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Focus paper 1

Fluorescent *in situ* hybridization of plant chromosomes: illuminating the *Musa* genome

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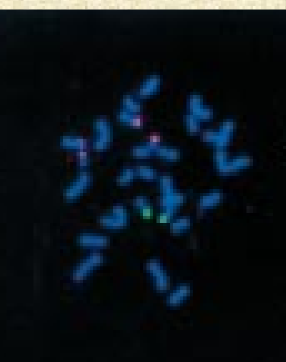
Introduction

Characterisation of banana and plantain germplasm has until now, been largely based on the use of phenotypic characters and more recently on molecular markers such as RFLP and RAPD (see INIBAP Annual Report 1996, p. 24-28). Cytogenetic studies have proved difficult in the genus *Musa* because of the small size of the genome (550 Mbp, Doležel *et al.* 1994), just 10% of the barley genome for example, and the large number of chromosomes (2n=3x=33 in most banana cultivars, compared to 2n=2x=14 in barley). Molecular cytogenetic studies, which link data about the molecular composition and organisation of the genome with the chromosomes, offer greater understanding of phylogenetic relationships and improved clarity of taxonomic discrimination, allowing the identification of aneuploids and assisting selection. In recent years, there have been rapid advances in the direct observation and analysis of banana chromosomes using molecular cytogenetic methods. This focus paper provides some information on the applications of such techniques in relation to banana and plantain research.

Figure 1. Double FISH showing the rDNA sites on somatic metaphase chromosomes of Narenga. The 18-25S rDNA site are visualized in green (FITC) and the 5S rDNA sites are visualized in red (Texas Red). The chromosomes are counterstained with DAPI (blue). (Courtesy of CIRAD)



In Situ hybridisation

The *in situ* hybridisation (ISH) technique, developed more than 30 years ago (Gall and Pardue 1969, John *et al.* 1969) allows genes or DNA sequences to be directly localised on chromosomes in cytological preparations. The development of user-friendly fluorescent techniques (Langer-Safer *et al.* 1982, Pinkel *et al.* 1986) has greatly increased the application of this technique during the last 15 years. Fluorescent *in situ* hybridisation (FISH) allows hybridisation sites to be visualised directly and moreover, several probes can be simultaneously detected with different fluorochrome, allowing the physical order on the chromosomes to be determined.

For the FISH technique, DNA sequences to be localised are first labelled to produce the probe. The probe is coated on the target chromosome which is spread in a hybridisation buffer. After treatment to denature the DNA into single strands, the probe and target are allowed to re-anneal. The probe will bind specifically to the complementary site on the chromosome. After washing and detection with a fluorescent reporter, a discrete fluorescent signal is visible at the site of probe hybridisation, which can be visualised using a fluorescent microscope (Figure 1).

One of the important modifications of the ISH technique is genomic *in situ* hybridization (GISH) (Schwarzacher *et al.* 1992). GISH is a genomic painting technique which allows parental genomes in interspecific hybrids to be distinguished (Figure 2). Total genomic DNA from one parent is labelled as a probe and unlabelled total DNA of the other parent is used as a block. Alternatively, total DNA from both parents is labelled and these are both used as probes, each one revealed with a different fluorochrome. This technique is based on the rapid evolution during speciation of repeated sequences, which represent the major part of plant DNA. If the species are distant enough, the repeat sequences allow the chromosomes from the two parental species to be differentiated.

Applications

Untangling the A, B, S and T genomes by genomic *in situ* hybridization

The classification of *Musa* cultivars into genomic groups has so far been based on chromosome numbers and morphological traits (Cheesmann 1947, Simmonds and Shepherd 1955) as well as

