Theor Appl Genet (2003) 106:594–598
DOI 10.1007/s00122-002-1077-z

F. Lheureux · F. Carreel · C. Jenny
B. E. L. Lockhart · M. L. Iskra-Caruana

Identification of genetic markers linked to banana streak disease expression in inter-specific *Musa* hybrids

Received: 8 February 2002 / Accepted: 24 May 2002 / Published online: 19 September 2002
© Springer-Verlag 2002

Abstract Recently-introduced inter-specific *Musa* hybrids, bred for improved yield and resistance to diseases, have been found to be widely infected with *banana streak virus* (BSV), the causal agent of banana streak disease (BSD). One hypothesis suggests: (1) that BSD occurrence in these inter-specific hybrids results from activation of BSV-Ol endogenous pararetrovirus sequences (EPRV) integrated into the *Musa* genome rather than from external sources of infection, and (2) that the process of genetic hybridisation may be one factor involved in triggering episomal expression of the BSV integrants. In order to test this hypothesis we carried out a genetic analysis of BSD incidence in a F1 triploid (*Musa* AAB) population produced by inter-specific hybridisation between virus and disease-free diploid *Musa balbisiana* (BB) and tetraploid *Musa acuminata* (AAAA) parents. Half of the F1 progeny of this cross expressed BSV particles. Using PCR amplification to determine the presence or absence of BSV-Ol EPRVs, it was determined that this endogenous sequence was specific to the *M. babisiana* genome and occurred in a homozygous state. Using bulk segregant analysis, ten AFLP markers co-segregating with the absence and/or presence of BSV infection were identified in the *M. balbisiana* genome, but were absent from the *M. acuminata* genome. Seven of these markers segregated with the presence of a BSV particle and three with the absence of BSV particles. Analysis of the segregation of these markers using a test-cross configuration allowed the construction of a genetic map of the linkage group containing the locus associated with BSV infection in the F1 hybrid population. These data indicate that a genetic mechanism is involved in BSV appearance, and suggest that a monogenic allelic system confers the role of carrier to the *M. balbisiana* parent.

Keywords Badnavirus · Banana streak disease · Endogenous pararetrovirus sequences (EPRV) · Inter-specific hybridisation · Allelic system

Communicated by C. Möllers

F. Lheureux (✉) · M.L. Iskra-Caruana
CIRAD-AMIS, UMR BGPI TA 40/02 Avenue Agropolis,
34398 Montpellier cedex 5, France
e-mail: lheureux@cirad.fr
Tel.: (33)-04.67.71.99, Fax: (33)-04.67.56.01

F. Lheureux · F. Carreel · C. Jenny
CIRAD-FLHOR, Station de Neufchâteau, 97130 Sainte-Marie,
Guadeloupe, France

B.E.L. Lockhart
Department of Plant Pathology, University of Minnesota,
St. Paul MN 55108, USA

Introduction

Banana and plantains (*Musa* spp.) are important local food sources in many countries of the humid tropics. Several diseases and pests cause significant losses in the production of these crops. Therefore, several breeding programs have been initiated in order to produce high yielding disease-resistant cultivars. These improved banana and plantain genotypes are inter-specific hybrids derived from crosses between *Musa acuminata* (A genome) and *Musa balbisiana* (B genome) parents. While many of these inter-specific *Musa* hybrids show impressive gains in yield and disease tolerance (Bakry 2002); most of them have been found to be infected with the *banana streak virus*, BSV Obino l'Ewai strain (BSV-Ol) (Ortiz 1996; Pasberg-Gauhl et al. 1996), and to develop symptoms of the banana streak disease. Epidemiological studies showed that BSV infection in these improved *Musa* hybrids could not originate from external sources (Ndowora et al. 1997), and it was then hypothesised that infections arose instead through a complex recombination pattern from viral sequences integrated into the *Musa* genome, called BSV-OL EPRVS endogenous pararetrovirus sequences EPRVs (Ndowora et al. 1999). Banana streak virus is a member of the plant pararetrovirus, genus *badnavirus*, in the *Caulimoviridae* family (Hull 1999). Like other pararetroviruses, BSV has a circular dsDNA genome (Lockhart 1990) which is repli-

