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Micropropagation by tissue culture triggers differential expression of infectious endogenous *Banana streak virus* sequences (eBSV) present in the B genome of natural and synthetic interspecific banana plantains

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SUMMARY

The genome of *Musa balbisiana* spp. contains several infectious endogenous sequences of *Banana streak virus* (eBSV). We have shown previously that *in vitro* micropropagation triggers the activation of infectious eBSOLV (endogenous sequences of *Banana streak Obino l'Ewai virus*) in the synthetic tetraploid interspecific hybrid FHIA21 (AAAB). In this work, we show that another synthetic tetraploid (AAAB) hybrid and two natural triploid (AAB) plantains are equally prone to the activation of infectious eBSOLV during tissue culture. These results are a strong indication that such activation is a general phenomenon in interspecific *Musa* cultivars, whether synthetic or natural. We also report the first in-depth study of the correlation between the duration of tissue culture and the level of activation of infectious eBSOLV, and show that specific and common activation patterns exist in these banana plants. We hypothesize that these patterns result from the concomitant activation of infectious eBSOLV and a decrease in the virus titre in neoformed plantlets, resulting from cell multiplication outcompeting virus replication. We provide experimental data supporting this hypothesis. No activation of infectious eBSGFV (endogenous sequences of *Banana streak Goldfinger virus)* by tissue culture was observed in the two natural AAB plantain cultivars studied here, whereas such activation occurred in the AAAB synthetic hybrid studied. We demonstrate that this differential activation does not result from differences in the structure of eBSGFV, as all banana genomes harbour eaBSGFV-7.

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INTRODUCTION

The nuclear genome of numerous plants is invaded by a large number of viruses mainly belonging to the family *Caulimoviridae*. These integrations are named endogenous pararetrovirus sequences (EPRVs), and are thought to be relics of ancient infection events. Pararetroviruses neither integrate their hosts' genome nor encode an integrase function during viral replication (Harper *et al*., 2002; Hohn *et al*., 2008). Therefore, EPRVs probably integrated their hosts' genome by illegitimate recombination between host and viral DNA. EPRVs range from small, truncated fragments to larger rearranged sequences containing more than one copy of the viral genome. Some EPRVs are infectious because they have the potential to reconstitute the functional viral genome following activating stresses that contribute to viral infection. This is the case for sequences of *Banana streak virus* (BSV) integrated in the *Musa balbisiana* banana genome (denoted B) and named eBSV (endogenous BSV sequences) (Staginnus *et al*., 2009). Infectious eBSV can be activated by abiotic stresses, such as micropropagation by *in vitro* culture processes (Dallot *et al*., 2001), temperature differences (Dahal *et al*., 1998, 2000) or water stress, and after genetic hybridization (Lheureux *et al*., 2003), leading to infectious virions.

Banana streak viruses are plant bacilliform pararetroviruses containing a double-stranded DNA genome of 7.4 kbp, belonging to the family *Caulimoviridae* and the genus *Badnavirus*, and are transmitted by mealybugs. Potentially infectious eBSV of four natural widespread BSV species have so far been identified in the B genome: *Banana streak Obino l'Ewai virus* (BSOLV), *Banana streak Imové virus* (BSImV), *Banana streak Mysore virus* and *Banana streak Goldfinger virus* (BSGFV) (Gayral *et al*., 2008; Geering *et al*., 2005; Harper *et al*., 1999; Iskra-Caruana *et al*., 2003; Ndowora *et al*., 1999). It is hypothesized that the

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emergence of BSV infection in many micropropagated triploid (AAB) and tetraploid (AAAB) interspecific improved *Musa* hybrids, which has been observed in the last 15 years, very probably results from the triggering of eBSV (Fauquet *et al.*, 2005; Hull *et al.*, 2000).

