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RAPID COMMUNICATION

Integration of Banana Streak Badnavirus into the *Musa* Genome: Molecular and Cytogenetic Evidence

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Breeding and tissue culture of certain cultivars of bananas (*Musa*) have led to high levels of banana streak badnavirus (BSV) infection in progeny from symptomless parents. BSV DNA hybridized to genomic DNA of one such parent, Obino l'Ewai, suggesting integration of viral sequences. Sequencing of clones of Obino l'Ewai genomic DNA revealed an interface between BSV and *Musa* sequences and a complex BSV integrant. *In situ* hybridization revealed two different BSV sequence locations in Obino l'Ewai chromosomes and a complex arrangement of BSV and *Musa* sequences was shown by probing stretched DNA fibers. This is the first report of integrated sequences that possibly lead to a plant pararetrovirus episomal infection by a mechanism differing markedly from animal retroviral systems.

Plantains and bananas (*Musa*) are the fourth most important global food commodity (*1*). The international trade in dessert bananas is about 15% of *Musa* production, much of the rest being an important staple crop for many tropical people. Breeding, particularly for pathogen resistance, and tissue culture are being used to improve and multiply both commercial and subsistence *Musa* cultivars. However, after sexual hybridization and/or tissue culture of certain symptomless parent or mother plants, several promising lines are showing high rates of infection with banana streak badnavirus (BSV).

Banana streak disease has been reported from many countries (*4, 12*) but, until recently, has not been considered to be a serious problem. However, the appearance of this disease in certain tissue culture and breeding lines is causing increasing concern. The disease is caused by a badnavirus, a pararetrovirus with a double-stranded DNA genome contained in bacilliform particles (*8, 12, 13*).

Obino l'Ewai, a triploid "French" plantain cultivar with an AAB genome constitution, has a propensity to produce a large proportion of BSV-infected progeny after tissue culture or crossing. The sequence of the episomal form of BSV DNA from a tetraploid hybrid derived from Obino l'Ewai (*9*) showed that it is a typical badnavirus (*14*) with all the features associated with episomally replicating plant pararetroviruses (Fig. 1a). PCR of Obino l'Ewai DNA with a primer pair based on the BSV sequence to amplify the region between nucleotides 4673 and 5317 resulted in a product of the predicted size of 644 bp that hybridized with a probe to the total BSV sequence (Fig. 1b). Southern blots of total cellular DNA of Obino l'Ewai and related hybrid cultivars probed with the full-length episomal BSV sequence showed hybridization to bulk phase high-molecular-weight DNA (Fig. 1c) as well as to DNA of the size expected for the episomal form (7.4 kbp). Restriction endonuclease digests of this DNA gave bands whose sizes did not fit those expected for the episomal DNA form. For example, *Bam*HI, which cuts once in episomal BSV DNA and gives a linear molecule of about 7.4 kbp (Fig. 1c, lane 4), gives a major band at about 5 kbp (Fig. 1c, lane 2).

To examine whether these BSV sequences in high-molecular-weight DNA were actually in the *Musa* nuclear chromosomes we conducted double-target *in situ* hybridization on more than 100 metaphase and prometaphase chromosomes from 20 root tips from two plants of Obino l'Ewai using three probes. Probe BSV4673-5317 covered the region of the viral genome containing amino acid sequence homologies to aspartate protease and reverse transcriptase, activities found in all retroelements but that show considerable differences in nucleotide sequences; probe BSV6294-1679 covered a region with little or no homology to other badnaviruses or retroelements (Fig. 1a). The MusaOL probe was to the sequence adjacent to the BSV insert (see below). These three probes were used in combination with one another and with an rDNA (*15*) probe. All probes gave hybridization signals on chromosomes of Obino l'Ewai (Fig. 2). A major hybridization site to BSV6294-1679 was detected

