

How to Control and Prevent the Spread of Banana Streak Disease when the Origin Could be Viral Sequences Integrated in the Banana Genome?

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Abstract

Banana streak viruses are among the most widely distributed viruses of banana and are responsible for banana streak disease. Natural field spread occurs by either mealybugs or use of infected planting material, such as suckers. Banana streak viruses are pararetroviruses belonging to the genus *Badnavirus*, in the family Caulimoviridae. Like all members of the *Badnavirus* genus, they have bacilliform virions, 30 × 150 nm in size, and a circular dsDNA genome of 7.4 kbp. Fifteen years ago, an increasing number of outbreaks of banana streak disease were reported worldwide. Many occurred in banana breeding lines and micropropagated interspecific banana hybrids. The origin of infections in new hybrids and tissue-cultured plants was linked to the presence of viral DNA sequences integrated into the *Musa balbisiana* genome. Although integration is not an essential step in the replication cycle of pararetroviruses, it is assumed that under stress conditions some endogenous banana streak viruses could become infectious by reconstituting a complete replication-competent viral genome. Several serological and molecular tools have been developed to detect either virions or endogenous banana streak viruses. Their specificity and potential to prevent and control outbreaks of banana streak disease is discussed.

INTRODUCTION

Banana streak disease is caused by a complex of banana streak viruses. All are mealybug-transmitted plant bacilliform pararetroviruses belonging to the genus *Badnavirus* within the family Caulimoviridae (Fauquet et al., 2005; Lockhart, 1986). Their genome consists of a double-stranded, non-covalently linked circular DNA of 7.4 kb, and they have a wide serological and molecular variability. Banana streak virus infections cause characteristic chlorotic and necrotic streaks on leaves with highly susceptible banana cultivars developing more severe symptoms, such as pseudostem splitting, which eventually leads to the death of infected plants (Lockhart and Jones, 2000; Fargette et al., 2006). Banana streak viruses are among the most widely distributed viruses of banana and have never been considered a serious threat until recently when a number of outbreaks of the disease occurred in promising banana breeding lines and micropropagated interspecific *Musa* hybrids (Lheureux et al., 2003; Dallot et al., 2001). Such infections were correlated with the presence of banana streak virus sequences integrated into the nuclear genome of *Musa balbisiana*. Integrated virus sequences exist in *M. acuminata* and *M. balbisiana* although the majority appear to be inactive due to premature stop codons, frame-shift mutations and perhaps incomplete genomes. From

field experience, only endogenous banana streak viruses (eBSVs) from *M. balbisiana* appear capable of being reconstituted into a complete replication-competent viral genome (Harper et al., 1999a; Ndowora et al., 1999; Dallot et al., 2001; Lheureux et al., 2003). It is thought that epidemics of banana streak arise as a consequence of both the activation of eBSVs and mealybug spread of exogenous forms of the virus.

Diagnostics for banana streak viruses are difficult because of the broad sequence diversity in the banana streak virus complex and the existence of sequences integrated in the banana genome. Their presence in the banana genome hampers the detection of cognate episomal viruses by PCR, since PCR amplifies viral DNA of both viral particles and integrated viral sequences, which leads to false positives (Harper et al., 1999b; Yang et al., 2003). Banana streak viruses are today an important constraint to banana germplasm movement, genetic improvement and mass propagation. Several serological and molecular techniques have been developed so far and detect either virions or eBSVs or both. A relevant generic diagnostic for all banana streak viruses is needed. We have compared several immunocapture PCR assays to determine which is best with regards to sensitivity and ability to detect a broad range of banana streak viruses.

MATERIAL AND METHODS

Banana plants were grown under insect-proof conditions in a tropical glasshouse at CIRAD in Montpellier, France. B.E.L. Lockhart kindly provided plants infected with different species/strains of banana streak virus, including *Banana streak OL virus* (BSOLV), *Banana streak GF virus* (BSGFV), *Banana streak Mysore virus* (BSMyV), *Banana streak Imové virus* (BSV-Im), *Banana streak Cavendish virus* (BSV-Cav) and *Banana streak Vietnam virus* (BSV-VN). Individual banana streak infections of plants of the Cavendish subgroup (AAA genome) were obtained by mealybug transmission using *Planococcus citri*.