The majority of cultivated bananas originate from inter- and intraspecific crosses between the two wild diploid species, *Musa acuminata* (denoted A genome) and *M. balbisiana* (denoted B genome). The B genome is the genome of many important cultivars, such as the famous plantain subgroup, which is a staple food for millions of people in West and Central Africa and in Latin America and the Caribbean (Jones, 2000). Nowadays, tissue culture remains the most appropriate method for supplying large quantities of clonal *Musa* planting material, the demand for which has been increasing steadily for the past 15 years, in particular for plantain. Unfortunately, the process of *in vitro* culture has been reported to systematically trigger the activation of infectious eBSV present in newly created interspecific hybrids. Indeed, Dallot *et al.* (2001) have shown that the proliferation stage of the *in vitro* micropropagation process triggers the expression of infectious eBSOLV in virus-free suckers of the improved tetraploid interspecific hybrid FHIA21 (AAAB). This process greatly affects the plantlets, as 58% of micropropagated lines were affected by BSV after six *in vitro* subcultures.

Nevertheless, the risk of activating eBSV following tissue culture has never been assessed in BSV species other than BSOLV and in natural interspecific cultivars containing the B genome. Several natural cultivars are regularly used to supply the increasing demand for diversification.

In this article, we report an extensive study of the spontaneous activation of eBSV of two distinct viral species (BSOLV and BSGFV) in natural plantain cultivars and synthetic interspecific hybrids by *in vitro* culture. We investigate the correlation between the duration of tissue culture and the level of activation of infectious eBSVs, and between the activation potential of infectious eBSVs and their molecular structure. We also establish the cleaning effect of *in vitro* culture in BSV multiplication from BSV-infected plants.

RESULTS

Micropropagation by tissue culture triggers the expression of infectious eBSOLV in both natural and synthetic interspecific plantain cultivars

In this study, we used two natural triploid plantains Kelong Mekintu (AAB) and Black Penkelon (AAB), and one synthetic tetraploid plantain CRBP 39 (AAAB). The activation patterns of infectious eBSOLV, monitored by multiplex immunocapture polymerase chain reaction (M-IC-PCR), during the regeneration process are shown in Fig. 1A,C,E, respectively. The patterns were similar for the three cultivars studied and showed three distinct phases. During the first subculture cycles, there was a steep increase in the percentage of infected plants, until the number of total produced shoots (TPSs) reached values between 300 and 400. Then the percentage of infected plants increased more slowly until the number of TPSs reached values of 1200 (CRBP 39, Fig. 1E) and 2500/2000 [Kelong Mekintu (Fig. 1A)/Black Penkelon (Fig. 1C)]. The highest percentage of infected plants was reached at the end of this second phase and ranged from 9% to 20% depending on the cultivar. Immediately afterwards, there was a decrease in the percentage of infected plants for high TPS values. This decrease was slower in the cultivars Kelong Mekintu and Black Penkelon than in cultivar CRBP39, in which values of zero (i.e. below the sensitivity threshold of M-IC-PCR) were observed for TPS values of 4000 and above (Fig. 1E).

Micropropagation by tissue culture triggers the expression of infectious eBSGFV in the synthetic hybrid CRBP39 only

The activation pattern of infectious eBSGFV was assessed in the three cultivars studied using the same plantain lines, experimental approaches and leaf extracts as above. No activation was observed in cultivars Black Penkelon and Kelong Mekintu (Fig. 1B,D). In contrast, activation of infectious eBSGFV was observed in the three lines of CRBP39. Figure 1F shows similar activation patterns as that described above for eBSOLV, whatever the cultivar. However, the maximum percentage of infected plants was lower for BSGFV than for BSOLV (5%–7% vs. 7%–15%; Fig. 1E,F).

Origin of the decrease in the percentage of BSV-infected plants observed for high TPS values

In order to explain the decrease in infected plants observed at high TPS values, four distinct BSOLV-infected CRBP39 proliferation clumps were selected and used as starting material to produce vitroplants. The percentage of BSOLV-infected vitroplants regenerated from each infected clump was monitored by M-IC-PCR. Figure 2 shows that the percentage decreased steadily over time in the four regenerated lines and reached zero values in all four lines for TPS values between 100 and 400.

eBSGFV signature in both natural and synthetic interspecific plantain species

The eBSGFV signature was established for Kelong Mekintu, Black Penkelon and CRBP39 using sets of eight PCR markers (Gayral,

