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Improved detection of episomal *Banana streak viruses* by multiplex immunocapture PCR

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Abstract

Banana streak viruses (BSV) are currently the main viral constraint to *Musa* germplasm movement, genetic improvement and mass propagation. Therefore, it is necessary to develop and implement BSV detection strategies that are both reliable and sensitive, such as PCR-based techniques. Unfortunately, BSV endogenous pararetrovirus sequences (BSV EPRVs) are present in the genome of *Musa balbisiana*. They interfere with PCR-based detection of episomal BSV in infected banana and plantain, such as immunocapture PCR. Therefore, a multiplex, immunocapture PCR (M-IC-PCR) was developed for the detection of BSV. *Musa* sequence tagged microsatellite site (STMS) primers were selected and used in combination with BSV species-specific primers in order to monitor possible contamination by *Musa* genomic DNA, using multiplex PCR. Furthermore, immunocapture conditions were optimized in order to prevent *Musa* DNA from interfering with episomal BSV DNA during the PCR step. This improved detection method successfully allowed the accurate, specific and sensitive detection of episomal DNA only from distinct BSV species. Its implementation should benefit PCR-based detection of viruses for which homologous sequences are present in the genome of their hosts, including transgenic plants expressing viral sequences.
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Keywords: *Banana streak virus* (BSV); Badnavirus; Endogenous pararetrovirus (EPRV); Episomal; Multiplex immunocapture PCR (M-IC-PCR); Diagnosis

1. Introduction

Banana streak viruses (BSV) are mealybug-transmitted members of the plant pararetrovirus genus *Badnavirus*, infecting banana and plantain worldwide (Hull et al., 2000; Fauquet et al., 2005). BSV infections cause characteristic chlorotic and necrotic leaf streak symptoms. Depending on infecting BSV species, highly susceptible banana cultivars can develop more severe symptoms, such as pseudostem splitting and necrosis, eventually leading to the death of infected plants (Lockhart and Jones, 2000; Daniells et al., 2001). Although originally not considered an economically important virus, BSV has raised strong concern over the past 15 years due to an increasing record of infections among new banana and plantain breeding lines and micropropagated hybrids. Interspecific *Musa acuminata* × *Musa balbisiana* genotypes, including a number of newly created hybrids, showed a tendency to produce BSV-infected propagules from virus-free source plants propagated by tissue culture (Dallot et al., 2001). Likewise, infected progeny were repeatedly obtained following genetic crosses involving virus-free *M. acuminata* and *M. balbisiana* parents (Lheureux et al., 2003). Such infections were correlated to the presence of BSV sequences integrated into the genome of *M. balbisiana*, a widespread progenitor of natural and created hybrid banana and plantain species. Similar pararetroviral sequences called endogenous pararetroviruses (EPRVs) have been identified in the nuclear genome of several other plants, including petunia, tobacco, potato, rice and tomato (Jakowitsch et al., 1999; Lockhart et al., 2000; Mao et al., 2000; Budiman et al., 2000; Richert-Pöggeler et al., 2003; Gregor et al., 2004; Kunii et al., 2004; Hansen et al., 2005). It was recently shown that some EPRVs have the potential to express infectious viral genomes

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and ultimately viral particles (Richert-Pöggeler et al., 2003). The presence of EPRVs in the genome of plants hampers the detection of cognate episomal viruses by PCR, since PCR will equally amplify encapsidated (episomal) viral DNA and integrated viral sequences, leading to false positives (Harper et al., 1999a; Yang et al., 2003). Therefore, an immunocapture-PCR (IC-PCR) method was developed for the detection of episomal BSV (Harper et al., 1999b). It combines immunological capture of BSV viral particles by a polyclonal antiserum (Ndowora, 1998) and amplification of the viral ORF3 domain encoding the RNaseH and reverse transcriptase (RT). Based on currently available nucleotide sequences (Harper and Hull, 1998; Geering et al., 2000, 2005a; Lheureux, pers. comm), specific primers are available for the detection of several BSV species by IC-PCR. The major drawback of the otherwise specific and sensitive diagnosis of BSV by IC-PCR is the persistent background encountered when indexing *Musa* species or hybrids harbouring the *M. balbisiana* genome that contains integrated BSV sequences. This situation is assumed to result from the binding of residual *Musa* genomic DNA to the walls of the thin-walled tubes or microplates used for IC-PCR and to lead to the amplification of EPRV BSV sequences. Therefore, a simple single step multiplex immunocapture PCR (M-IC-PCR) assay was developed for the sole detection of episomal BSV. *Musa* sequence tagged microsatellite site primers (STMS) were used in combination with BSV species-specific primers in order to allow the detection of false positives resulting from the presence of *Musa* genomic DNA, and immunocapture conditions were optimized in order to limit contamination by residual *Musa* genomic DNA.