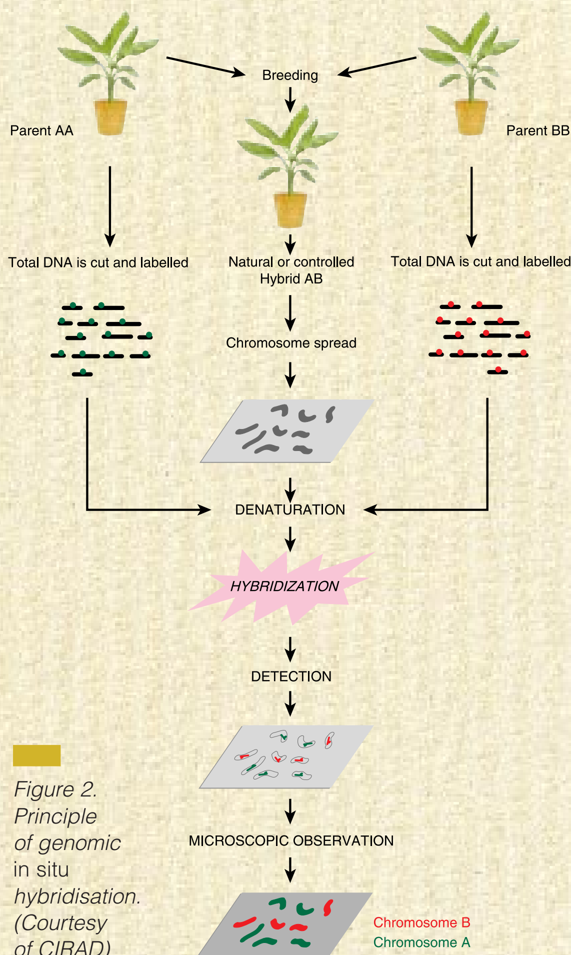


Figure 2. Principle of genomic in situ *hybridisation. (Courtesy of CIRAD)*

more recently, on molecular markers. GISH however provides a powerful complementary tool to molecular markers, enabling the portion of the genome contributed by each parental species in interspecific hybrids and their derivatives to be visualised (Figure 3). This technique has allowed the chromosomes from the four wild *Musa* species, *M. acuminata*, *M. balbisiana*, *M. schizocarpa* and the *Australimusa* species, involved in the origins of cultivated bananas to be differentiated (Osuji *et al.* 1997, D'Hont *et al. in press*).

The exact genome structure of several interspecific cultivars has been examined using GISH. The results were in most cases consistent with the chromosome constitution estimated through phenotypic descriptors, with one notable exception. The clone 'Pelipita', was found to contain 8A and 25 B chromosomes, instead of the 11A and 22 B predicted (Figure 4).

Using molecular markers, it was recently confirmed that the species *M. schizocarpa* (S genome) and species of the *Australimusa* section (T genome) have contributed to the origin of some cultivars (Carreel 1994). However, it was not possible to determine what proportion of these species are present in the genome. Using GISH it was possible to demonstrate for example, that the S genome contributed a full set of S chromosomes to the cultivar Wompa. Similarly, GISH showed that one basic set of T chromosomes are present in the cultivars 'Karoina'and 'Yawa 2'and established their genome constitution as AAT and ABBT, respectively (Figure 5).

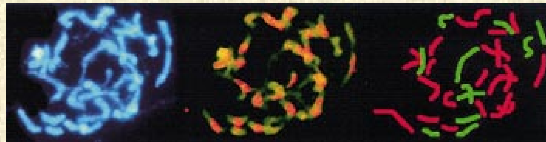


Figure 3. Metaphase of triploid plantain Mbi Egome (AAB): a. The 33 chromosomes stained blue with the DNA stain DAPI; b. In situ *hybridisation of genomic A DNA (red) and B genomic DNA (green); c. Interpretation shows the red labelled regions on 22 chromosomes; the other 11 chromosomes are labelled only with green. (Courtesy of John Innes Center)*

Figure 4. GISH on somatic metaphase chromosomes of 'Pelipita'using total DNA from a BB clone revealed in red with Texas Red and total DNA from an AA clone revealed in green with FITC. (Courtesy of CIRAD)

Identifying individual *Musa* chromosomes and visualising DNA sequences

Individual chromosomes are difficult to identify conventionally because they are so similar. However individual chromosomes can be defined by the hybridisation of specific cloned or synthetic repetitive DNA sequences (Osuji *et al.* 1998, Doležělová *et al.* 1998). For example the 18S-25S rDNA is present at a single site in each genome and can be used to define that chromosome. This has a further significant use as this single site in each genome enables easy assessment of basic ploidy levels in hybrid or tissue culture material. The hybridisation pattern obtained can also provide indicators of recent and evolutionary rearrangements in the genomes (Figure 6).