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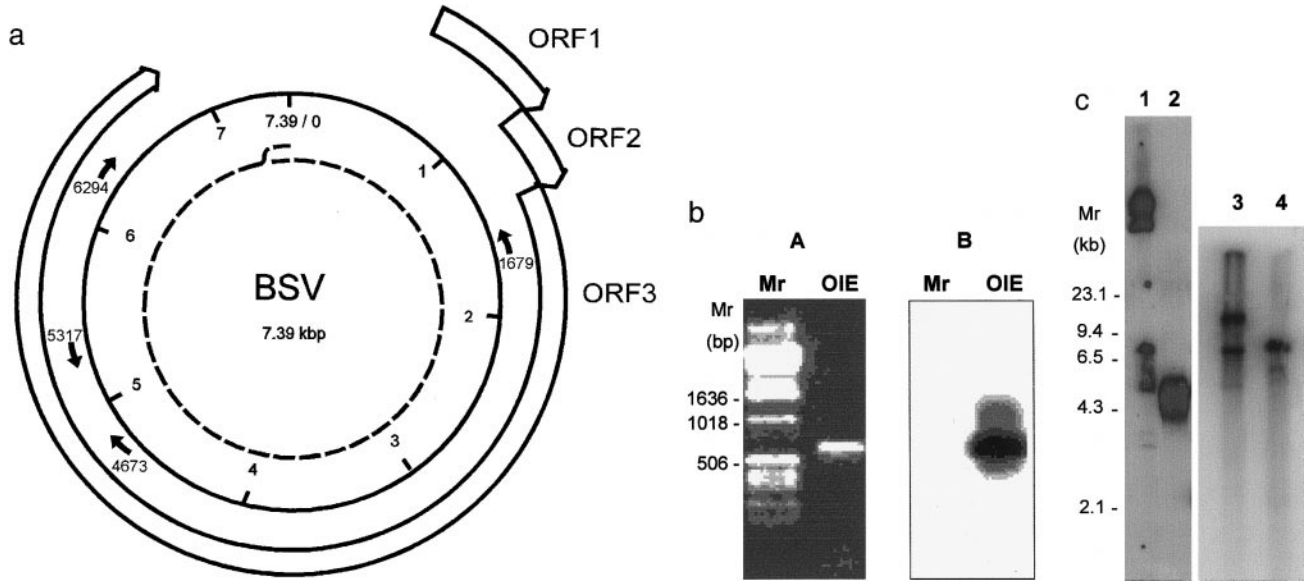


FIG. 1. (a) Genome organization of episomal BSV DNA showing regions of primers and probes. Complete circle represents the 7389-bp double-stranded DNA genome (*9*). The arrows indicate the relative positions of the two sequences used as probes, 644 bp at 4673–5317 and 2775 bp at 6294–1679. Outer arcs indicate the position of the three large open reading frames. Inner broken line depicts 35S RNA transcript; 5′ end position is known and the position of the 3′ end is estimated. (b) PCR of Obino l'Ewai DNA using primers BSV4673 and BSVr5317, giving a characteristic 644-bp band. Panel A shows the ethidium bromide stained gel, with the 1-kb ladder (Gibco) in lane Mr and the PCR product in lane 01E; panel B is an autoradiograph of a Southern blot of the gel probed with radiolabeled BSV DNA. (c) Genomic Southern blot of DNA from *Musa* TMP115BC and BSV isolated from Obino l'Ewai, probed with full-length BSV DNA. Lane 1, undigested TMP115BC DNA; lane 2, *Bam*HI digested TMP115BC DNA; lane 3, undigested BSV DNA; lane 4, *Bam*HI digested BSV DNA.