2. Materials and methods

2.1. Plant materials

All *Musa* plant materials were obtained from the Guadeloupe outstation of the Centre International de Recherche en Agronomie pour le Développement (CIRAD), located in the French West Indies. Some material was maintained under insect-proof tropical greenhouse conditions in Montpellier (France).

2.2. Total DNA extraction and Southern blot hybridisation

Total DNA was extracted from *Musa* leaf tissue by the method of Gawel and Jarret (1991). Ten micrograms of undigested DNA (100 μg for accession Pisang klutung wulung, PKW) were separated overnight by electrophoresis through a 1% agarose gel at 40 V in 0.5× Tris–borate–EDTA buffer. Following ethidium bromide staining and UV light visualisation, DNA was transferred by capillarity onto nylon membrane (Hybond N, Amersham). A ^{32}P-labelled probe was prepared by random priming of the 2.1 kbp PCR product encompassing the intergenic region of BSV species Obino l'Ewaï (BSOLV) described by Lheureux et al. (2003). Filters were pre-hybridized in 15 ml of hybridisation buffer (5× SSC, 5× Denhardt's solution, 0.5% SDS and 10 μg ml^{-1} of sheared herring sperm DNA) for 4 h at 65 °C, then incubated overnight at 65 °C in 15 ml of fresh hybridisation buffer containing heat-denatured radio-labelled probe. Following hybridisation, membranes were washed for 5 min at room temperature in washing buffer 1 (2× SSC, 0.5% SDS), then for 10 min at 65 °C in washing buffer 2 (2× SSC, 0.1% SDS), and finally for 10 min at 65 °C in washing buffer 3 (1× SSC, 0.1% SDS), dried and placed under Konica medical X-ray film (Konica, Tokyo, Japan) for 3 days at −80 °C.

2.3. Multiplex-immunocapture PCR (M-IC-PCR)

Immunocapture was performed using a rabbit polyclonal antiserum raised against a cocktail of purified BSV species and *Sugarcane bacilliform virus* (SCBV) species (kindly provided by B.E.L. Lockhart). This antiserum can detect multiple BSV species (Ndowora, 1998; Ndowora and Lockhart, 2000). Sterile polypropylene thin-walled 0.2 ml microfuge tubes (Axygen, Union City, USA) or 96-well plates (Starlab, Paris, France) were coated overnight at 4 °C with 25 μl of IgG purified from this anti-BSV polyclonal antiserum, and diluted at 2 μg/ml in carbonate coating buffer (15 mM sodium carbonate, 34 mM sodium bicarbonate, pH 9.6), then washed three times with 100 μl of PBT washing buffer (136 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 8 mM NA_2HPO_4, 0.05% Tween-20, pH 7.4). Plant extracts were prepared by grinding 0.5 g leaf samples in 5 ml of grinding buffer (2% polyvinylpyrrolidone 40, 0.2% sodium sulfite and

Table 1
Nucleotide sequence of primers used for IC-PCR and PCR experiments

Target	Name of primer	Primer sequence (5′–3′)	Size of PCR product (bp)
BSOLV	RD-F1[a]	ATCTGAAGGTGTGTTGATCAATGC	522
	RD-R1[a]	GCTCACTCCGCATCTTATCAGTC	
BSGFV	GF-F1[a]	ACGAACTATCACGACTTGTTCAAGC	476
BSMysV	GF-R1[a]	TCGGTGGAATAGTCCTGAGTCTTC	589
	Mys-F1[a]	TAAAAGCACAGCTCAGAACAAACC	
	Mys-R1[a]	CTCCGTGATTTCTTCGTGGTC	
STMS	AGMI 025[b]	TTAAAGGTGGGTTAGCATTAGG	248[c]
	AGMI 026[b]	TTTGATGTCACAATGGTGTTCC	

[a] Published in Geering et al. (2000).
[b] Published in Lagoda et al. (1998).
[c] Size of PCR fragment amplified from *M. balbisiana* genomic DNA.