The development of similar markers (repeated sequences, BAC, etc.) for the various linkage groups will enable the different chromosomes to be assigned to respective linkage groups and will thus efficiently complement genetic mapping efforts. This would also open the way for the investigation of structural rearrangements which are reported to be frequent in bananas (Faure *et al.* 1993). These rearrangements result in important irregularities in meiosis and irregular chromosome transmission and may have been involved in the development of sterility, a prerequisite for edible fruit.

Understanding BSV

FISH can be used to analyse the numbers and loci of other chromosomal sequences and it has been used to analyse the integration of banana streak

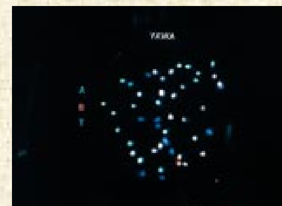
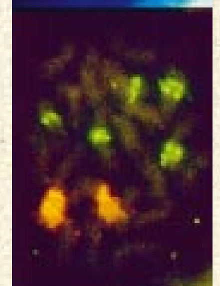


Figure 5. GISH on somatic metaphase chromosomes of 'Yawa 2' using total DNA from a AA clone revealed in green with FITC, total DNA from a BB clone revealed in red with Texas Red and DAPI counterstaining (blue). (Courtesy of CIRAD)



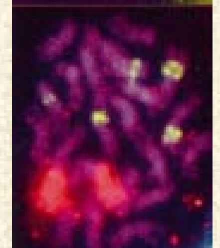


Figure 6. In situ *hybridisation to chromosomes of an AA* Musa *hybrid: a. The 22 chromosomes stained blue with DNA stain DAPI; b. Five sites of hybridisation to 5S rDNA probe (green); c. Single site hybridisation to 18S-25S rDNA probe on each of the two genomes. (Courtesy of John Innes Center)*

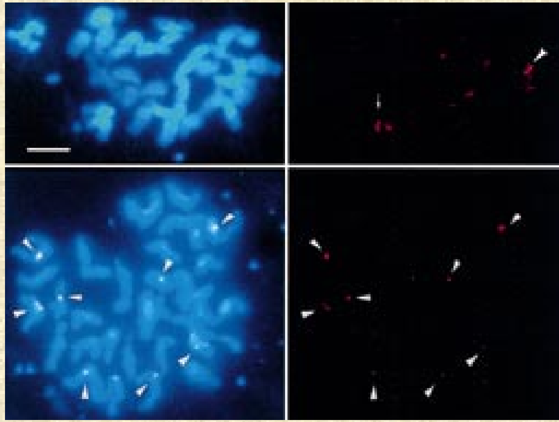


Figure 7. Musa *genotypes Cavendish (AAA) and Obino L'Ewai (AAB) showing hybridising (integrated) BSV sequences.*
In situ *hybridisation to metaphase spreads of Obino L'Ewai:*
a. The 33 chromosomes stained blue with the DNA stain DAPI.
b. Hybridisation sites of BSV (red) showing one major site in each metaphase (arrowhead) and at least one minor site (arrow).
In situ *hybridisation to metaphase spreads of Dward Cavendish:*
c. The 33 chromosomes stained blue with the DNA stain DAPI.
d. Hybridisation sites of BSV (red) showing at least eight major site in each metaphase (arrowhead). (Courtesy of John Innes Center)
Bar = 5 µm

virus (BSV) DNA into the *Musa* genome. Numerous lines of evidence including PCR and genomic Southern analysis pointed to the possible integration of BSV sequences (LaFleur *et al.* 1996, Ndowora *et al.* 1997, Harper and Hull 1998). To examine whether these BSV sequences in high molecular weight DNA were actually in the *Musa* nuclear chromosomes, double target *in situ* hybridisation was conducted on chromosomes from the plantain cultivar Obino L'Ewai, using a probe specific to BSV and a probe specific to a *Musa* sequence. Both probes gave hybridisation signals on chromosomes of Obino L'Ewai. A major hybridisation site to BSV was detected on both chromatids of one chromosome in each metaphase and at least one weaker hybridisation site was regularly seen. This clearly demonstrates that viral sequences are integrated in the nuclear genome. The *Musa* probe showed hybridisation to multiple sites throughout the genome, including near the major BSV site, but was not uniformly dispersed.