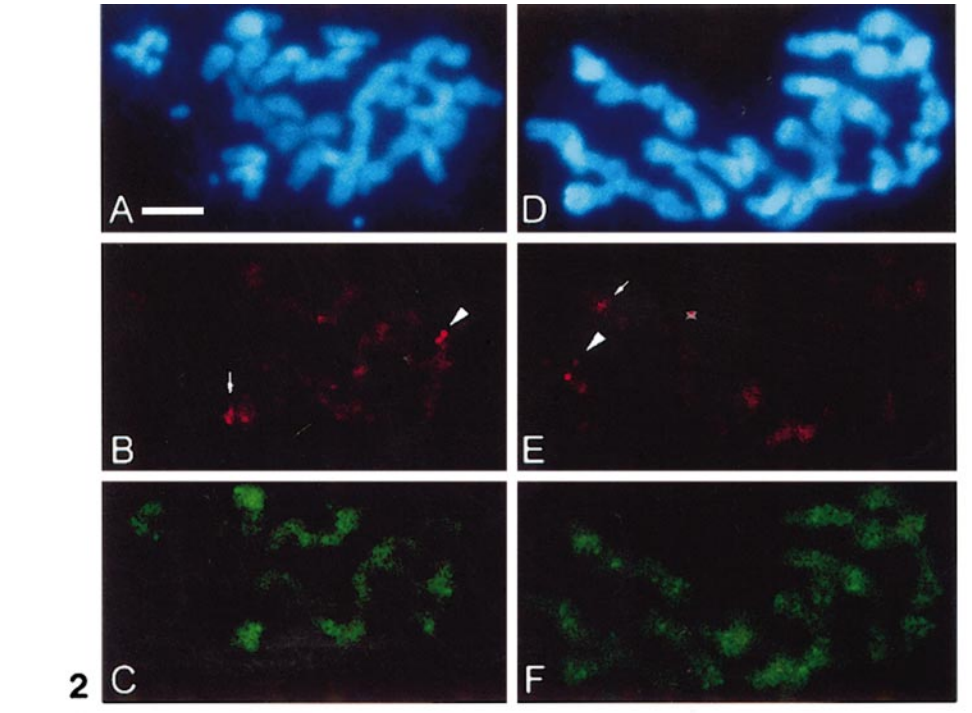
on both chromatids of one chromosome in each metaphase (Figs. 2b and 2e) and at least one weaker hybridization site was regularly seen. Hybridization of the shorter probe, BSV4673-5317, was weak and less clearly discriminated from diffuse background although a single site was normally detected, colocalizing with BSV6294-1679 in double-target experiments. The MusaOL probe showed hybridization to multiple sites throughout the genome (including near the major BSV site; Figs. 2c and 2f), but was not uniformly dispersed. Neither of the two BSV signals was on the same chromosome that hybridized with the rDNA probe.

Since fragments of the reverse transcriptase gene of *Ty1-copia*-like retrotransposons show a dispersed distribution on chromosomes of all plant species examined so far (*10*), it is clear that, at the high hybridization stringency used, the BSV4673-5317 probe is not hybridizing to other reverse transcriptase-like sequences in the genome, while no sequences homologous to the BSV6294-1679 probe have been reported from plant genomes. Therefore, these observations are direct evidence for the integration of BSV sequences into the *Musa* genome.

A crucial stage in the replication of retro- and pararetroviruses is the transcription of the template for reverse transcription that covers the viral genome together with a terminal redundancy, frequently termed the 35S RNA. Such a molecule is considered to be an essential intermediate for the expression of episomal BSV from an integrated form. Thus, the sequence-specific amplification polymorphism (S-SAP) approach (*21*) with a specific primer downstream of the 5′ end of the BSV 35S RNA (*9*) and *Taq*I primers was used to detect BSV/*Musa* interfaces in nuclear DNA. This approach gave a number of products that hybridized with BSV (Fig. 3a) and that were cloned. Twenty of the clones were sequenced and these fell into three families. Clones in one of these families, e.g., clone pS-SAP17, 338 bp, contained both BSV and non-BSV (shown later to be *Musa*) sequences that showed an interface between the position of the 5′ nucleotide of BSV 35S RNA and the *Musa* sequence (Fig. 3b). The *Musa* sequence has no significant homology to any EMBL/GenBank database sequences, either as nucleic acid or as protein.

Another family of S-SAP products (pS-SAP2) contained a mosaic of BSV sequence (Fig. 3c). This indicates a rearrangement of this BSV sequence that is compatible with the sequenced insert (see accompanying paper by Ndowora *et al.* (*22*)) and shows that the 35S RNA transcription start site of BSV occurs in more than one arrangement. The third family of S-SAP products comprised various linear sequences of BSV.