cated by reverse transcription involving an RNA intermediate (Medberry et al. 1990). In the case of human and animal retroviruses, integration of the entire viral dsDNA genome into the host genome is compulsory for its replication (Hindmarsh and Leis 1999). In contrast, integration of viral sequences into a plant host genome is considered to be an accidental event and this is not a normal feature of the viral life cycle (Bejarano et al. 1996). Nevertheless, truncated viral sequences have been recently reported for several members of the *Caulimoviridae* family such as tobacco vein clearing virus, TVCV, petunia vein clearing virus, PVCV, and tobacco pararetrovirus-like (NtEPRV) (Richert-Pöggler et al. 1996; Jakowitsch et al. 1999; Lockhart et al. 2000) as well as *banana streak virus* (Ndowora et al. 1999). Then, two kinds of loci containing endogenous badnavirus sequences have been identified in the *Musa* genome. The first one (*Musa* 1) carries a truncated *badnavirus* genome unable of producing a functional viral transcript (Lafleur et al. 1996). The second locus contains two sequences whose junction can reconstitute a full-length BSV-Ol genome. A model by which these two elements could recombine and produce a complete viral genome has been proposed by Ndowora et al. (1999).

In Guadeloupe (French West Indies), BSD was observed in several F1 hybrid populations resulting from inter-specific crosses between virus-free *M. acuminata* and *M. balbisiana* parents. There was no evidence of a natural field spread of BSV. The study reported here was undertaken in order to provide a genetic basis for BSD expression in inter-specific hybrids, and to test the hypothesis that *de novo* BSV infection and BSD expression in inter-specific *Musa* hybrids can arise from viral sequences integrated in the parental genome as pathogenic EPRVs.

Materials and methods

Plant population

A population consisting of 249 F1 allotriploid hybrids (AAB) was used in this study. This population was derived from inter-specific hybridisation between a virus-free autotetraploid *M. acuminata* male parent (IDN 110 4*x*, AAAA) and a virus-free diploid *M. balbisiana* female parent [Pisang Klutuk Wulung (PKW), BB].

Disease and virus detection

Visual observation

BSD incidence was scored visually. Incidence of BSV was determined by immunosorbent electron microscopy (ISEM) and by immunocapture PCR (IC-PCR) using partially purified extracts. Extracts were prepared from 10 g leaf samples as described by Ahlawat et al. (1996).

Immunosorbent Electron Microscopy (ISEM)

Carbon-coated Formvar grids were floated for 30 min at room temperature on 10 µl of 10 µg/ml anti-BSV IgG (antiserum PMX2RC (Ndowora 1998) diluted in 60 mM carbonate buffer, pH 9.5. The coated grids were rinsed with distilled water and incubated over night at 4C on 10 µl of partially purified leaf tissue extract, and stained with 2% sodium phosphotungstate, pH 7.0, for electron microscopy examination.

Immunocapture PCR

IC-PCR assays using the primers pairs BSVCL1: 5′ATGGCCTTAATAGTCTTTCGTGAT3′ and BSVCl2: 5′GGTGGCGCTGAGGATGTG3′ were performed according to Dallot et al. (2001).

PCR detection of integrated viral sequences

Total *Musa* DNA was extracted from young leaf tissue, according to Lanaud et al. (1995). The presence of the BSV-Ol EPRVs (Ndowora et al. 1999) was checked by PCR using the mixed primers (*Musa* T3-2 5′GGCTTATGATGCTGACCACAT3′ and BSV510 5′TTCTCGACCATAAATTGTAT3′) located on the *Musa* and BSV-Ol genome respectively, as described by Ndowora (1998). The specificity of the amplification was confirmed by Southern-blot hybridisation according to Sambrook et al. (1989) using a probe specific to BSV-Ol and the *Musa* flanking fragment.

AFLP and linkage analysis

Bulked segregant analysis (BSA) was performed according to the procedure described by Michelmore et al. (1991). AFLP analysis was performed according to Vos et al. (1995), using the enzyme combination *Eco*RI/*Mse*I. Selective amplification was carried out using *Eco*RI and *Mse*I primers having three selective nucleotides. AFLP fingerprinting was performed on pools and on individuals. Two pools (each containing DNA from five individuals) were used, one representing a disease phenotype, the other a healthy phenotype. Candidate markers were identified visually from the fingerprint data obtained from the pooled DNA and were tested on the individual progeny of each pool. Markers showing a good correlation were tested on all individual progeny of the PKW × IDN 110 4*x* cross. Linkage analysis was performed with MAPMAKER version 3.0 (Lander et al. 1987). The linkage group was computed using a minimum LOD of 3.0.