Partial purification of the viruses was obtained by grinding 10 g of banana leaf tissue in liquid nitrogen. The powder was extracted in 20 ml of 200 mM Tris-HCl (pH 7.4) containing 1% of Na₂SO₃ and was then filtered through cheesecloth. After centrifugation at 13,000-14,000 x g for 10-15 min, 1 ml of triton X100 at 33% was added to the filtrate before layering it over 6 ml of 30% sucrose in 15 mM Tris-HCl (pH 7.4). The centrifugation was done for 60 min in a Beckman 50.2 rotor at 30,000 rpm (109,000 x g max). After centrifugation and elimination of the supernatant, the sides of the tube were rinsed to eliminate residual triton X100. The pellet was resuspended in 200 µl of 1.5 mM Tris HCl (pH 7.4) and clarified by centrifugation at 8000 g for 5 min before being transferred into microtubes to be kept at 4°C.

All immunocapture PCRs were performed using the protocol of Le Provost et al. (2006). All PCR mixtures contained 50 ng of DNA (prepared using a Qiagen Plant Genomic DNA kit), 1 × Taq DNA polymerase buffer of 20-mM Tris-HCl (pH 8.4), 50 mM KCl, 0.1 mM each dNTP, 1.5 mM MgCl₂, 10 pmol each of reverse and forward primers and 1U Taq DNA polymerase (Eurogentec, Seraing, Belgium) in a final volume of 25 µL. DNA was amplified following specific amplification cycles related to the sets of primers used (Table 1 and 2). Amplicons were separated on a 1.5-% agarose gel in 0.5 × TBE (45 mM Tris-borate, 1 mM EDTA, pH 8), stained with ethidium bromide, and the expected bands visualised on a UV transilluminator.

All multiplex IC-PCR and multiplex PCR assays were undertaken using the common mix described above. The ratio between the banana streak virus- or badnavirus-

specific sets of primers and the STMS primers was 10 pmol: 30 pmol. The amplification cycles were as described in Tables 1 and 2.

RESULTS AND DISCUSSION

Capacities of Existing Tools for Detecting BSV

Several sets of primers are available to detect banana streak virus sequences (Table 1), some of which are specific to each banana streak virus while others are badnavirus-generic. The sensitivities of these PCR assays to detect banana streak virus virions were compared by IC-PCR using the same viral concentration of leaf extracts from banana plants infected by BSOLV and BSGFV. Fig. 1 shows that the BSOLV- and BSGFV-specific primers were able to detect virus over a dilution of 1:100,000. The Badna 1A/4' badnavirus-generic primers did not detect BSGFV at any dilution while BSOLV could only be detected down to a dilution of 1:100. Badnavirus RP/FP, the alternative badnavirus-generic primers, were as sensitive as the primers specific to a banana streak virus species, but unfortunately this set of primers also reacted with the uninfected banana leaf samples, indicating that the immunocapture step did not exclude all unencapsidated DNA.

Generic Diagnostic of BSV by IC-PCR

The ability of the badnavirus-generic primers to detect a range of banana streak virus species were assessed against all six different viruses used in this study. The Badna 1A/4' primers detected all viruses by IC-PCR after partial purification of the viruses (data not shown). All six banana streak viruses were also detected with the Badna FP/RP primers (Fig. 2), although relative band intensities were different according to banana streak virus species detected. This did not appear to be due to differences in viral concentration since the plant samples are controlled using species-specific sets of primers (data not shown). Strong amplification was observed for BSV-Cav and only slight reactions with BSOLV, BSMYV and BSV-VN (data not shown). Unexpected PCR bands were observed with virus-free banana samples used as negative controls, Cavendish plants and the wild *M. balbisiana* diploid 'Pisang Klutuk Wulung' (PKW). These reactions result from the binding of residual *Musa* genomic DNA to the walls of tubes or microplates used for IC-PCR leading to the amplification of integrated viral sequences.