Using a primer based on the *Musa* sequence (M2) and reverse primers based on BSV sequence, the linear extent of this BSV insert was explored by PCR (Fig. 3d). The expected size of fragments was found with primers up to position 5.8 kbp on the BSV genome, but with primers to positions 6.1 kbp and beyond, the fragments decreased in size. This indicates that the integrant is contiguous



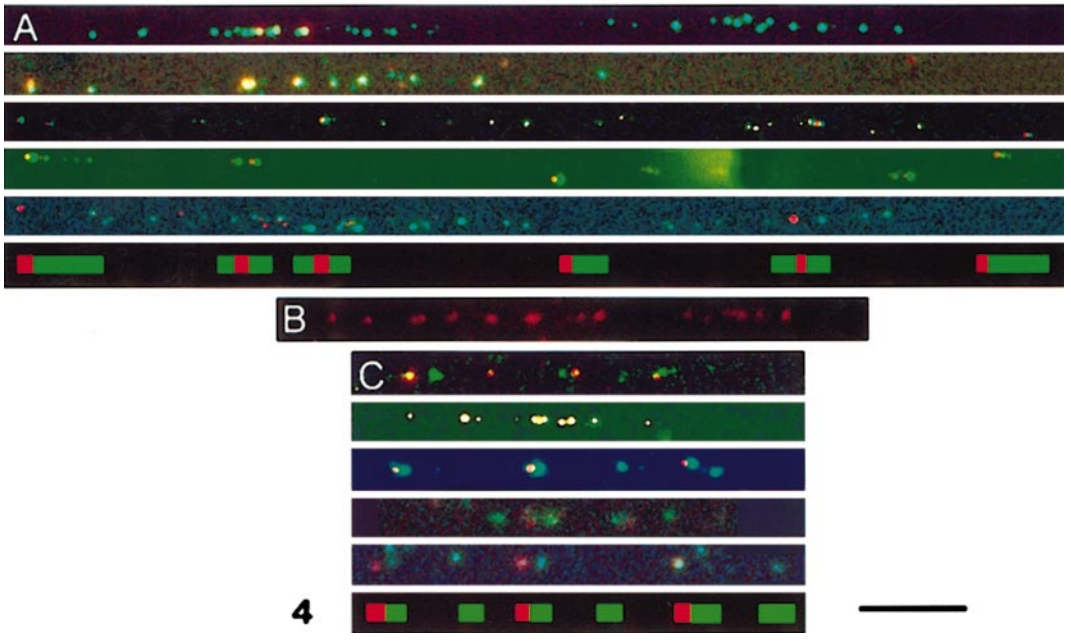


FIG. 2. *In situ* hybridization to chromosomes from two metaphase spreads of Obino L'Ewai plantain ($2n = 3 \times 33$). (A and D) Chromosomes stained blue with the DNA stain DAPI. (B and E) Hybridization sites of BSV6294-1679 (red) showing one major site in each metaphase (arrowhead) and at least one minor site near the limits of hybridization sensitivity (arrow). X is overstain precipitate not associated with a chromosome. (C and F) The dispersed but uneven hybridization pattern of the MusaOL probe including some sites associated with BSV6294-1679. Bar, 5 μm.

FIG. 4. *In situ* hybridization to extended DNA fibers from Obino L'Ewai nuclei. Three different hybridization patterns of chains of dots, representing probe hybridization sites, were detected with BSV6294-1679 (green) and MusaOL (red). (A) Five independent and aligned long fibers above a consensus diagram of hybridization pattern showing red sites and chains of green signals. Both the MusaOL and the BSV6294-1679 sequences are present in multiple copies in the structure of 150 kb, in at least two different relative orientations, and are separated by gaps with no hybridization (no homology to probes). (B) Chains of red dots representing sites of MusaOL not associated with the BSV sequence. (C) Five aligned short fibers above a consensus diagram, showing a pattern that can be interpreted as three subrepeats. Under the hybridization, detection, and imaging procedures used, individual signal sizes are larger than expected from the probe length and may be slightly displaced from the fiber axis, and some supposed target sites may not have a detectable signal. Bar, 5 μm, corresponding to a 15-kb DNA fiber length.