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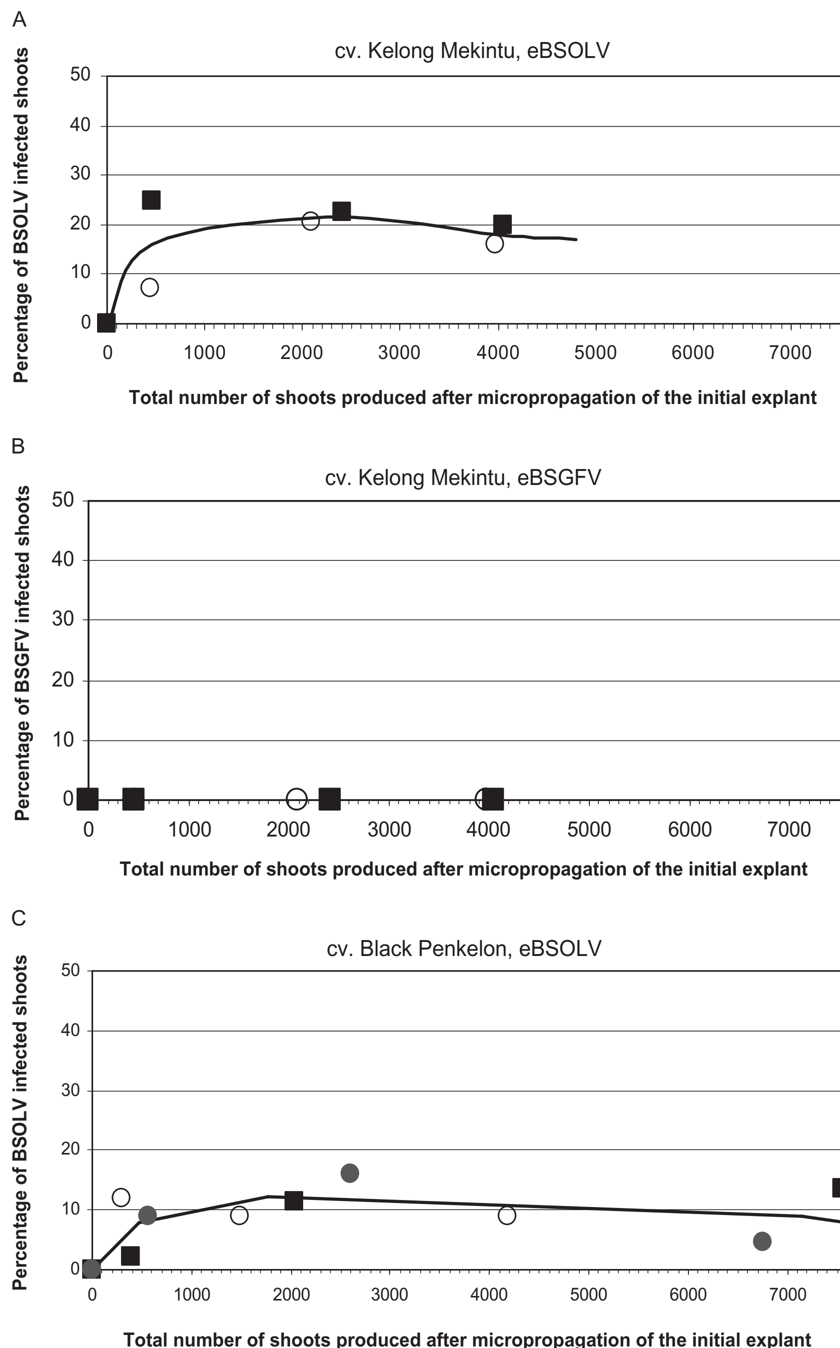

Fig. 1 Activation patterns of infectious eBSOLV (endogenous sequences of *Banana streak Obino l'Ewai virus*) (A, C, E) and eBSGFV (endogenous sequences of *Banana streak Goldfinger virus*) (B, D, F) in cultivars Kelong Mekintu (A, B), Black Penkelon (C, D) and CRBP39 (E, F). For each cultivar, the results are shown for three (Black Penkelon, CRBP39) or two (Kelong Mekintu) distinct lines.