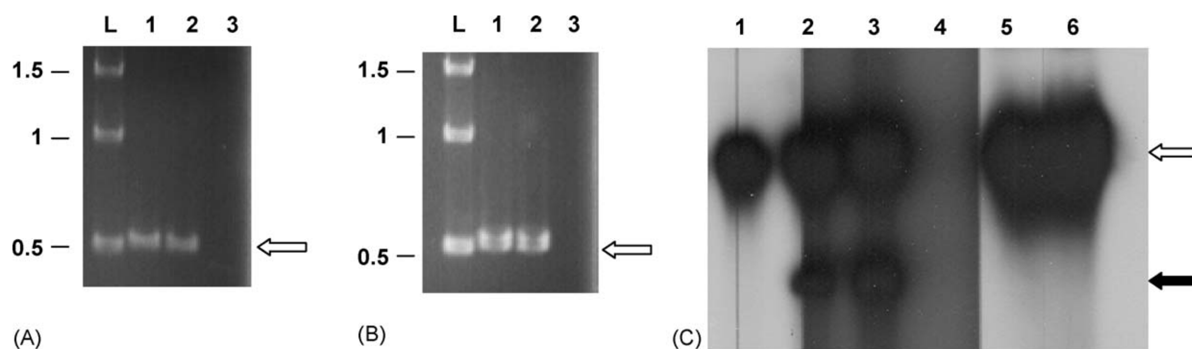

Fig. 1. IC-PCR, PCR and Southern blot analysis of uninfected and BSOLV-infected *Musa* hybrids and species. (A) IC-PCR performed on leaf extracts from distinct virus-free PKW (BB) (lanes 1 and 2) and Grande Naine (AAA, lane 3) plants using primer pair RD-F1/RD-R1, following overnight immunocapture at 4 °C. (B) PCR detection of BSOLV EPRVs performed on 50 ng of genomic DNA purified from uninfected PKW (lanes 1 and 2) or Grande Naine (lane 3) plants using primer pair RD-F1/RD-R1. Open arrows indicate BSOLV PCR product. (C) Southern blot analysis of various *Musa* hybrids and accessions. Ten micrograms (lanes 1–4) or 100 μg (lanes 5 and 6) of total undigested DNA extracted from an uninfected AAB hybrid (lane 1), a BSOLV-infected AAB hybrid (lane 2), a BSOLV-infected AAB plantain (lane 3), an uninfected AA accession (lane 4) and two distinct virus-free BB PKW accessions (lanes 5 and 6) were hybridized to a ^{32}P-radiolabelled BSOLV probe. The filled arrow indicates the linear form of BSOLV episomal DNA, and the open arrow indicates *Musa* genomic DNA. L: 1 kbp DNA ladder (Sigma) with indicated marker sizes in kbp.

0.2% bovine serum albumin, prepared in PBT), using a manual bead grinder and plastic grinding bags (Biorad Phytodiagnostics, Marnes-la-Coquette, France). One milliliter of plant extracts was transferred to microfuge tubes and clarified by centrifugation at room temperature for 2 min at 21,000 × *g*. Then 25 μl aliquots of the supernatant were loaded in IgG-coated tubes or wells and incubated at room temperature. Tubes or wells were washed three times with 100 μl of PBT, one time with 100 μl of sterile water, and then dried briefly. Multiplex PCR was carried out directly in tubes or wells using BSV species-specific primers (see Table 1) and *Musa* sequence tagged microsatellite site primers AGMI025 and AGMI026 (Lagoda et al., 1998). PCR reaction mix of 25 μl contained 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 100 mM each dNTP, 1.5 mM $MgCl_2$, 10 pmol of each primer and 1 U *Taq* DNA polymerase (Eurogentech, Seraing, Belgium). PCR cycle conditions were an initial denaturation step at 94 °C for 3 min, then 35 cycles (94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min) followed by a final elongation step of 5 min at 72 °C. Ten microliters of PCR products were analysed by standard electrophoresis on a 1.5% agarose gel in 0.5× TBE (45 mM Tris–borate, 1 mM EDTA, pH 8.0) followed by ethidium bromide staining and visualization under UV light.