Representatives of AA, AAA and BB genome *Musa* were analysed by FISH and all showed clear hybridisation of BSV sequences. The strength of the signals indicates that multiple copies of the target sequence were integrated at most of the observed sites (Figure 7.) This is further compelling evidence that BSV sequences are integrated into the *Musa* genome and that this integration must have been an ancient event.

Visualisation of fine scale DNA structure
The organisation of gene and DNA structures can be visualised by a relatively new method, that of *in situ* hybridisation of probes to DNA fibres extended to their full molecular length (Fransz *et al.* 1996, Brandes *et al.* 1997, see also Schwarzacher *et al. in press*). Theoretical considerations of the length of the extended DNA molecule and calibration from hybridisation with probes of known length and interspersion pattern (Fransz *et al.* 1996, Sjöberg *et al.* 1997) can relate the lengths of observed fibres to the numbers of bases (Figures 8 and 9).

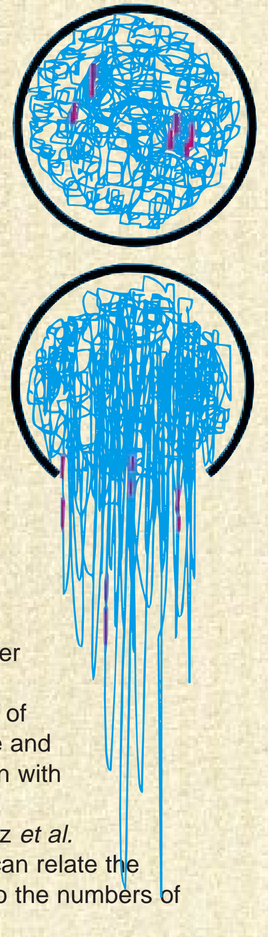


Figure 8. Cartoon of an interphase nucleus fixed to a slide: a. The blue chromatin labelled at eight sites by a red in situ *hybridisation probe; b. After lysis of the nucleus and tilting of the slide the DNA fibres are stretched to their full molecular length. They can hybridise with the same probe and now clearly show the relationship between probe and fibre. (Courtesy of John Innes Center)*

This technique was used to investigate the structure of the integrated BSV sequence. A genomic clone (Ndowora *et al. in press*) and PCR-based methods (Harper *et al. in press*) had shown that the integrated sequence adjacent to a *Musa* interspersed sequence was complex, containing an inverted region and some very highly rearranged stretches. Stretched DNA fibres were prepared on slides from Obino L'Ewai nuclei. Double-target hybridisation with the genomic *Musa* sequence and BSV showed long rows of hybridisation sites ('dots') along stretched DNA fibres. The *Musa* sequence was present at sites associated with the BSV hybridisation sites and also independently as variable lengths of rows of dots (Figure 10). It was apparent that there were two different lengths of *Musa*-BSV chains of dots present in approximately equal numbers. Some were 50 µm long, representing structures containing multiple copies of BSV sequences (150 kb long) and others were 17 µm long (about 50 kb structures). Each group of fibres, long and short, showed common patterns of red and green signal sites and gaps, with repeating units of BSV sequence adjacent to *Musa* sequence. The longer structure is considered to correspond to the major hybridising site seen on metaphase chromosomes while the shorter structure, corresponded to the minor hybridisation site.