2008). These PCR markers are specific to the allelic eBSGFV of the natural diploid *M. balbisiana* Pisang Klutuk Wulung—PKW (BB), named eaBSGFV-7 and eBSGFV-9 (Gayral *et al*., 2008; Staginnus *et al*., 2009). One set of primers (VV5F/R) was specific to eBSGFV-9. All natural cultivars and the synthetic hybrid, including FHIA21 as control, displayed the same patterns characteristic of an eaBSGFV-7 signature (Fig. 3). No PCR was recorded with either VV5F/R or VV5bisF/R primers.

DISCUSSION

Among the growing number of EPRVs characterized so far in the genomes of crops, only endogenous sequences of *Petunia vein clearing virus* (ePVCV), *Tobacco vein clearing virus* (eTVCV) and *Banana streak virus* (eBSV) have the potential to generate infectious viral particles on activation by abiotic stresses (Staginnus and Richert-Pöggeler, 2006). Although substantial progress has

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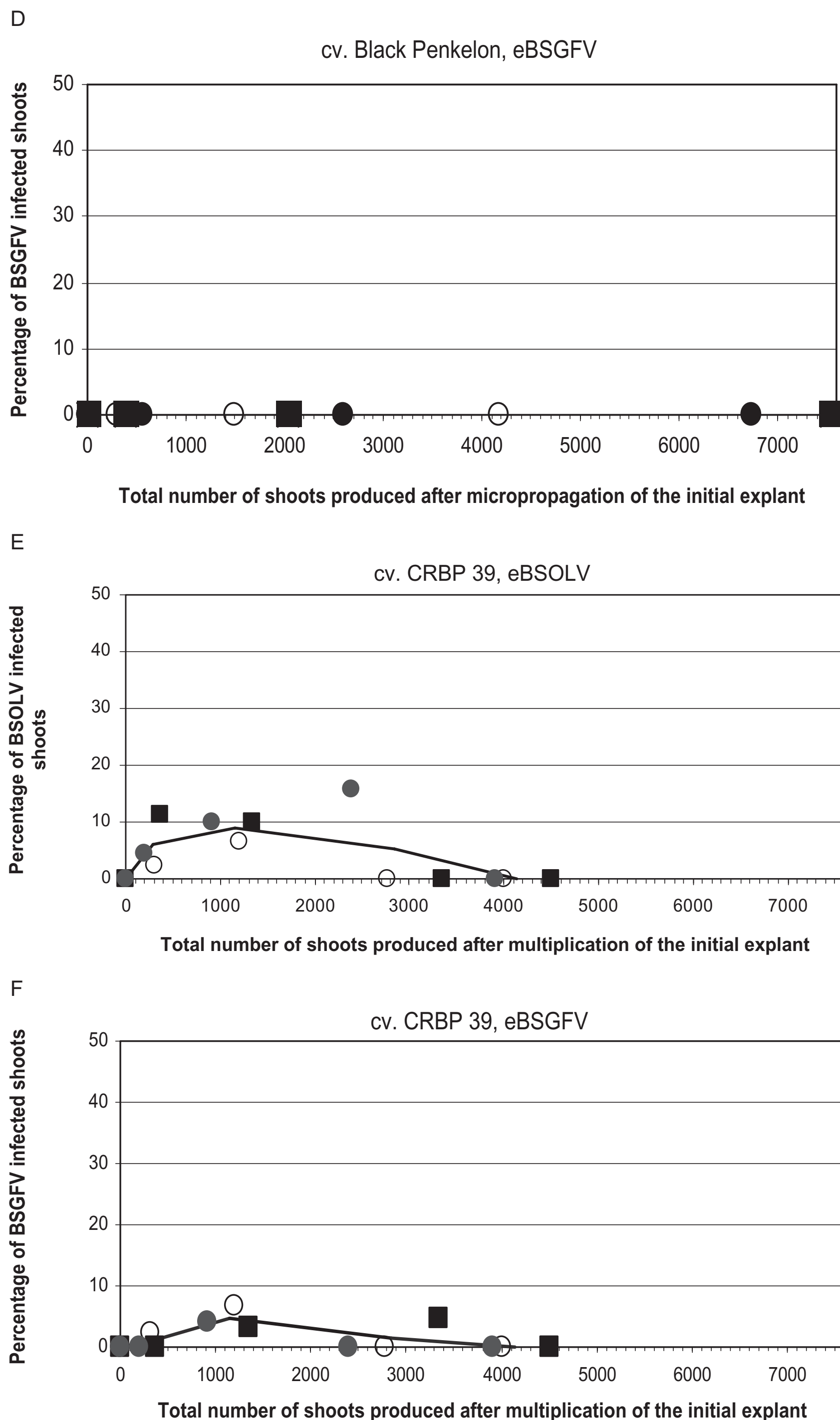

