A global Programme for Musa Improvement

First meeting of the PROMUSA working groups’ convenors

A first meeting of the convenors of the PROMUSA working groups was held in Montpellier on 18-20 April. Up-to-date news of the activities of each of the five groups were shared and it was agreed that the Genetic improvement working group should continue to operate through two subgroups and not divide into two independent groups as had been proposed. A formulation for two levels of participation in the working groups has been conceived:

- those who are interested in receiving information in order to develop research in general, and
- those whose participation is more proactive and involved in the development of priority areas of research in the group.

The convenors will have the responsibility to familiarize themselves with the work of participants and identify those who are most active, and to stimulate information-sharing and the use of the list server. Working group members will be encouraged to send regular updates on publications, meetings, training events and to collaborate in writing project proposals. The PROMUSA secretariat will assist in proposal-writing by making available information on donors, proposal-writing guidelines, background information on banana and plantain production and by assisting in editing and English, if necessary. The responsibilities of INIBAP’s regional coordinators in stimulating participation from all banana-growing regions was emphasized, and the responsibilities of the secretariat in assisting the convenors were also consolidated.

A database of PROMUSA participants will be set up, using and linking to the INIBAP databases, BRIS and MUSALIT. The scope of the database will be relatively broad and participants will be asked to provide information on:

- Materials, tools and methods available for distribution
- Availability of biological materials and conditions to obtain them
- Information about current collaborative activities and novel collaborative areas
- Current training activities, and also areas of expertise and facilities for training.

Changes were suggested for the PROMUSA Web site. Each working group will have its own page containing information on:

- Members (with a link to the proposed database above)
- Research priorities
- Any relevant databases on aspects of research (e.g. Foc database)
- Protocols and methodologies available (with contact details)
- Useful publications: fact sheets, technical guidelines, handouts (Word or PDF versions)
- Links to other relevant homepages

It was also proposed that posters be prepared for scientific meetings, both on PROMUSA in general and on the work of the different working groups. The individual benefits of the global PROMUSA meetings and working group meetings were discussed. Future global meetings should invariably be scheduled back-to-back with another major scientific meeting. The following schedule has been tentatively suggested:

- Nematology working group (24-25 May 2001) after the International Symposium on Nematology meeting in South Africa (21-23 May 2001)
- Sigatoka working group (March 2002) in Latin America back-to-back with an International symposium on banana leaf spot diseases.

What is PROMUSA?

The Global Programme for Musa Improvement (PROMUSA) is a broad-based programme which aims at involving all the major players in Musa improvement. It was developed as a means to link the work carried out towards addressing the problems of export banana producers, with those initiatives directed towards improving banana and plantain production at the subsistence and smallholder level. The global programme builds upon existing achievements and is based upon ongoing research initiatives. PROMUSA is therefore a mechanism to further maximize the outputs and accelerate the impact of the overall Musa improvement effort. The programme is an innovative mechanism to bring together research carried out both within and outside the CGIAR, creating new partnerships between National Agricultural Research Systems (NARS) and research institutes in both developing and developed countries. The formation of such partnerships will also contribute to strengthening the capacity of NARS to conduct Musa-related research.

The major thrust of PROMUSA is to develop a wide range of improved banana varieties from which growers worldwide can select those most suited to their needs. The programme brings together conventional breeding based on hybridization techniques with genetic engineering and biotechnological breeding approaches. This broad-based genetic improvement effort is supported by research being carried out on specific pests and diseases within the various PROMUSA working groups. An efficient mechanism for evaluating new varieties produced within the framework of PROMUSA is also an essential component of the programme.
• Genetic improvement working group + Breeding strategies meeting on banana after the 3rd International Symposium on Molecular and Cellular Biology on Banana in Leuven, Belgium (September/October 2002)

• Fusarium working group meeting is to be decided - suggestions welcome

• Virology working groups meetings to be defined - suggestions welcome

It is also suggested that global meetings should take place every three years, to allow more time for working groups to meet independently and make significant progress.

The next PROMUSA meeting would therefore take place in 2003 and may possibly be held back-to-back with an International Musa Congress.

2nd International Symposium on the Molecular and Cellular Biology of Banana

The inaugural Symposium on the Molecular and Cellular Biology of Banana held in March 1999 in Ithaca, New York, USA, was organized by the Boyce Thompson Institute for Plant Research. The concept was to open a forum for all people involved in molecular and cellular biology to have an opportunity to meet and exchange information about their research activities. The meeting was a resounding success, and it was therefore suggested to continue the concept under the auspices of PROMUSA.