Results

Identification of markers linked to the disease

The F1 allotriploid progeny from the PKW × IDN 110 4*x* cross was scored for BSD expression. Because symptoms of BSD may appear only periodically in some plants due to their temperature dependence (Dahal et al. 1997, 1998) and the variable range of expression symptoms, the presence of virions was also required by ISEM and IC-PCR (Table 1). The expression of the disease was scored during 2 years for the presence of viral particles; 130/249 plants of the F1 population showed severe and mild symptoms of BSD and were confirmed as infected by ISEM and IC-PCR. Two symptomless plants were identified both by ISEM and IC-PCR as infected plants and contained BSV particles. The data recorded confirmed: (1) the presence of the viral particles as the best reliable disease marker, and (2) the repartition of the disease in half of the progeny. These observations indicate that the expression of the disease could result from a Mendelian segregation phenomenon.

Table 1 Disease analysis in the progeny of PKW × IDN110 4*x*

Item	% of BSV-positive hybrids[a]	% of BSV-negative hybrid	Total
Symptom	130/249 = 52.2%	0	130
No symptom	2/249 = 0.8%	117/249 = 47%	119
Total	132/249 = 53%	117/249 = 47%	249

[a] Measured by ISEM and IC-PCR

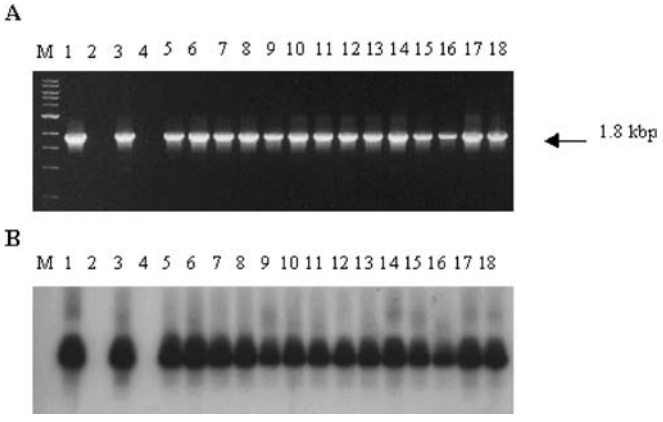

Fig. 1A, B PCR analysis of the F1 progeny from the PKW × IDN 110 4*x* cross, using the mixed primer pair (Musa T3.2 and BSV 510). **A** EtBr-stained agarose-gel analysis of the PCR product. **B** Southern-blot hybridization of the gel using a BSV-Ol specific probe. *Lanes 5 to 11* F1 hybrids showing no sign of BSD. *Lanes 12 to 18* F1 hybrids showing symptoms and BSV particles. *Lane M* 1-kbp ladder; *lane 1* Obino l'Ewai (positive control); *lane 2* Cavendish (AAA, Negative control), *lane 3* PKW, *lane 4* IDN 110 4*x*

Table 2 Segregation of markers in the progeny of PKW × IDN 110 4*x*

Markers	Parents		Progeny		$P\chi^{2a}$
	PKW	IDN 110 4*x*	Observed +:–	Expected +:–	
Virion	–	–	120/109	1:1	0.47
ACTCTA1[c]	+[b]	–	99/127	1:1	0.06
ACTCTA2	+	–	127/99	1:1	0.06
AACCAG	+	–	134/94	1:1	0.01
ACACAC	+	–	113/116	1:1	0.84
ACGCTC	+	–	114/115	1:1	0.95
AAGCAT	+	–	104/124	1:1	0.19
ACGCAC	+	–	130/98	1:1	0.03
ACACAG	+	–	115/109	1:1	0.69
AAGCTT[c]	+	–	118/111	1:1	0.64
AACCAC[c]	+	–	123/105	1:1	0.23

[a] $P\chi^2$ was calculated to test that the data fitted an expected ratio of 1:1
[b] Presence of fragment; – absence of fragment
[c] Markers segregating with absence of viral particles

The BSV-Ol EPRV as a genetic marker candidate of the disease segregation

The presence of BSV-Ol EPRVs among parental and progeny genotypes was investigated by PCR amplification, using the mixed primer pair (Musa T3-2 and BSV510). A product of the predicted size (1,800 bp) was obtained from total genomic DNA of both the *M. balbisiana* parent PKW and the entire F1 population (Fig. 1A). No product was obtained from genomic DNA of IDN 110 4*x*, the *M. acuminata* parent, confirming the results already obtained by Ndowora et al. (1999) and Geering et al. (2001) in the Calcutta-4 cultivar and other *M. acuminata* genotypes. These results were confirmed by Southern-blot hybridisation (Fig. 1B). PKW appears homozygous for the BSV-Ol EPRVs tested. Hence, the set of primers used are not reliable to test whether these BSV-Ol EPRVs co-segregate with the disease.