Fig. 1 *Continued.*

been made in the genomic characterization of eBSV (Gayral *et al*., 2008) and the molecular regulation of the expression of ePVCV (Noreen *et al*., 2007), little is known about the exact mechanisms underlying the expression of functional viral genomes from eBSV.

The pioneering work of Dallot *et al*. (2001) demonstrated that shoot tip micropropagation triggers the expression of infectious eBSOLV in the synthetic tetraploid interspecific hybrid FHIA21 (AAAB). It has since been shown that the genome of *M. balbisiana* hosts distinct infectious eBSV originating from at least four BSV species, including BSOLV (Iskra-Caruana *et al*., 2003; Ndowora *et al*., 1999). Whether the activation of eBSOLV occurs in other synthetic interspecific hybrids and in natural interspecific cultivars, and whether all infectious eBSVs are equally

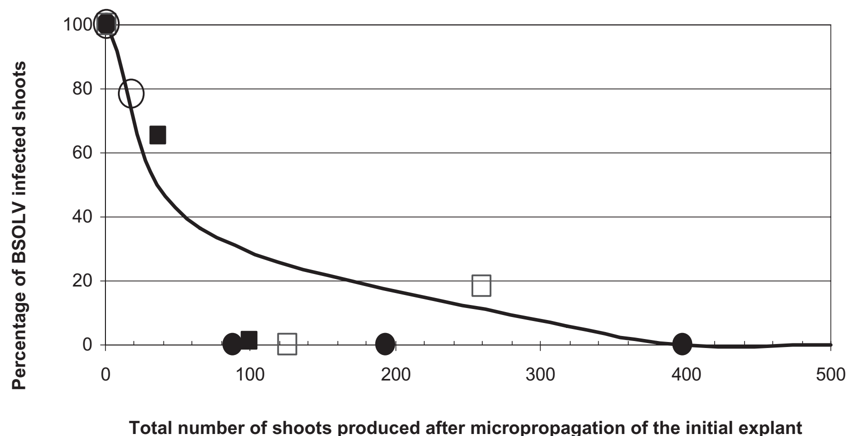

Fig. 2 Infection patterns of BSOLV (*Banana streak Obino l'Ewai virus*) during micropropagation of CRBP39 plants infected by BSOLV. Symbols in the figure are the results for four distinct lines. The curve represents the mean curve of these lines.