Generic Diagnostic by Multiplex IC-PCR

To alert the user of residual *Musa* genomic DNA while still retaining sensitivity of detection, a multiplex IC-PCR using *Musa* sequence target microsatellite site primers (STMS), as developed by Le Provost et al. (2006), was undertaken. A multiplex PCR was firstly performed in order to test the reactivity of the combination of both STMS and badna FP/RP within the same tube. Several combinations to multiplex the two sets of primers were set up. The best one is illustrated in Fig. 3. The two controls were correct: the microsatellite PCR amplification was recorded for both DNA from Cavendish plants and PKW while the eBSVs amplification was recorded for DNA from PKW only. Surprisingly, BSOLV, BSV-Im and BSV-VN, which were detected in previous tests, did not react in this test, whereas the microsatellite amplification is correct at least for two of them.

A multiplex IC-PCR was undertaken with the same multiplex conditions and results confirmed (Fig. 4A). Fig. 4B and 4C show that the infected leaf samples used to

realise the IC step are infected since a strong amplification was recorded in multiplex IC-PCR using specific sets of primers for both BSOLV and BSGFV, respectively.

DISCUSSION AND CONCLUSIONS

Among the several molecular tools existing to detect banana streak viruses, the species-specific PCR primers are clearly the best for detecting individual species by IC-PCR. However, broader specificity is required and a relevant generic molecular banana streak virus diagnostic does not exist today. Even if the range of detection for the Badna 1A/4' tool is correct, they do not have sufficient sensitivity in IC-PCR unless a partially purified virus sample is used. Partial purification to concentrate the virus in the sample is very time-consuming and requires expensive equipment, which must be available if this process is to be used in the routine diagnosis of banana streak virus. The Badna FP/RP primer set provides greater sensitivity, although this extra sensitivity revealed problems with the immunocapture step, as residual *Musa* genomic DNA could be detected. This last experience reveals the need to systematically develop a multiplex IC-PCR approach in order to allow the detection of false positive resulting from the presence of residual *Musa* genomic DNA containing eBSVs. Multiplex PCR assays containing controls for DNA have been developed (Le Provost et al., 2006), although such assays are problematic when the badnavirus primers are too degenerate. An alternative is to develop a molecular test specific to the circular viral genome, such as those based on rolling-circle and long PCR amplification. Even if this test works, it is expensive for diagnostic use. The last solution is to design a set of primers which does not react with any of the eBSVs and which only amplifies the circular viral form. This requires a wide knowledge of all patterns of integration existing in banana.

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Tables

Table 1. Nucleotide sequence of primers used in IC-PCR and PCR experiments to test for banana streak viruses.

Name of primer	Primer sequence (5'-3')	Size of PCR product (bp)	Target
RDOL-F1 ¹	ATCTGAAGGTGTGTTGATCAATGC	522	BSOLV
RD-R1	GCTCACTCCGCATCTTATCAGTC		
GF-F1 ¹	ACGAACTATCACGACTTGTTC AAGC	476	BSGFV
GF-R1	TCGGTGGAATAGTCCTGAGTCTTC		
Badna 1A ²	TAAAAGCACAGCTCAGAACAAACC	589	Badnavirus
Badna 4'	CTCCGTGATTTCTTCGTGGTC		
Badna RP ³	CCA YTT RCA IAC ISC ICC CCA ICC	570	Badnavirus
Badna FP	ATGCCITTYGGIAARAAYGCICC		
AGMI 025 ⁴	TTAAAGGTGGGTAGCATTAGG	248*	STMS
AGMI 026	TTTGATGTCACAATGGTGTTCC		

*: size of PCR fragment amplified from *M. balbisiana* genomic DNA.

¹Geering et al., 2000; ²Geering et al., 2005; ³Yang et al., 2003; ⁴Lagoda et al., 1998.

Table 2. PCR cycles of amplification for each set of primers used.