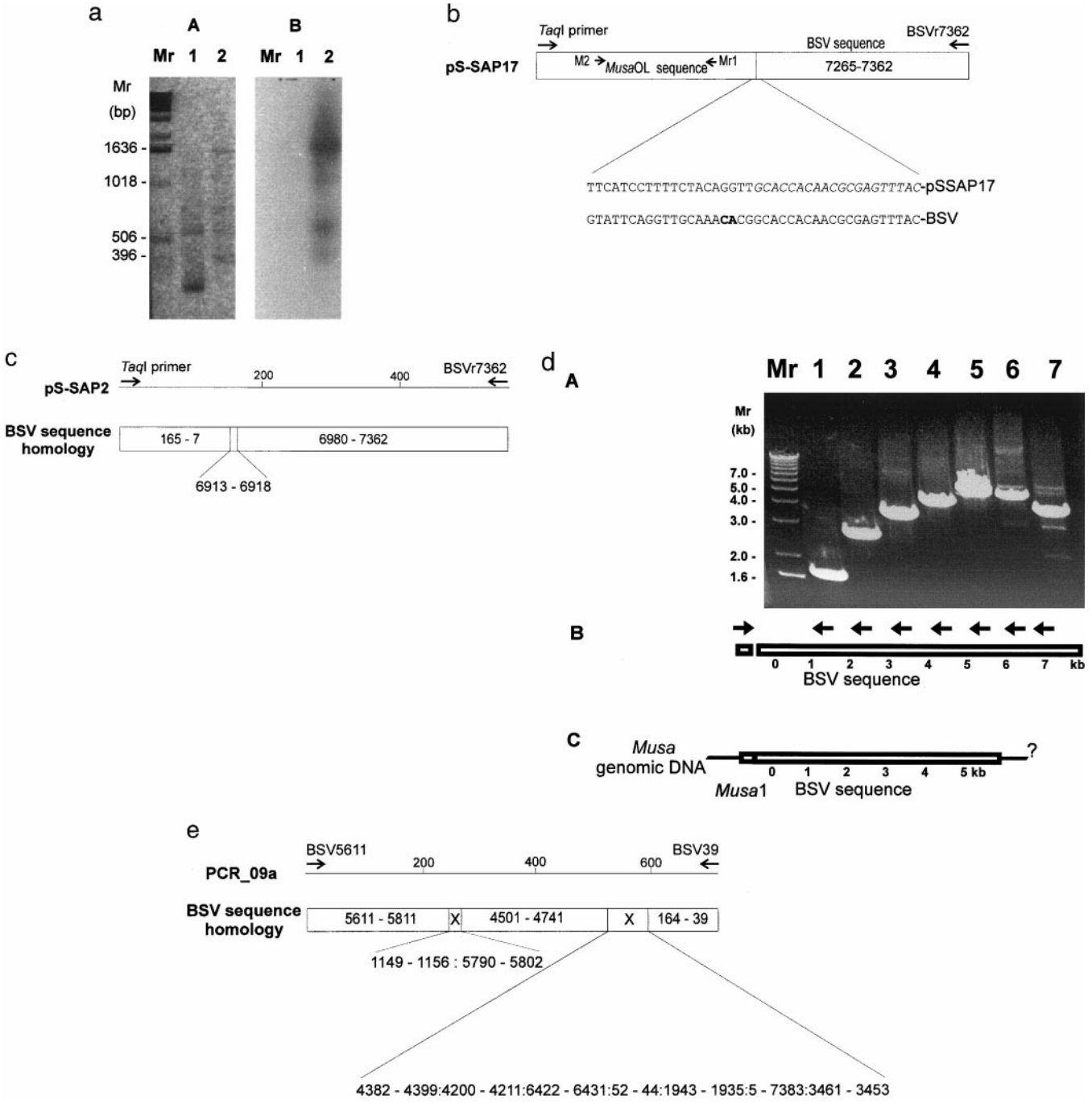


FIG. 3. (a) S-SAP analysis of Obino l'Ewai DNA. Panel A shows an ethidium bromide stained gel and panel B an autoradiograph of a blot of the gel probed with radiolabeled BSV DNA. Lane 1, *Taq*I primer alone; lane 2 secondary amplification with nested BSVr7353 and *Taq*I primers. (b) Diagram derived from the sequence of the 338-bp fragment of pS-SAP17. The sequence of pS-SAP17 contains 285 bp of *Musa* sequence adjoining a BSV sequence at a position corresponding to the 35S RNA transcription start site. The relative positions of primers *Taq*I and BSVr7362 used to generate the S-SAP fragment and of Mr1 and M2 used to amplify the probe MusaOL are shown. The expanded segment shows the sequence of the interface between *Musa* and BSV with the pS-SAP17 sequence identity to BSV indicated in italics and the mapped 5′ ends of the BSV 35S RNA in boldface type. (c) Diagram from sequence of pS-SAP2, which has a mosaic of BSV sequences. The upper line represents the 548-bp sequence of pS-SAP2 from *Taq*I to BSVr7362 primers. The rectangle below represents the corresponding BSV sequence. (d) Progressive PCR along BSV integrants with *Musa* primer and reverse BSV primers at various positions. (Panel A) Ethidium bromide stained gel of PCR products using M2 primer and the following BSV primers; lane 1, BSVr1438; lane 2, BSVr2473; lane 3, BSVr3539; lane 4, BSVr4557; lane 5, BSVr5488; lane 6, BSVr6407; lane 7 BSVr7099. (Panel B) Diagram showing positions of PCR primers. (Panel C) Diagram showing interpretation of results. (e) Diagram derived from sequence of 664-bp product (PCR_09a) generated from Obino l'Ewai DNA with BSV5611 and BSV39 primers. The "X regions" are interpreted as very rearranged BSV sequences of 8 bp or greater as shown in the expanded sections.

BSV sequence from the position of the 5′ end of the 35S transcript to about 5.5 kbp; further experiments showed that after BSV 5.8 kbp the sequence becomes perturbed, as is shown in the sequencing of the product from PCR of genomic DNA using BSV5611 and BSV39 primers (Fig. 3e). It is also in accord with the nucleotide sequence of the insert (see accompanying paper by Ndowora *et al.* (*22*)).

To examine the structure of the *in situ* hybridization sites in the *Musa* genome, stretched DNA fibers were prepared on slides (*3, 6*) from Obino l'Ewai nuclei. Following hybridization with BSV6294-1679, conspicuous rows of punctate hybridization sites ("dots") were observed, with a low level of randomly placed dots representing background or nonspecific hybridization; this is the result obtained in successful hybridization to plant clone and