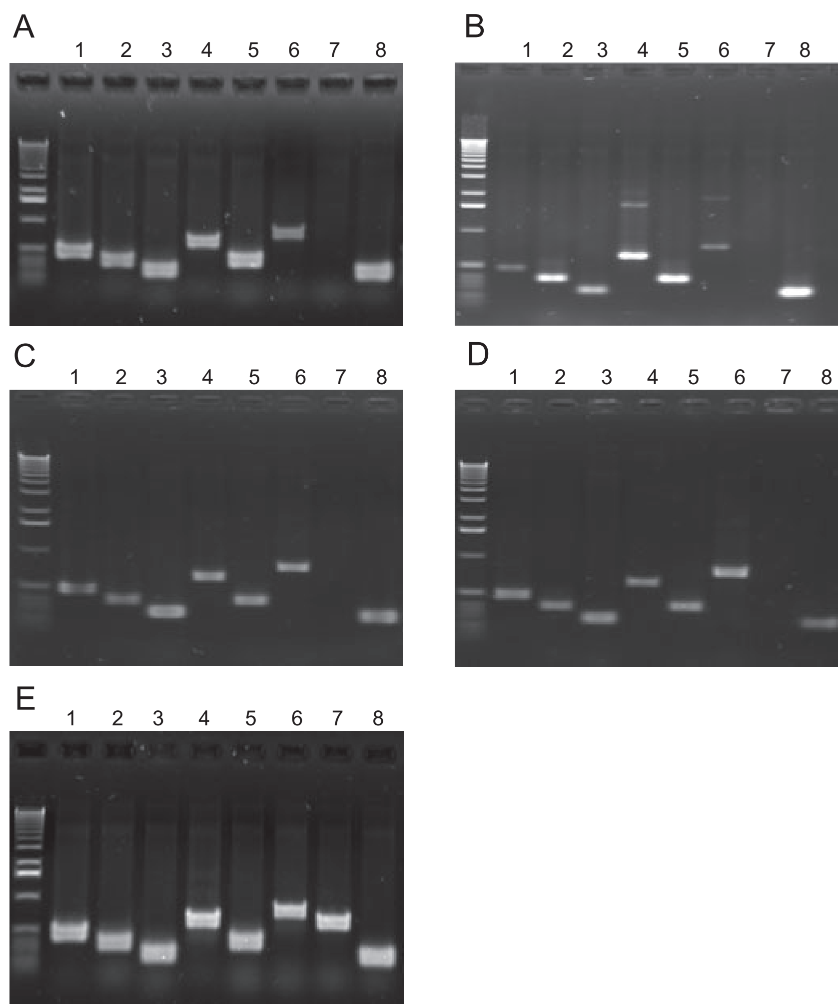

Fig. 3 Molecular signature obtained by polymerase chain reaction (PCR) analysis of infectious eBSGFV (endogenous sequences of *Banana streak Goldfinger virus*) in cultivars CRBP39 (A), FHIA21 (B), Kelong Mekintu (C), Black Penkelon (D) and Pisang Klutuk Wulung (E). Primer pair numbers refer to Table 1; 1–8 refer to primer pairs VM1F/R, VM2F/R, VV1F/R, VV2F/R, VV3F/R, VV4F/R, VV5F/R and VV6F/R.

triggered during tissue culture, is still unknown. The work reported here provides answers to some of these questions.

Our results showed that infectious eBSOLV was expressed during tissue culture in CRBP39, a synthetic tetraploid interspecific hybrid (AAAB) other than FHIA21, as well as in Kelong Mekintu and Black Penkelon, two natural triploid plantains (AAB). The maximum percentages of BSOLV-infected plantlets monitored in our assays ranged from 5% to 20% depending on

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the cultivar, but were consistently below 30%. These values are lower than those reported by Dallot *et al.* (2001) for the FHIA21 hybrid, but are in agreement with those reported by Meyer *et al.* (2008) for the same hybrid. The activation of infectious eBSOLV took place in a limited number of cells and did not occur in the entire shoot tip.

Dallot *et al*. (2001) studied the activation of infectious eBSOLV over six proliferation cycles. Our results confirmed that a strong increase in the percentage of infected plants occurred during the first subcultures. Extending the time frame of our study to 9–12 proliferation cycles revealed a decrease in the percentage of BSOLV-positive plantlets for high TPS values in all of the lines of the three cultivars used in this study. The TPS values at which this decrease occurred varied among the cultivars. We hypothesize that this decrease results from the conjunction of two distinct biological phenomena: (i) the activation of infectious eBSOLV, resulting in an increase in BSOLV-infected plants; and (ii) a concomitant decrease in the episomal particle titre in neoformed plantlets, resulting from cell multiplication outcompeting virus replication. Our experimental data support this hypothesis. They showed that the proportion of infected vitroplants regenerated by tissue culture from a BSOLV-infected CRBP39 plant decreased steadily over time, and that no infected plantlet could be detected from TPS values between 100 and 400 (see Fig. 2). Thus micropropagation by tissue culture results in a cleaning of BSV in banana, usually referred to as the dilution effect. This phenomenon is often exploited to regenerate healthy plantlets from virus-infected mother plants, e.g. to regenerate healthy banana plants from *M. acuminata* mother plants (Helliot *et al*., 2002). However, it is important to note that all *Musa* genotypes harbouring infectious eBSV still retain their capacity to exhibit virus infection during *in vitro* culture. The virus-free regenerated CRBP39 plants displayed activation patterns of both infectious eBSOLV and eBSGFV, similar to those shown in Fig. 1E,F (data not shown).