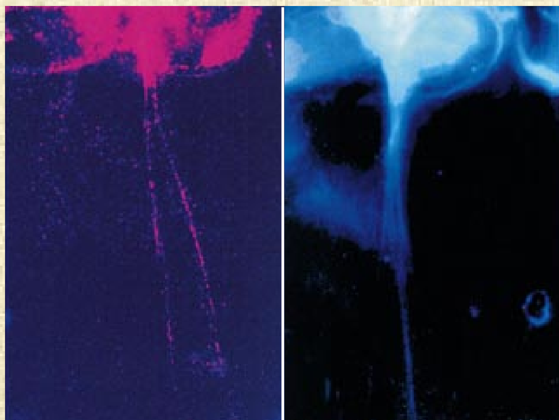


Figure 9. Rye interphase nucleus:
a. DAPI staining shows the strechted DNA as blue fibres running downwards.
b. A highly repetitive ribosomal DNA probe labels multiple sites on some but not all of the fibres. Here, the fibres are too bundled for detailed analysis of the gene structure, but the relationship between nucleus, the fibres and the probe is evident. (Courtesy of John Innes Center)

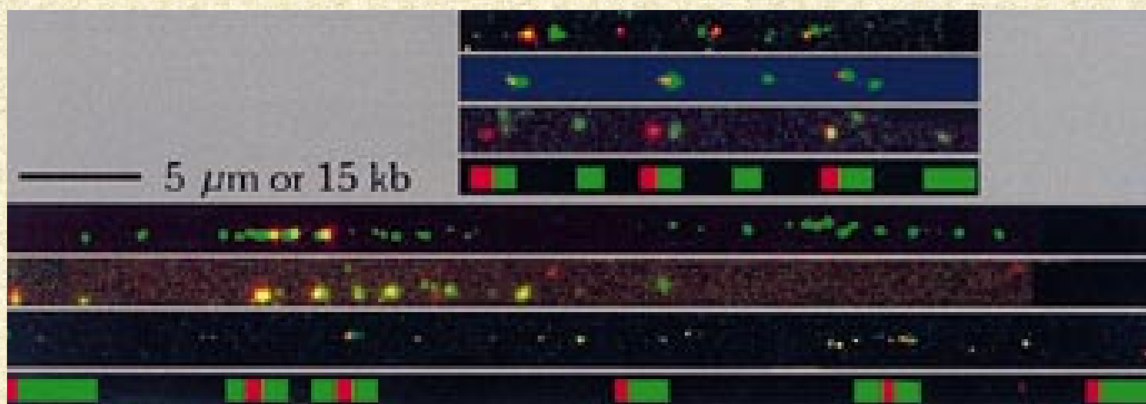


Figure 10. Fibre stretches of Musa *Obino L'Ewai AAB showing hybridising BSV and associated* Musa *sequences.* In situ *hybridisation to extended DNA fibres from Obino L'Ewai nuclei. Green and red dots represent probe hybridisation sites to BSV sequences and associated* Musa *sequences respectively. Two different patterns of chains of dots were detected:*
a. Three independent and aligned long fibres above a consensus diagram of hybridisation pattern showing red sites and chains of green signals. Both the Musa *and BSV 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 hybridisation (no homology to probes).*
b. Three aligned short fibres above consensus diagram, showing a pattern that can be interpreted as three sub-repeats. Under the hybridisation, detection and imaging procedures used, individual signals are larger than expected from the probe length, may be slightly displaced from the axis, and some supposed target sites may not have a detectable signal. (Courtesy of John Innes Center)
Bar = 5 μm, corresponding the 15 kb DNA fibre length.

Conclusion

Molecular cytogenetic methods are adding a powerful set of tools to those already available to study genome organisation, evolution and recombination. GISH has immense potential for identification of chromosome origin and can be used to characterise cultivars and hybrids produced by *Musa* breeding programmes. Repetitive and single copy DNA probes are yielding insights into the relationship between genetic and physical maps of *Musa* and genome evolution. Finally, fibre *in situ* hybridisation can be used to examine the organisation of genes and DNA sequences. Together, these techniques provide data for *Musa* breeders, which can be used to tackle the challenges caused by banana streak virus, tissue culture and somaclonal variation, the use of wild germplasm in breeding and the irregular transmission of chromosomes during meiosis. *In situ* hybridisation therefore holds great potential to help scientists develop optimum breeding strategies in order to create high quality and disease resistant bananas.

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