RD-F1/RD-R1	GF-F1/GF-R1	Badna 1/4	Badna FP/RP
94°C -3 min	94°C -3 min	min 94°C -3 min	94°C -2 min
30 cycles (94°C-30s, 58°C-30s, 72°C-30s)	30 cycles (94°C-30s, 58°C-30s, 72°C-30s)	5 cycles (94°C- 30s, 37°C-30s, 72°C-1 min 30)	35 cycles (94°C-30s, 55°C-30s, 72°C-30s)
72°C – 5 min	72°C – 5 min	30 cycles (94°C-30s, 50°C-30s, 72°C-30s)	72°C – 10 min
		72°C – 10 min	

Figures

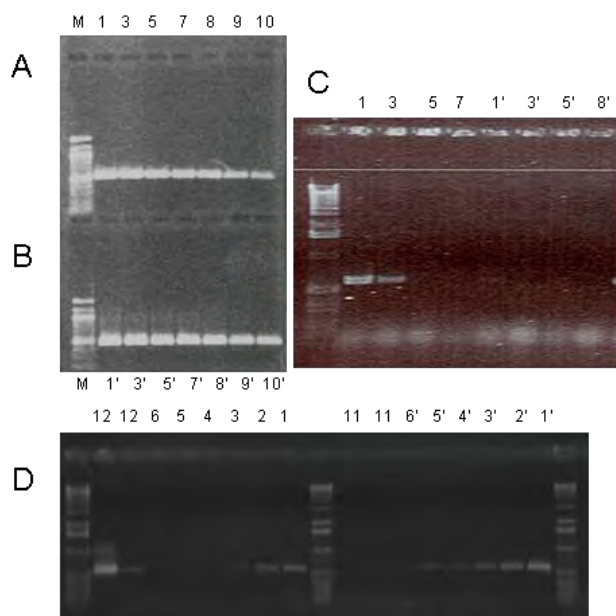


Fig. 1. Comparative sensitivities of molecular tools to detect banana streak viruses by IC-PCR: A and B: BSOLV- and BSGFV-specific primers, respectively; C: BSV-specific primers (Badna 1A/4'); D: Badnavirus-specific primers (Badna FP/FR). Leaf extracts from BSOLV (1 to 10) and BSGFV (1' to 10') infected plants diluted at 1:10, 1:20, 1:100, 1:500, 1:1,000, 1:2,000, 1:5,000, 1:10,000, 1:50,000, 1:100,000; (11) Leaf extracts from virus-free banana plant; (12) Total DNA from the virus-free 'Pisang Klutuk Wulung' (BB genome). M: 1Kb DNA marker (Invitrogen).

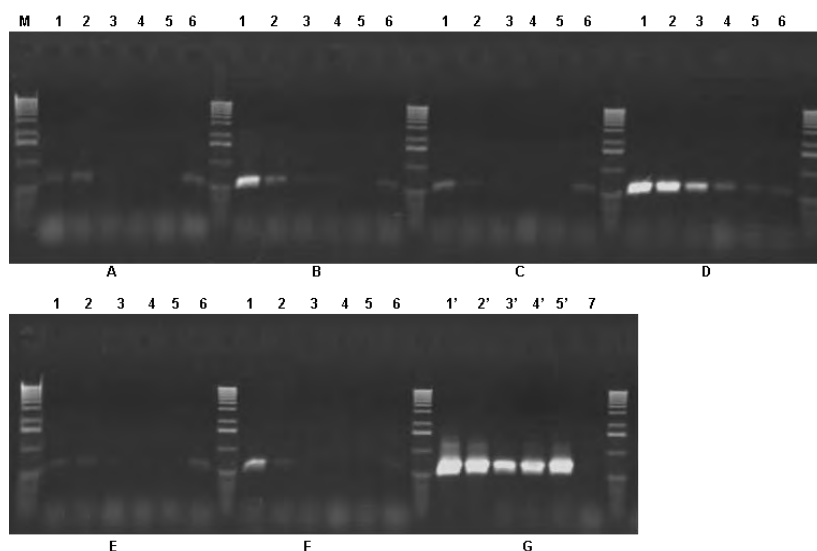


Fig. 2. Detection range of banana streak viruses by the Badna FP/RP set of primers by IC-PCR: Banana streak virus-infected banana leaf samples with A: BSOLV; B: BSGFV; C: BSV-Im; D: BSV-Cav; E: BSMYV; F: Uninfected banana leaf

samples from 'Pisang Klutuk Wulung' (BB genome); G: Total DNA extract from 'Pisang Klutuk Wulung'.