2.4. Multiplex direct binding PCR (M-DB-PCR)

For M-DB-PCR, 25 μl aliquots of the plant extracts used for M-IC-PCR were incubated for 3 h at room temperature in uncoated sterile polypropylene 0.2 ml thin-walled tubes or 96-well plates. Subsequent washes and PCR reactions were performed as described for IC-PCR.

3. Results

3.1. Background PCR amplifications from genomic Musa DNA during IC-PCR

The effect of immunocapture conditions on the accuracy of BSV detection by IC-PCR was assessed. Various temperatures and immunocapture times were evaluated. Fig. 1A shows that standard overnight incubation at 4 °C of leaf extracts prepared from virus-free *M. balbisiana* accession Pisang Klutuk Wulung (PKW) results in background PCR amplification when using the BSOLV species-specific primer pair RD-F1/RD-R1 (see lanes 1 and 2). Control PCR experiments showed that similar BSOLV PCR products are amplified from genomic DNA purified from the same uninfected PKW plants (Fig. 1B, lanes 1 and 2). No BSOLV PCR product can be amplified from uninfected *M. acuminata* leaf extract or purified genomic DNA (Fig. 1A and B, lane 3). When indexing other *Musa* accessions harbouring the *M. balbisiana* genome for BSGFV and BSMysV species by IC-PCR following overnight immunocapture, similar background amplifications were observed (data not shown). Southern blot experiments performed on total DNA extracted from various BSOLV-infected or uninfected accessions confirmed that the PKW plants tested, for which up to 100 μg of purified DNA was loaded, are devoid of BSOLV episomal DNA (Fig. 1C, lanes 5 and 6). This result was confirmed by observation under electron microscope. It is likely that background amplification of BSV sequences from BSV EPRVs arose from non-specific binding of *Musa* genomic DNA to thin-walled tubes or microplates over extended immunocapture times.

3.2. Optimized immunocapture multiplex PCR conditions for the detection of BSV episomal DNA

Immunocapture conditions were optimized in order to prevent residual *Musa* genomic DNA from binding to thin-walled tubes or microplates. Furthermore, in order to detect potential contamination by *M. balbisiana* genomic DNA, a multiplex IC-PCR (M-IC-PCR) assay was designed. *Musa* sequence tagged microsatellite site primers were used in combination with BSV species-specific primers. STMS primer pair AGMI 025/AGMI 026 was selected because (i) its Tm is compatible with that of BSV species-specific primers, (ii) its sensitivity threshold for *M. balbisiana* DNA is identical to that of BSV species-specific