We observed that all CRBP39 plantlets generated after TPS = 4000 were BSOLV free. This was not the case in the two other cultivars. The percentage of BSOLV-infected shoots during the first phase of activation was slower in CRBP39 than in Kelong Mekintu and Black Penkelon (Fig. 1A,C,E). Whether such differential activation of eBSOLV results from structural differences, such as the number of infectious eBSV activated or the localization in the B genome, or differences in the regulation of expression, remains to be investigated.

Nevertheless, taken together, these results are a strong indication that the activation of infectious eBSOLV by tissue culture is a general phenomenon in interspecific *Musa* cultivars, whether synthetic or natural.

Conversely, our work showed that infectious eBSGFV was activated in all CRBP39 lines, but not in Kelong Mekintu and Black Penkelon. This indicates that differential activation does not result from structural differences in infectious eBSGFV insertions among the cultivars studied, as they all harboured the infectious eaBSGFV-7 allele at the same locus in the B genome. The observed differential activation pattern might result from differences in the regulation of expression of eaBSGFV-7, as infectious eBSOLV is activated in Kelong Mekintu and Black Penkelon. Whether such differences are related to the ploidy of the cultivars—triploid vs. tetraploid—or to their respective *M. acuminata* background remains to be investigated.

In CRBP39, infectious eBSOLV and eBSGFV displayed different maximum activation levels, indicating that the activated cells could express BSOLV or BSGFV, or both BSOLV and BSGFV. This indicates an independent regulation of the expression of each infectious eBSV in CRBP39.

These differences indicate that the activation potential differs between cultivars for a given infectious eBSV and between infectious eBSV within given interspecific *Musa* genotypes.

Our results have profound implications for the management of eBSV in interspecific *Musa* cultivars. They show that the mass micropropagation technique cannot be considered to be safe because of the risk of activating infectious eBSV. This work highlights the risk of promoting outbreaks of BSV by large-scale distribution of risky or infected vitroplants of interspecific banana and plantain genotypes. Considering the increasing demand for *Musa* germplasm worldwide, there is a need for more research efforts to identify alternative mass propagation of interspecific *Musa* germplasm and to further investigate the activation mechanisms of infectious eBSV in order to develop and implement inactivating strategies.

EXPERIMENTAL PROCEDURES

Plant material

Two natural triploid (AAB) plantain varieties, 'Kelong Mekintu' and 'Black Penkelon', and one synthetic tetraploid (AAAB) hybrid plantain, CRBP39, were used for this study. These varieties are part of the *ex situ Musa* collection of the Centre Régional de Recherche sur Bananier et Plantain, Cameroon (CARBAP), and were kindly provided by CARBAP.

Virus indexing

Leaf samples from all suckers used for tissue culture and from their respective mother plants were symptomless and, prior to tissue culture, were subjected to full indexing for all known viruses that infect *Musa* spp. using the appropriate enzyme-linked immunosorbent assay (ELISA) or PCR-based detection techniques. Indexing for BSV was performed on leaf tissues or shoots of proliferation clumps by M-IC-PCR using primers specific for each BSV species, analysed as described by Le Provost

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et al. (2006). The absence of viral particles in the suckers used for tissue culture was further investigated by observations of semi-purified extracts from all leaf samples by electron microscopy.

Tissue culture and kinetics of expression of infectious eBSOLV during micropropagation

Vitroplants were produced from selected virus-free suckers according to the *in vitro* budding method described by Côte *et al.* (1990). Distinct suckers were used to establish at least two independent lines for each banana cultivar. The kinetics of activation of infectious eBSOLV and eBSGFV were studied in each line during the multiplication (proliferation) process. Forty-four shoots were randomly selected from proliferation clumps for virus indexing at each subculture step. After grinding, each shoot was tested for both BSOLV and BSGFV expression by M-IC-PCR using primers specific for each BSV species, analysed as described by Le Provost *et al.* (2006). The shoots not selected for virus indexing were divided into two parts: one-half was removed and the other half was transferred to fresh culture medium for multiplication.