The 2nd International Symposium on the Molecular and Cellular Biology of Banana held 29 October-3 November 2000 in Byron Bay, Australia, was organized by the Queensland University of Technology (QUT) with the local collaboration of CRCTTP (Cooperative Research Center for Tropical Plant Pathology) and QDPI (Queensland Department of Primary Industries). The local organizing committee also received major assistance internationally from INIBAP, Zeneca and DNAP (DNA Plant Technology Corporation, USA). This second symposium allowed participants from both developing and developed countries to present their research activities, covering a broad range of subject areas.

The symposium was structured around the following sessions: genomics; gene expression in transgenic plants; plant pathology and disease resistance; intellectual property and genetically modified organisms; biodiversity and evolution; and biochemistry and fruit ripening. Thanks to the support received from the participating institutions, international scientists were invited to attend and addressed keynotes on "Genomics and banana" (Colin Bird, Zeneca) and "Intellectual property and GMOs" (Dianne Nicoll, University of Tasmania). Participants from the CSIRO (Commonwealth Scientific and Industrial Research Organization) Plant Industry also delivered keynotes introducing the sessions on gene expression in transgenic plants (Peter Waterhouse), plant pathology and disease resistance (Jeff Ellis), and biochemistry and fruit ripening (Simon Robinson).

With 50 papers presented and 60 participants from 17 countries attending the symposium, this event ranks among the most important scientific fora on Musa.

As an additional contribution, INIBAP publishes hereunder a special PROMUSA supplement containing the abstracts of presentations made at the symposium.

Abstracts of presentations

Genomics

Induction, detection and use of aneuploids for genetic studies in Musa spp.

N.S. Roux, A. Toloza, J. Dolezel and F.J. Zapata-Arias

Polypliod and aneuploid banana plants were obtained after gamma radiation and colchicine treatments. Variation in chromosome number was also observed in plants regenerated via organogenesis or somatic embryogenesis from tissue cultures, which were not exposed to any mutagenic treatment. Regenerated off-type plants were analyzed by flow cytometry as described by Dolezel et al. (1997) to estimate their ploidy levels and to check sensitivity of the method to detect aneuploidy in Musa. Chicken red blood cell (CRBC) nuclei were used as internal reference standard and the DNA index was calculated by comparing peak positions of CRBC nuclei and nuclei of the sample. At a triploid level, the minimal difference between euploid (3x) and aneuploid plant (3x ± 1) should be approximately 3%. Thus, all plants with DNA index differing by more than 1.5 % from the index established for control (3x) plants were considered aneuploid. The results obtained by flow cytometry were verified by chromosome counting in meristem root-tip cells (Dolezel et al. 1998). The results indicated that flow cytometry was sensitive enough to detect aneuploidy in Musa. However, detection of aneuploidy with ± 1 chromosome accuracy required high-resolution analyses with coefficient of variation of DNA peaks lower than 2%. The advantage of flow cytometric assay was that abnormalities in DNA content could be detected at an early stage of plant growth, and also during in vitro culture. Moreover, flow cytometry enabled detection of mixoploidy. Thus, in several cases differences in ploidy levels between leaf tissue and root tissue of the same plant were detected. Aneuploids have been particularly useful in genetic studies of many plant species such as maize, tomato, tobacco and wheat (Khush 1973). Following the work of Sears, the collection of aneuploid lines has been made possible to define the relationship between chromosomes of hexaploid wheat in terms of their origin and function (Law et al. 1987). In Musa spp., aneuploids are relatively frequent and viable in triploid clones. Being sterile, their value for genetic analyses is limited. Nevertheless, they could be very useful for physical mapping and to link genetic and physical maps using already available molecular markers.

References


high resolution chromosome studies in Musa spp. InfoMusa 7:3-4.


Acknowledgements

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Molecular cytogenetic and cytometric analysis of Musa genomes