(1 to 5) Dilutions of banana leaf extract at 1:10, 1:20, 1:100, 1:500, 1:1000; (1' to 5') Dilutions of total DNA 1 μ l, 0.5 μ l, 0.2 μ l, 0.1 μ l, 0.05 μ l; (6) Uninfected banana leaf samples from a Cavendish cultivar (AAA genome); (7) H₂O.

M: 1Kb DNA marker (Invitrogen).

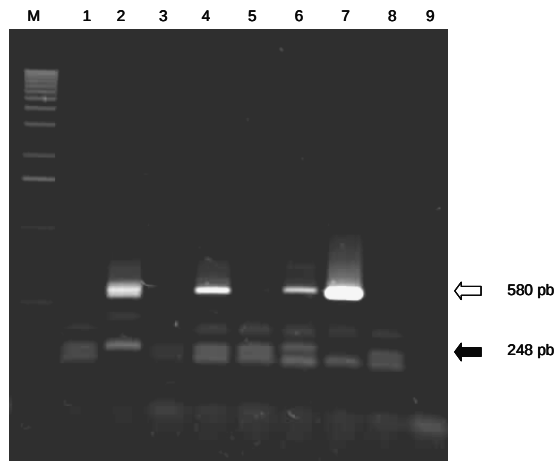


Fig. 3. Detection range of banana streak viruses by the Badna FP/RP set of primers and multiplex PCR.

(1) Uninfected banana leaf samples from a Cavendish cultivar (AAA genome); (2) Uninfected banana leaf samples from 'Pisang Klutuk Wulung' (BB genome); (3 to 8) Banana leaf samples from a Cavendish cultivar infected by BSOLV, BSGFV, BSV-Im, BSMYV, BSV-VN and BSV-Cav; (9) H₂O.

M: 1Kb DNA marker (Invitrogen).

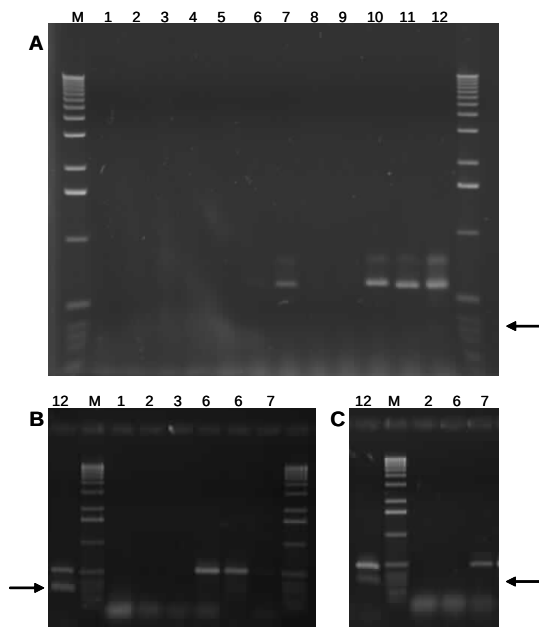


Fig. 4. Multiplex IC-PCR to detect banana streak viruses: A: multiplex of Badna FP/RP and STMS primers; B: multiplex of BSOLV-specific and STMS primers; C:

multiplex of BSGFV-specific and STMS primers. Arrows indicate the STMS amplification.

Banana leaf samples from (2) uninfected banana plants of a Cavendish cultivar (AAA genome), (3) 'Pisang Klutuk Wulung' (BB genome), (4) 'Penkelon' (AAB genome), (5) 'CRBP 39' (AAAB genome); (6 to 11) Cavendish infected by BSOLV, BSGFV, BSV-Im, BSV-Cav, BSV-VN; (12) DNA extract from 'Pisang Klutuk Wulung'; (1) H₂O.

M: 1Kb DNA marker (Invitrogen).