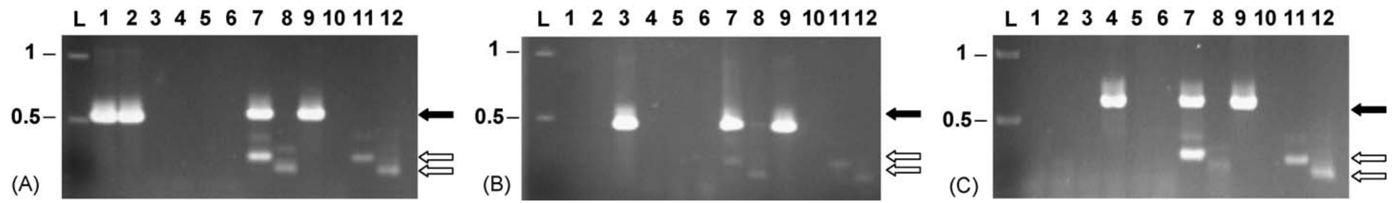

Fig. 2. Detection of episomal BSOLV, BSGFV and BSMysV by M-IC-PCR. Multiplex PCR reactions were carried out on immunocaptured viral particles using STMS primer pair AGMI 025/AGMI 026 and either BSOLV species-specific primer pair RD-R1/RD-F1 (A), BSGFV species-specific primer pair GF-R1/GF-F1 (B) or BSMysV species-specific primer pair Mys-R1/Mys-F1 (C). Prior to PCR, viral particles were immunocaptured for 3 h at room temperature from 25 μl of leaf samples prepared from infected accessions Figue pomme (AAB, lane 1), Grande naine (AAA, lane 2), Guindy (AAB, lane 3), Langka (AAAB, lane 4), and uninfected PKW (BB, lane 5) and IDN-110 (AA, lane 6). Control PCR experiments were carried out on 50 ng of genomic DNA purified from cvs PKW (lanes 7, 9 and 11) or IDN-110 (lanes 8, 10 and 12) using BSV species-specific primer pairs and STMS primer pair (lanes 7 and 8), BSV species-specific primer pairs only (lanes 9 and 10) or STMS primer pair only (lanes 11 and 12). The filled arrow indicates BSV product, and the open arrows indicate STMS products. L: 1 kbp DNA ladder (Sigma) with indicated marker sizes in kbp.

primers and (iii) BSV and *M. balbisiana* PCR products amplified by either set of primers can be easily distinguished from each other under standard agarose gel electrophoresis conditions (see lane 7 in Fig. 2A–C). Likewise, the size difference between *M. acuminata* and *M. balbisiana* PCR products amplified by the STMS primer pair makes them easily distinguishable (see lanes 11 and 12 in Fig. 2A–C).

Limiting the immunocapture step to 3 h at room temperature allowed the elimination of any background PCR amplification when using BSOLV, BSGFV or BSMysV species-specific primers on a range of infected and uninfected plants of distinct genotypes, including PKW (see lanes 1–6 in Fig. 2A–C). Therefore, these immunocapture conditions were selected for further experiments. In order to confirm that the absence of background amplification resulted from a lack of unspecific binding of *Musa* DNA to thin wall tubes or microplates, multiplex direct binding PCR was performed on the same samples, under the same incubation conditions (3 h at room temperature) and using the same primer combinations. The results shown in Fig. 3 unambiguously demonstrate that the optimized incubation conditions prevent non-specific binding of residual genomic DNA, or limits such binding below the detection threshold, since no *Musa* sequence could be amplified by STMS primers from any of the tested cultivars, including PKW (see lanes 1–6). Moreover, *Musa* sequences were not amplified by STMS primers from a sample prepared by mixing leaf extract from a healthy IDN-110 plant (AAA) and PKW genomic DNA to a final concentration of 100 ng/μl, and subjected to direct binding (lane 7), whereas PCR amplification from PKW genomic DNA used as a PCR control raised the expected products (lanes 8–10). Hence, BSV amplification products of the expected sizes obtained by M-DB-PCR from plant samples infected by BSOLV (Fig. 3A, lanes 1 and 2), BSGFV (Fig. 3B, lane 3) and BSMysV (Fig. 3C, lane 4) originate from episomal BSV DNA. These results confirm previous data showing that BSV viral particles can themselves bind non-specifically to polypropylene thin-walled tubes or microplates (Harper et al., 2002a), even under the optimized incubation conditions that were used in this work. It is likely that this non-specific binding accounts for part of the PCR amplification of episomal BSV sequences during M-IC-PCR.

3.3. Comparison of M-IC-PCR and M-DB-PCR for the detection of BSV

Direct binding PCR (DB-PCR) was reported previously as a straightforward sample processing procedure for plant virus detection (Rowhani et al., 1995; Hema et al., 2003), including that of BSV (Harper et al., 2002a). Since episomal BSV can be amplified by M-DB-PCR (see Fig. 3), the usefulness of this technique for the detection of episomal BSV was assessed, and its sensitivity was compared to that of M-IC-PCR. Fig. 4 shows that