animal DNA targets (e.g., *3, 6, 19*). Double-target hybridization showed that the MusaOL sequence was present at sites associated with the BSV6294-1679 hybridization sites and also independently (Fig. 4). In total, more than 50 photographs of fibers from 20 slides were analyzed quantitatively, and further fibers were examined and compared in the microscope. It was immediately apparent that there were two different MusaOL-BSV6294-1679 associated structures present in approximately equal numbers. The longer structure, represented by rows of dots 51 $\pm$ 14 μm long (Fig. 4a), was considered to correspond to the major hybridizing site seen on metaphase chromosomes (Figs. 2b and 2e), while the shorter structure, 17 $\pm$ 6 μm long (Fig. 4c), corresponded to the minor hybridization site. Based on theoretical considerations of the length of the extended DNA molecule and calibration from hybridization with probes of known length and interspersion pattern (*6, 19*), these structures are estimated to be 150 and 50 kb long. Each group of fibers, long and short, showed evidence for common patterns of red and green signal sites and gaps. For perfectly uniform patterns to be seen, the technique requires the detection of complete hybridization of short labeled probes to homologous segments of linearly stretched, but unbroken, DNA molecules that have been completely denatured. Nonlinear stretching, incomplete denaturation, reannealing of target DNA, DNA nicking (for example by fixation chemicals), incomplete probe hybridization to target sites and partial detection of probe mean that there is substantial variation between fibers, with most showing a subset of the maximum hybridization that would be possible. Given these caveats, the elements revealed a consensus pattern of BSV hybridizing signals, MusaOL signals, and gaps in which there was no signal. The longer dot pattern (Fig. 4a) is interpreted as comprising six elements, each with a MusaOL hybridizing signal at one end; the overall structure had the MusaOL signal at one end and not at the other. Most gaps represent regions where there was BSV sequence not complementary to the 2.7-kbp BSV probe or *Musa* chromosomal sequence not homologous to the MusaOL probe. The shorter dot pattern was relatively simple with three subrepeats (Fig. 4c). Although both types of element show the presence of repeating BSV sequence adjacent to MusaOL sequence, it is not possible at this stage to relate in detail any one element to the sequence given in the accompanying paper (*22*). The shorter dot pattern was relatively simple with three MusaOL sites interspersed by BSV6294-1679 sites and gaps.