Considering that *Musa* accessions and even suckers of a given accession display different proliferation rates, resulting in different numbers of plantlets being produced after a given number of subcultures, the results were expressed as the percentage of BSV-infected plantlets per number of TPSs. This facilitated the comparison of the percentages of infected plantlets. TPS is defined here as TPS $= p^n$, where p is the proliferation rate and n is the number of subcultures. TPS is therefore directly proportional to the number of subcultures. The percentage of BSV-infected plantlets was measured for TPS values between 200 and 8000, which correspond to 6–12 proliferation subcultures, depending on the cultivar and on the sucker from which the different regenerated lines originated.

Micropropagation of BSOLV-infected vitroplants

To assess and quantify the sanitation effect of tissue culture, a selection of BSOLV-infected proliferation clumps was used to generate plantlets as described above. The progeny of four lines regenerated from these infected clumps were indexed for BSOLV throughout the regeneration process as described above.

Genotyping of eBSGFV

Total genomic DNA was extracted from banana leaf tissue by the method of Gawel and Jarret, (1991). The quality of DNA was estimated visually after migration of 5 µL of DNA in a 0.8% agarose gel, stained with ethidium bromide, and visualized under UV light and by PCR using housekeeping primers of the *Musa* actin gene.

PCR was performed on plant DNA extracts using specific primers (Gayral, 2008) which amplified the eight characterized junctions of eaBSGFV-7 and eBSGFV-9 in PKW shown in Table 1 (Gayral *et al.*, 2008). To ensure that the absence of amplification did not result from single nucleotide polymorphisms, PCRs were performed with a set of primers designed as external to the first set on the same DNA fragment (labelled 'bis' in Table 1).

Table 1 Primers used in the polymerase chain reaction (PCR) screen of endogenous *Banana streak Goldfinger virus* (eBSGFV).

Primer name	Primer sequence (5′–3′)*	Expected product size (bp)
VM1-F†	TTGTCCAAAATCTGCTCGTG	481
VM1-R	TGTAATTCCTGCTCCTGCAA	
VM2-F†	TTCTCCCTTTTCGATCCGTA	374
VM2-R	TTTTGATGCATCTCCAGCAG	
VV1-F	ACAGCTCCAGGAGATTGGAA	268
VV1-R	CTGAAGTGTGCCTGTGGAGA	
VV2-F	TCTGAGATCTCCAGCCAGGT	639
VV2-R	GACAGTTCCAGCACAGCAGA	
VV3-F	TTGCCAAGAATTCCTCCAAG	376
VV3-R	AAGTTCTTGTCGGCAAGGTG	
VV4-F	GAGCAACACGAGTCAACGAA	784
VV4-R	TCTCCACAGGCACACTTCAG	
VV5-F‡	CCATGGAGGTTGACCTGTCT	588
VV5-R	ACCCCTCTGTCTTCCCAACT	
VV5bis-F§	CGCACCTTCATCACAGAAGA	628
VV5bis-R	TACCAGATGGGGAGAAATCG	
VV6-F	GCATGAAGCATGACTGGAGA	264
VV6-R	AATGCATAAGGGCCTCGAAT	

*Primer annealing temperature: 60 °C.
†Primer pairs 'VM' amplify the junction between the *Musa* genome and endogenous *Banana streak virus* (eBSV); primer pairs 'VV' amplify the internal fragment junctions within eBSGFV.
‡Primer pairs characterize eBSGFV-9.
§Primer pairs external to primer ‡ on the same DNA fragment.

PCRs were performed with 5–20 ng of DNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 100 mM of each deoxynucleoside triphosphate (dNTP), 1.5 mM $MgCl_2$, 10 pmol of each primer and 1 U Taq DNA polymerase (Eurogentech, Seraing, Belgium) in a total reaction volume of 25 µL. PCRs were performed as follows: one cycle at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and one elongation cycle at 72 °C for 10 min. PCR products were visualized under UV light after migration of 10 µL of PCR products on a 1.5% agarose gel in 0.5 × TBE [45 mM Tris-borate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8] stained with ethidium bromide.

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