Mapping of the BSV infection locus linked to the disease

Bulked segregant analysis (BSA) was used to identify AFLP markers co-segregating with the presence or absence of BSV infection. For BSA analysis, we identified two pools of AFLP templates composed of five individuals selected from healthy and diseased F1 progeny respectively. Both templates pools were fingerprinted by AFLP using 64 combinations of *Eco*RI + 3/*Mse*I + 3 primer extensions. BSA yielded seven fragments specific for the diseased pool and three fragments specific for the healthy pool. All ten markers were tested on each individual of the bulks, and on the rest of the progeny individually. The ten markers were present in PKW only, the *M. balbisiana* parent, in a heterozygous condition and absent in IDN 110 4*x*, the *M. acuminata* parent. All ten markers segregated in a 1:1 ratio in the F1 progeny following a test-cross configuration. This result was validated by a χ^2 test to check the null hypothesis of a 1:1 segregation (Table 2). The data obtained from the progeny using these markers permitted the construction of a maternal genetic map of the BSV expressed locus (BEL) as shown on Fig. 2. Markers followed by "r" are linked in the repulsion phase. The repulsion phase was found by inverting the markers scored and comparing them to non-inverted scores using Mapmaker. All markers were linked to the BEL. The direct distance between BEL and the nearest marker (ACACAC) was 1.2 cM. The marker covered 69.8 cM and the genetic distances between markers varied in size from 0.6 to 39.2 cM. The 1:1 disease segregation ratio, and the joint occurrence of markers segregating with disease and markers segregating with the absence of viral particles, suggest a monogenic allelic system, which confers the role of the carrier to PKW.

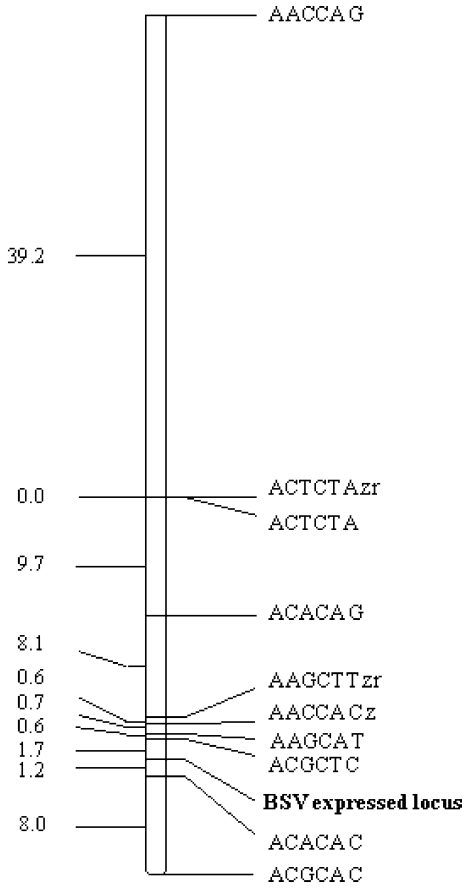

Fig. 2 Genetic map of the BSV-expressed locus area. Markers (abbreviation of the selective nucleotide extension in the 3′-end of each primer) are indicated on the right. Map distances (in centimorgans) are given to the left. z, markers segregating with absence of viral particle. r, markers linked in repulsion phase

Discussion

The results of our study to test the BSV-OL EPRVS as a candidate gene for BSD expression were inconsistent because it occurs in a homozygous state for the PKW parent. However, the identification of the origin of viral particles remains essential for the understanding of the BSV appearance mechanism. In this study, we also revealed the existence of a unique locus for the PKW genome and in an heterozygous state, which is responsible for BSD expression in 50% of the F1 progeny. Genetic data suggest that the release mechanism depends on a monogenic system. This could be inhibited by the allele situated on the homologous chromosome. Such a system confers the role of a free carrier on the *M. balbisiana* parent. The inhibition of this mechanism would be triggered during breeding hybridisation allowing disease expression in part of the F1 progeny.