With the MusaOL probe alone, variable lengths of rows of dots were detected (Fig. 4b) as were smaller sites, while no conspicuous rows of dots were visualized with BSV4673-5317 probe alone (data not shown). In double-target combinations, no probes were colocalized, but rows of BSV6294-1679 dots on the longer dot structure were interspersed with, typically, three BSV4673-5317 sites, which were normally seen as only one or two dots.

Although all the circumstantial evidence points to episomal BSV infections arising from the activation of integrated sequences in certain *Musa* cultivars, it is not possible at this stage to firmly implicate either of the BSV integrants described here as being the activatable integrant. The evidence for an integrant being activatable is that its sequence is essentially identical to that of the episomal form and that it is present in other cultivars and hybrids that have a history of BSV (unpublished observation). On the other hand, although the *Musa*/BSV interface is exactly at the 5′ end of the replication template RNA, the integrant does not appear to have the full structure to express this RNA in a straightforward manner.

It is generally considered that plant reverse transcribing viruses (pararetroviruses) differ from animal retroviruses in that they do not involve integration into the host genome in their replication cycle (*11*). Pararetroviruses do not encode the integrase function or make the long terminal repeats necessary for insertion of the viral genome into the host chromosome. The only reported exception for this is petunia vein clearing virus, in one of the gene products of which an integrase motif was recently described to be found (*17*). Partial viral sequences of a single-stranded DNA geminivirus have been reported to be integrated at random into a plant genome (*2*). It is most likely the perennial nature of *Musa* and its vegetative propagation have given close contact of the BSV and plant genome over a long period of time, enabling a potentially active form of the viral sequence to integrate but not in the conventional retroviral way.

The observations in this and in the accompanying paper (*22*) raise numerous questions. Apart from the need to gain an understanding of the molecular biology of integration and activation there are the implications that this has for *Musa* improvement. It would appear that only certain *Musa* cultivars have the potential for activa-

tion of BSV integrants, which could cause problems in tissue culture and/or breeding programs especially in relation to germplasm movement and quarantine. Once there is an understanding of the nature of the active integrant, it should be possible to design diagnostics to identify *Musa* cultivars at risk. However, some of these cultivars may have important agronomic characters and it may also be possible to suppress the activation event by transformation technology and by improved *in vitro* techniques.

Suckers of plantain, Obino l'Ewai, and TMP115BC, were obtained from the International Institute for Tropical Agriculture, Ibadan, Nigeria. These plants were maintained in glasshouses at the John Innes Centre at 28–32°C and high humidity and showed no BSV symptoms over a period of 3 years.