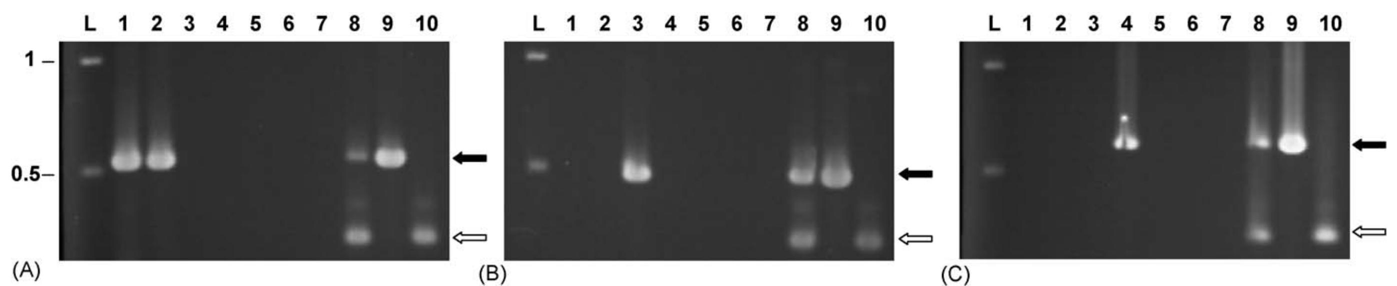

Fig. 3. Detection of BSOLV, BSGFV and BSMysV by M-DB-PCR. Twenty-five microliters of the same banana leaf extracts as the ones used for IC-PCR (lanes 1–6) or 25 μl of leaf extract from a healthy IDN-110 (AAA) plant mixed with 2.5 μg of purified DNA extracted from cv PKW (lane 7) were incubated for 3 h at room temperature in uncoated thin-walled 0.2 ml polypropylene tubes. Fifty nanograms of genomic DNA extracted from cv PKW was used as a PCR positive control (lanes 8–10). Multiplex PCRs were performed under similar conditions as the ones used for IC-PCR using STMS primer pair AGMI 025/AGMI 026 and either BSOLV species-specific primer pair RD-R1/RD-F1 (panel A, lanes 1–8), BSGFV species-specific primer pair GF-R1/GF-F1 (panel B, lanes 1–8) or BSMysV species-specific primer pair Mys-R1/Mys-F1 (panel C, lanes 1–8). Lanes 9 and 10 show control PCR performed using BSV species-specific primers only or STMS primers only, respectively. The filled arrow indicates BSV product, and the open arrows indicate STMS products. L: 1 kbp DNA ladder (Sigma) with indicated marker sizes in kbp.