At least two hypotheses can be considered for the origin of viral particles. In the first one, the viral particles originate from the BSV-OL EPRVS identified by mixed primers and occurs as a homozygous state in the *M. balbisiana* parent. In order to explain the observed segregation, the PKW genome contains a genetic factor (revealed by AFLP markers) as a heterozygous state because the homologous recombination model proposed by Ndowora et al. (1999) cannot account for BSD appearance in 50% of the F1 hybrids. This hypothesis also implies that the BSV-OL EPRVS and the BSV expressed locus are not genetically linked. In our second hypothesis the viral particles would originate from a BSV-Ol EPRV occurring in a heterozygous state linked to the releasing factor.

These two hypotheses are totally compatible with the silent expression observed in the PKW parent. Our results show that an allelic interaction occurring in-trans can prevent the action of the releasing factor involved in homology dependent gene silencing. This phenomenon appears an attractive hypothesis with regard to the regulation of the transposable-elements activity (Hirochika et al. 2000; Matzke et al. 2001); and the epigenetic inhibition of the releasing factor will be irreversible in PKW. In fact, PKW, but also IDN 110 4*x*, show no sign of infection and have never developed disease in the past. The most appropriate explanation could be that BSV infection in our cross results from the allopolyploidisation phenomenon which leads to the formation of triploid hybrids AAB in which one *M. balbisiana* (B) genome is associated with two *M. acuminata* (A) genomes. Therefore, during breeding hybridisation, 50% of the F1 progeny would contain the releasing factor and become active by the absence of the inhibitor mechanism. This type of mechanism has been hypothesised for transposable-element (TE) activity in which integrated TE sequences could both change the expression pattern and the methylation level (Matzke et al. 2000). Finally, a recent study has shown that allopolyploidy can also induce the elimination of a specific sequence in noncoding regions of the plant genome (Eckardt 2001). The integrated BSV sequences could be the target of such a phenomenon, causing their excision and making possible the regeneration of the viral genome.

These evoked mechanisms are complex but seem to occur related to the emergence of a new disease and new hybrids resulting from the co-adaptation/co-evolution of the plant and viral genomes. During the last 2 years, other cases of EPRVs have been reported. Multiple integrations of truncated sequences from uncharacterised tobacco pararetrovirus-like (Nt EPRV) have been identified into the *Nicotiana tabacum* genome (Jacowisch et al. 1999). A recent report has shown that an episomal form of *tobacco vein clearing virus* may arise from viral integrants in the *Nicotiana edwardsonii* genome (Lockhart et al. 2000). Understanding the BSV expression mechanism from BSV-OL EPRVS could lead to a useful model system for evaluating the risk of disease expression from EPRVs for other pararetroviruses. Indeed, today the BSD is the unique described pathosystem with economic consequences. Our study confirm that BSD is currently the major constraint for improvement programs and appears today as an important genetic disease for the new banana

hybrids involving the *M. balbisiana* progenitor. Therefore, the co-dominant marker identified in this study might prove extremely useful for the marker-assisted selection of new hybrids devoid of pathogenic BSV EPRVs.

Acknowledgements We thank Ange-Marie Risterucci and Dr. Laurent Grivet for their generous help with molecular genetic analysis and Dr. Frederic Bakry for *Musa* genetic information. This Work partially funded by INIBAP (International Network for the Improvement of Banana and Plantain).

References

Ahlawat YS, Pant RP, Lockhart BEL, Srivastava M, Chakraborty NK, Varma A (1996) Association of a badnavirus with citrus mosaic disease in India. Plant Dis 80:590–592

Bakry F (2002) Banana genetic diversity and perspectives for breeding. New varieties: why and how? Comp Rend l'Académie d'Agric France (in press)

Bejarano ER, Khashoggi A, Witty M, Lichtenstein C (1996) Integration of multiple repeats of geminiviral DNA into the nuclear genome of tobacco during evolution. Proc Natl Acad Sci USA 93:759–764

Dahal G, Gauhl F, d'A Hugues J, Pasberg-Gauhl C, Nokoe KS (1997) Symptomatology and development of banana streak, a disease causes by *banana streak badnavirus*, under natural conditions in Ibadan, Nigeria. Acta Hort 126:25–31

Dahal G, Pasberg-Gauhl C, Gauhl F, Thottapilly G, d'A Hugues J (1998) Effect of temperature on symptom expression and reliability of *banana streak badnavirus* detection in naturally infected plantain and banana (*Musa* sp.) Plant Dis 82:16–21