The following primers were based on the episomal BSV sequence (*9*; EMBL Accession No. AJ002234), with the number being the position of the 5′ nucleotide of the primer on the viral sequence and "r" indicating sequence reverse and complementary to the published sequence: BSV4673, 5′GGAATGAAAGAGCAGGCC; BSVr5317, 5′AGTCATTGGGTCAACCTCTGTCCC; BSVr7362, 5′ATCTTGCGCTCTACTCGC; BSVr7353, 5′CTCTACGTCGACAAAGTGAACTCGC; BSVr1438, 5′CTGAGAAGTTTGTAGGGG; BSVr2473, 5′AACATGCTCTATTGTGGC; BSVr3539, 5′ATAAACCATGTACTGGCG; BSVr4557, 5′TTCCTTGGCCTCAGAGCG; BSVr5488, 5′TGCTACTTGATGAAACCC; BSV5611, 5′ CTTTAGAGGGACAGAGG; BSV39, 5′ GTAATTCCTTTAGATAGGAGC; BSVr6407, 5′ TCTCAAGACCTGTCCCGG; and BSVr7099, 5′ CTAGACAACGCACACCG. The *Musa* primers were Mr1 (5′ ACCATCTAATGCAATTTATCTATCTCC) from 33 bases upstream of the *Musa:*BSV interface and M2 (5′ AACTTATGATATTTACAAAATACCTC) from position 185 from the *Musa:*BSV interface (indicated in Fig. 3B). The *Taq*I primer was 5′ ATGAGTCCTGAACGA.

Probe BSV4673-5317 was produced by PCR of BSV DNA using primers BSV4673 and BSVr5317 to give a 644-bp fragment. The probe from nucleotide 6294 via 1 to 1679 (BSV6294-1679) was prepared by PCR from a pBluescript *Sac*I subclone of episomal virus using primers to the T3 and T7 promoters of the vector. The MusaOL probe was derived from pS-SAP17 as a 153-bp PCR product using Mr1 and M2. Radioactive ^{32}P-labeled probes for Southern hybridization were synthesized by random priming (*5*) using the full-length BSV PCR product as template. Probes for *in situ* hybridizations were also random prime labeled with biotin-11–dUTP or digoxigenin-11–dUTP.

High-molecular-weight DNA, isolated from *Musa* by the method of Gawel and Jarret (*7*), was digested with various restriction endonucleases and the products were separated by electrophoresis through a 0.75% agarose gel. Southern blotting was as described in Sambrook *et al.* (*18*).

Metaphase chromosome spreads were prepared from fixed, unpretreated, root tips. *In situ* hybridization followed standard protocols for banana (*16*); following hybridization, the most stringent wash was in 0.1× SSC (15 mM NaCl, 1.5 mM sodium citrate) and 40% formamide at 42°C, enabling probe:target hybrids with more than approximately 85% homology to remain hybridized. Extended DNA fiber preparations were made using methods modified from (*3, 6*). Briefly, isolated nuclei were dried on microscope slides and treated in buffer containing SDS, and the DNA was extended to essentially its molecular length (*3, 6*) by tilting the slides and allowing the drop of buffer to run out. Denaturation and hybridization then followed similar methods to those for metaphases. In all experiments, *in situ* hybridization sites were detected with streptavidin-Cy3 (Sigma) or anti-digoxigenin-FITC conjugates and counterstained with DAPI. Micrographs were taken on Fuji color negative film, scanned to PhotoCD, and processed with Adobe Photoshop using only functions applied to the whole image equally.

Obino L'Ewai genomic DNA (0.5 μg) was prepared as described by (*20*) except digestion used *Taq*I for 4 h at 65°C. *Taq*I adaptors were ligated overnight and final dilution to 150 μl with water and S-SAP (*21*) was conducted. The BSV-specific primer (BSVr7362) was 5′ biotin labeled (Genosys). Touchdown PCR with 3 μl diluted template was performed exactly as described (*20*) using BSVr7362 and *Taq*I primers.

Biotinylated PCR products were separated from each reaction with streptavidin-coupled paramagnetic particles (Promega) and reamplified using a nested primer (BSVr7353) and *Taq*I primer. PCR product bands containing BSV sequences were separated by electrophoresis on a 1% agarose gel, Southern blotted, and identified by hybridization with ^{32}P-labeled full-length BSV probe. Appropriate bands isolated from agarose were purified with Wizard PCR Prep (Promega) and cloned into pCR II vector (TA cloning kit, Invitrogen).

One hundred nanograms of genomic DNA was amplified with an Expand Long Template PCR System using the supplier's (Boehringer) protocol. The primers were selected from different points through the BSV sequence. The PCR cycle conditions were as suggested in the supplier's protocol, with an initial elongation time of 5 min and an annealing temperature of 50°C.

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