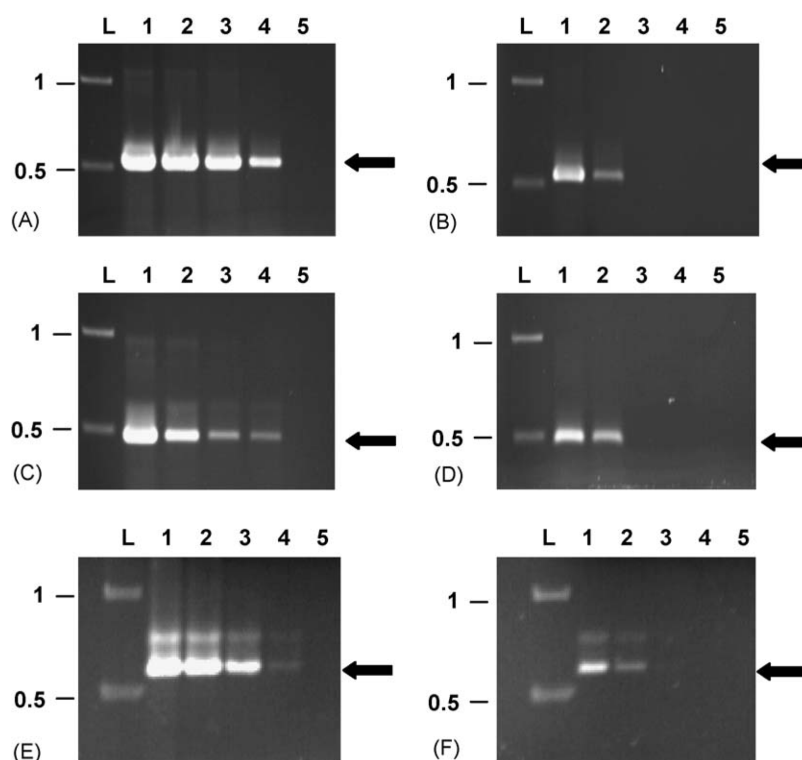

Fig. 4. Comparative sensitivities of M-IC-PCR and M-DB-PCR for the detection of BSOLV, BSGFV and BSMysV. Twenty-five microliters of undiluted (lane 1) or diluted 1:10 (lane 2), 1:100 (lane 3), 1:1000 (lane 4) or 1:10,000 (lane 5) leaf extracts from BSOLV-infected Figue Pomme plant (AAB, panels A and B), BSGFV-infected Guindy plant (AAB, panels C and D) or BSMysV-infected Langka plant (AAAB, panels E and F) were used for multiplex PCR detection of the BSV species following immunocapture (panels A, C, E) or direct binding (panels B, D, F). Primer pairs AGMI 025/AGMI 026 was used with either RD-R1/RD-F1 (panels A and B), GF-F1/GF-R1 (panels C and D) or Mys-R1/Mys-F1 (panels E and F) primer pairs for multiplex PCR. Incubation of leaf extracts and PCR conditions were as described above. Dilutions were prepared in leaf extract from an uninfected IDN-110 (AAA) plant. The filled arrow indicates BSV product. L: 1 kbp DNA ladder (Sigma) with indicated marker sizes in kbp.

M-IC-PCR allows the detection of BSV species in leaf extracts of diseased plants diluted up to 1:1000 in leaf extract from a healthy plant (see panels A, C and E). On the contrary, M-DB-PCR does not allow the detection of BSV species above a 1:10 dilution range for the same samples and primer combinations (see panels B, D and E), nor from undiluted extracts from plants with low virus titres (data not shown). These results clearly indicate that M-DB-PCR is not suitable for sensitive detection of BSV, and should not be considered as a diagnostic tool, since it is likely to result in false negatives for samples with low virus titre.

4. Discussion

A growing number of EPRVs are being identified in the genome of economically important crops, many of them vegetatively propagated (Harper et al., 2002b). Although integration is not an essential step in the replication cycle of pararetroviruses, and seems to occur during viral replication via illegitimate recombination, Richert-Pöggeler et al. (2003) have shown that virions can be released from an infectious *Petunia vein clearing virus* (PVCV) EPRV and induce viral symptoms. It is postulated that other infectious EPRVs, including several BSV EPRVs, could release infectious virions and induce viral symptoms under stress conditions. Infectious BSV EPRVs corresponding to several widespread BSV species are indeed present in the genome of *M. balbisiana* but not in that of *M. acuminata* (Piffanelli, pers. comm.). Since episomal infectious viral particles can originate from infectious EPRVs, serious concerns have been raised about the risk of spreading viral diseases through mass distribution of clonal plant material prone to EPRV activation, such as *M. acuminata* × *M. balbisiana* interspecific hybrids or cultivars. In fact, due to the widespread occurrence of infectious BSV EPRVs in *Musa* species and interspecific hybrids, BSV has now become the main viral constraint to *Musa* germplasm movement and genetic improvement. Setting up a tight control on *Musa* germplasm movement is currently the only efficient strategy for controlling the spread of BSV resulting from horizontal transmission by its mealybug vectors or vertical transmission by infectious BSV EPRVs. Therefore, accurate diagnosis methods are needed for the unambiguous detection of episomal BSV. Traditional methods include visual leaf inspection for symptoms, which can be misleading due to intermittent expression of symptoms or possible confusions with symptoms caused by other viruses such as *Cucumber mosaic virus* (CMV), and immunosorbent electron microscopy (ISEM), which is time consuming and requires sophisticated equipment. Enzyme linked immunosorbent assay (ELISA) has been shown to lack sensitivity due to high backgrounds (Thottapilly et al., 1998; Dahal et al., 1998). Therefore, PCR-based diagnosis was developed. Its use unveiled