Dallot S, Acuña P, Rivera C, Ramìrez P, Côte F, Lockhart BEL, Caruana ML (2001) Evidence that the proliferation stage of micropopagation procedure is a determinant in the expression of *banana streak virus* integrated into the genome of FHIA 21 hybrid (*Musa* AAAB). Arch Virol 146:2179–2190

Eckardt NA (2001) A sense of self: the role of DNA elimination in allopolyploidization. Plant Cell 13:1699–1704

Geering ADW, Olszewski NE, Dahal G, Thomas JE, Lockhart BEL (2001) Analysis of the distribution and structure of integrated *banana streak virus* DNA in a range of *Musa* cultivars. Mol Plant Pathal 2:207–213

Hindmarsh P, Leis J (1999) Retroviral DNA integration. Microbiol Mol Biol Rev 63:836–43

Hirochika H, Okamoto H, Kakutani T (2000) Silencing of retrotransposons in *Arabidopsis* and reactivation by ddm1 mutation. Plant Cell 12:357–368

Hull R (1999) Classification of reverse transcribing elements: a discussion document. Arch Virol 144:209–214

Jakowitsch J, Mette MF, Van der Winden J, Matzke MA, Matzke AJM (1999) Integrated paraetroviral sequences define a unique class of dispersed repetitive DNA in plants. Proc Natl Acad Sci USA 96:13241–13246

Lafleur DA, Lockhart BEL, Olszewski NE (1996) Portions of the *banana streak badnavirus* genome are integrated in the genome of its host *Musa* sp. Phytopathology (supplement) 86:100

Lanaud C, Risterucci AM, N'Goran JAK, Clement D, Flament M, Laurent V, Falque M (1995) A genetic linkage map of *Theobroma cacao L.* Theor Appl Genet 91:987–993

Lander ES, Green P, Abrahamson J, Barlow A, Daly M, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Lockhart BEL (1990) Evidence for double-stranded circular DNA genome in a second group of plant viruses. Phytopathology 80:127–131

Lockhart BEL, Menke J, Dahal G, Olszewski NE (2000) Characterization and genomic analysis of *tobacco vein clearing virus*, a plant pararetrovirus that is transmitted vertically and related to sequences integrated in the host genome. J Gen Virol 81: 1579–1585

Matzke MA, Mette MF, Matzke AJM (2000) Trangene silencing by the host genome defense: implication for the evolution of epigenetic control mechanisms in plants and vertebrates. Plant Mol Biol 43:401–415

Matzke MA, Mette MF, Jakowitsch J, Kanno T, Moscone EA, Van der Winden J, Matzke AJM (2001) A test for transvection in plants: DNA pairing may lead to trans-activation or silencing of complex heteroalleles in tobacco. Genetics 158:451–461

Medberry S, Lockhart BEL, Olszewski NE (1990) Properties of commelina yellow mottle virus complete DNA sequence, genomic discontinuities and transcript suggest that it is a pararetrovirus. Nucleic Acids Res 18:5505–5513

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Ndowora TC (1998) Development of an enzyme immunoassay to detect serologically diverse isolates of *banana streak virus* and characterization of viral sequences integrated into the *Musa* genome *Musa* sp. PhD thesis, University of Minnesota, USA

Ndowora TC, Lockhart BEL, Olszewski NE (1997) Relationship between integrated and episomal *badnavirus* genomic sequences in *Musa*. Phypathology 86:69 (Supplement)

Ndowora T, Dahal G, Lafleur D, Harper G, Hull R, Olszewski NE, Lockhart BEL (1999) Evidence that *badnavirus* infection in *Musa* can originate from integrated pararetroviral sequence. Virology 255:214–220

Ortiz R (1996) The potential of AMMI analysis for field assessment of *Musa* genotypes to virus infection. Hortscience 31:829–832

Pasberg-Gauhl C, Gauhl F, Schill P, Lockhart BEL, Afreh-Nuamah K, Osei JK, Zofa K (1996) First report of *banana streak virus* in farmer's fields in Benin, Ghana, and Nigeria, West Africa. Plant Dis 80:224

Richert-Pöggeler KR, Shepherd RJ, Casper R (1996) Petunia vein-clearing virus, a pararetrovirus that also exists as a retroelement in the chromosome of its host. In: Abstract of the International Congress of Virology; Jerusalem Israel, p 16

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Vos P, Hoger R, Bleeker M, Reijan M, Van de Lee T, Hornes M, Frijters A, Pot J, Pelemenan J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414