts indicated; Ct: threshold cycle.

Development of real-time assay. All PCR performed from BSV-infected material produced typical fluorescent curves with Ct values starting from 17.51, as well as the expected band of 1,336 bp after agarose gel electrophoresis. The real-time assay was as sensitive as the detection of the amplicon by gel electrophoresis (Fig. 3). Both techniques detected episomal BSV from 10^5-fold dilutions of DNA purified from an infected plant. Using purified virus particles, the sensitivity of the real-time assay was fixed between 1 and 5 pg (Fig. 4). The assay proved to be highly reproducible, as demonstrated by low Ct standard deviation values between triplicates and a high correlation coefficient (r = 0.995) of the standard curve (intra-assay variability; Fig. 5). Similar Ct values also were obtained in independent experiments, showing a good interassay reproducibility (*data not shown*).

Real-time assay using crude extracts. To simplify the experimental protocol and avoid a nucleic acid extraction step, the effectiveness of the real-time PCR assay was assessed on crude extracts of infected plants prepared in various extraction buffers. The lower Ct values were obtained using leaf extracts prepared in 137 mM NaCl, 8 mM $Na_2HPO_4{\cdot}12H_2O$, 1.5 mM KH_2PO_4, 2.7 mM KCl, 3 mM NaN_3, 0.05% Tween 20, 80 mM Na_2SO_3, pH 7.2 to 7.4, and diluted 10 times. Using this dilution, no inhibitory effect of *Musa* crude extracts (i.e., polyphenols or polysaccharides) on PCR amplification was observed. This was confirmed by comparable Ct values when amplifying viral purifications diluted either in sterile water or in crude extracts of a noninfected plant (cv. Cavendish) diluted 10 and 100 times (*data not shown*). The use of undiluted extracts proved to have an inhibitory effect on the amplification (*data not shown*).

Compared with amplifications on purified nucleic acid preparations, PCR on crude extracts generated fluorescence curves with later Ct and lower ΔRn values (Fig. 6).

Comparison between real-time assay on crude extracts and serology (ELISA).

To compare detection of BSV by real-time PCR and TAS-ELISA, 59 supposedly noninfected plantlets (cv. Obino l'Ewai) were analyzed in parallel by the two detection assays (*data not shown*). Both techniques used the same grinding buffer; therefore, a single crude extract and appropriate dilution was used for each plant. BSV was detected by real-time PCR and gel electrophoresis in eight plants, whereas none of these plants were tested positive by ELISA. The fluorescent curves of the positive plants presented Ct values varying from 27.95 to 47.66.

DISCUSSION

Musa breeding programs urgently require information and understanding on BSV infection in plants that carry integrated viral sequences. So far, biological studies on BSV infection are rare because current methods of detection of episomal BSV are relatively insensitive, laborious, and not suitable to large-scale tests.

Real-time PCR has revolutionized the field of molecular diagnostics due to its rapidity, sensitivity, reproducibility, and reduced risk of carryover contamination (18). To date, applications of real-time PCR and particularly 5′ nuclease assay in plant virology are increasing, but the genomic variability of the virus can impair the fluorogenic process with a single TaqMan probe. Recently, Salmon et al. (23,24) described a 5′ nuclease assay based on 3′ MGB probes for the real-time detection of plant viruses (*Apple chlorotic spot virus* and *Apple stem pitting virus*). The chemical modification of the probe allows the use of suitable melting temperatures with shorter probes; this property is critical to target-conserved sequences in viruses with high genomic variability.

In this article, we report the development of a real-time PCR assay to rapidly detect episomal BSV in *Musa* plants. The assay is optimized for the most characterized strain of BSV, BSV-OL, and constitutes a new tool to study BSV activation. The primers chosen specifically amplify episomal BSV even in the presence of viral sequences integrated in *Musa* spp., avoiding an immunocapture step before PCR. The MGB-fluorescent probe targets a unique short conserved sequence of 14 nt and allows the fluorogenic detection of unusually long PCR products (1,336 bp).

The assay was reproducible and as sensitive as gel electrophoresis. The virus target sequence was detected in as few as 5 pg of purified virus. The assay also provides a crude estimation of the sample viral concentration because the Ct values increase linearly while the amount of target decreases. The use of crude extracts as starting material allowed a further simplification of the test.