the widespread presence of BSV EPRVs in the genome of *Musa* (Harper et al., 1999a; Ndowora et al., 1999; Geering et al., 2005b), and ultimately led to the development and implementation of an IC-PCR diagnostic tool, using primers designed to sequences in the RT/RNase H region of BSV ORFIII (Harper and Hull, 1998). Unfortunately, BSV EPRVs can also interfere with IC-PCR diagnosis, and lead to background amplification of non-episomal BSV sequences, as shown Fig. 1. This phenomenon results from residual trapping on polypropylene thin-walled tubes or microplates of host *Musa* genomic DNA, whose presence in leaf extracts was confirmed by PCR run on leaf extracts (data not shown). Since nucleotide sequences of the BSV EPRVs characterized so far display very high levels of sequence identity with that of their cognate BSV species (Ndowora et al., 1999; Iskra-Caruana, pers. comm.), no primers specifically targeted against episomal BSV sequences and unable to amplify BSV EPRVs can be designed. It is therefore so far technically impossible to discriminate between EPRVs and their cognate viruses by PCR. As an alternative, real time PCR (Delanoy et al., 2003) and partial purification of viral particles by ion-exchange chromatography prior to PCR (Harper et al., 2003) were developed for the specific detection of episomal BSOLV and PVCV, respectively. But these time consuming and expensive techniques are not well suited for indexing large numbers of samples or implementation in developing countries where BSV occurs, contrary to the more straightforward IC-PCR. Furthermore, real time PCR requires sequence data that is currently only available for a limited number of BSV EPRVs.

The work reported here presents a simple and comprehensive multiplex immunocapture PCR (M-IC-PCR) method for the accurate and sensitive detection of episomal BSV. BSV species-specific primers designed to sequences in the RT/RNase H region of ORFIII from BSOLV, BSGFV or BSMysV species were used in this study, but it should be noted that similar primers exist for other BSV species such as Imové (BSImV) and Vietnam *acuminata* (BSAcVNV) (Lheureux et al, pers. comm.). Species-specific primers were preferred to degenerate primers that were designed to amplify the consensus RT/RNase H region (Lockhart and Olszewski, 1993; Yang et al., 2003). We found that degenerate primers were not sensitive enough for use in routine indexing. This lack of sensitivity probably results from the high molecular diversity in the conserved RT/RNase H region of badnavirus ORF3, which is especially high in the case of BSV (Harper et al., 2005; Jaufeerally-Fakim et al., 2005). Although detection of individual BSV species was presented here for clarity, simultaneous detection of several BSV species from mixed infections is technically possible, using distinct BSV species-specific primers and STMS primers in the same multiplex PCR reaction, as previously reported for the simultaneous detection of three other viruses infecting banana (Sharman et al., 2000). Limiting the immunocapture step time to 3 h at room temperature was found to eliminate background amplification of BSV EPRV sequences during IC-PCR (Fig. 2), probably by decreasing the binding of residual *Musa* genomic DNA to polypropylene below the detection threshold by PCR. Interestingly, even the optimized immunocapture conditions described in this work allow the binding of viral particles to thin-walled tubes or microplates (Fig. 3), and this non-specific binding certainly contributes to the PCR amplification of episomal BSV sequences by M-IC-PCR. Our work also shows that contamination by *Musa* genomic DNA leading to false positives in the course of IC-PCR can be conveniently detected by multiplex PCR assay using STMS primers (Fig. 2). The use of internal transcribed spacer (ITS) nuclear DNA sequences was previously described for the detection of petunia genomic DNA contaminations during multiplex PCR diagnosis of PVCV (Harper et al., 2003). In that particular case, virus replication was monitored by measuring changes in the ratio of virus sequence amplified by PVCV-specific primers, to marker DNA sequence amplified by ITS-specific primers. Thanks to the availability of an anti-BSV antiserum (Ndowora, 1998; Ndowora and Lockhart, 2000) the BSV M-IC-PCR assay presented here does not require such a ratio quantification of PCR products, and instead provides straightforward positive or negative results.

Overall, IC-PCR-based detection of plant or animal viruses whose hosts harbour endogenous viral sequences should benefit from the methodological improvements described here. The availability of a steadily growing number of microsatellite primers for many living organisms should help designing M-IC-PCR detection protocols. M-IC-PCR detection techniques are particularly relevant to plant pararetroviruses and geminiviruses, for which integrated sequences are widespread in the genome of their host plants (Harper et al., 2002b), and for other viruses whose genomic sequences are present in the genome of their hosts (Tanne and Sela, 2005). They are also relevant on the study of virus resistant transgenic plants (VRTPs) expressing viral sequences, since the evaluation of their level of resistance against viruses requires highly sensitive detection techniques showing no interference with the viral transgenes.

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