In conclusion, the real-time assay presented here detected rapidly and reproducibly episomal BSV-OL, the most characterized strain of this virus at the time of development. The test constitutes a high throughput detection method dedicated to an accurate study of the activation process of viral sequences integrated into the *Musa* genome. Furthermore, the quantitative property of the assay could be useful to monitor viral replication kinetics such as the progress of an infection, the response to antiviral therapy (11), and the evaluation of viral tolerance levels in new breeding programs (2,21).

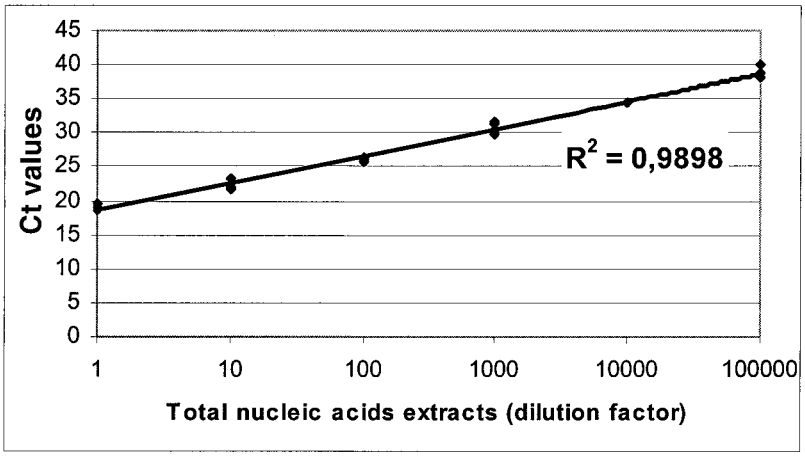


Fig. 5. Reproducibility and standard curve of threshold cycle (Ct) values of 10-fold dilutions of total nucleic acids extracts from *Banana streak virus*-infected cv. FHIA 21 (377 ng/µl), tested in triplicates.

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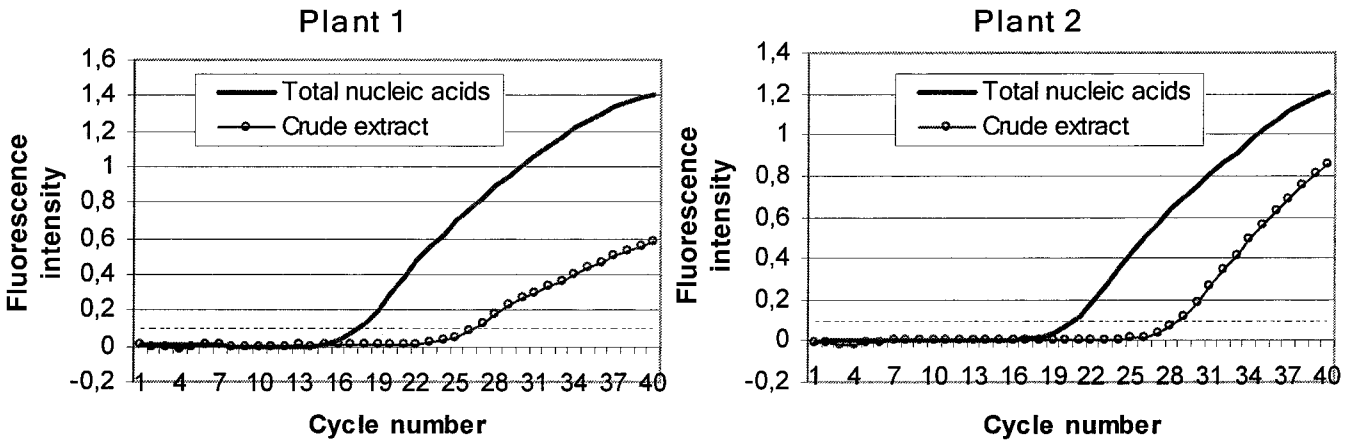

Fig. 6. Comparison of real-time polymerase chain reaction using purified nucleic acids (–––) and crude extracts (–θ–) from two *Banana streak virus*-infected plants (cv. FHIA 21, AAAB group). The horizontal dotted line indicates the threshold.

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