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The application of flow cytometry and molecular cytogenetics stimulated progress in understanding of Musa genome at nuclear and chromosomal level. Flow cytometric analysis was found a convenient method for estimation of nuclear DNA content in Musa (Dolezel et al. 1994) and has been used for ploidy verification in existing germplasm collections, characterization of newly collected materials, and evaluation of karyological stability in vitro. Due to its high throughput, the method may be easily incorporated into existing breeding programmes. Samples can be sent to laboratories equipped with a flow cytometer, as only a small amount of plant tissue is needed. The method also permits determination of the size of nuclear genome. It was found that Musa genomes are small with the B genome being smaller compared to the A genome (Lysák et al. 1999). The development of procedures for reliable and rapid detection of aneuploidy and for chromosome flow sorting remains a major challenge. Given the small size and poor morphological differentiation of Musa chromosomes (Dolezel et al. 1998), molecular cytogenetics holds major promise for karyotype analysis and the study of chromosome organization. While genomic in situ hybridization is suitable for determination of genomic constitution in hybrids (D’Hont et al. 2000), fluorescent in situ hybridization (FISH) permits physical mapping of DNA sequences to chromosomes. Several classes of repetitive DNA sequences, including ribosomal RNA genes, retrotransposon and BSV sequences have already been localized to Musa chromosomes (Balint-Kurti et al. 2000, Dolezelová et al. 1998, Harper et al. 1999). More DNA sequences need to be isolated and mapped to unravel the molecular structure of chromosomes and to establish mechanisms of genome differentiation in Musa. Identification of individual chromosomes using physically mapped DNA sequences will allow analysis of their behaviour and segregation during evolution and in breeding programmes. Physically mapped single- and low-copy DNA sequences will provide anchor sites needed to integrate physical and genetic maps.

References


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Markers for determining genomic integrity: somaclonal variants in bananas as a model system

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Somaclonal variation has long been recognized as a by-product of the propagation of plant cells through one or more cycles of disorganized cell growth. Most of the transformation processes used in the generation of transgenic plants include at least one step where cultured cells are grown and then plants are subsequently regenerated. Therefore all of the individuals that are transgenic and have arisen by this method have the potential to contain some of this variation, even in the absence of any visible mutation. Many genomic alterations in transgenic plants have already been demonstrated using RAPDs and AFLPs. In spite of the observation that similar polymorphisms repeatedly arise, none of the variants have proved useful as predictors of the level of genomic variation that has taken place. The well-documented off-types arising from tissue culture of bananas have been used as a model system to identify the regions of the genome that may be especially susceptible to change and to develop markers to determine the extent of that change. Representational difference analysis was used to isolate genomic differences between two sets of normal and variant banana cultivars – between Williams and a masada/chlorotic off-type, and a normal Curare Eno individual and a dwarf off-type (the latter pair supplied by Dr R. Swennen). In both instances difference clones were identified. Many of the sequences were common to both sets of difference products, in spite of the fact that they were different aberrant phenotypes. One of the difference products identified was a mini-satellite sequence that also appeared to be labile in date palms. These results add more evidence for the presence of a labile segment of the genome that is preferentially modified during the generation of somaclonal variants. These difference products are being further characterized with a view to developing a series of markers that can be used to identify early genomic changes and also as diagnostics for specific phenotypes arising in the tissue culture process.

Identification of AFLP and ISSR markers associated with dwarf somaclonal variants in Cavendish banana

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Somaclonal variation is a common feature of some micropropagated banana cultivars, caused by undetermined reasons. Early detection of variants is desirable for commercial micropropagation or to establish methods to increase variability for breeding. Molecular markers offer a great potential to detect and to disclose causes of somaclonal variation. The objective of this study was to test Amplified Fragment Length Polymorphism (AFLP) and Inter-Simple Sequence Repeat (ISSR) assays, using polyacrylamide gels and silver staining, comparing a Cavendish cultivar “Nanicão Jangada” with its somaclonal dwarf variant. Twelve ISSR primers were tested, and two (16.6%) presented 3 polymorphic fragment present only in the dwarf variant. All AFLP primer combinations from kit AFLP System I (Life Technologies, Rockville, MD, USA) were tested, amplifying a total of 1665 bands. Each primer combination amplified an average of 26.4 fragments, ranging from 7 to 44 bands. Forty-three polymorphic fragments (2.6%) were identified, with 19 (1.1%) present only in the dwarf variant. Polymorphic fragments were stable between assays. Methylation-sensitive AFLP assay, based on the differential ability of a pair of isoschizomers to restrict methylated cytosine, was also tested. A combination of 24 primers were used to amplify DNA from both genotypes. An average of 24.8 fragments were amplified from HpaII-treated DNAs and 22.1 fromMspI-treated, comparable to the regular AFLP. Twelve polymorphic bands (2.1%) were present only in “Nanicão Jangada” in HpaII-digested, while eight fragments (1.6%) were polymorphic forMspI-treated. Only three polymorphisms (0.5%) might have derived from differences in methylation. Other dwarf variants are being tested using the same primer combinations, and polymorphic fragments will be cloned and sequenced.

Application of the amplified fragment length polymorphism (AFLP) and the methylation-sensitive amplified polymorphism (MSAP) techniques for the detection of DNA polymorphisms and changes in DNA methylation in micropropagated bananas

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The effect of the explant source on DNA polymorphisms and changes in methylation in the leaves of micropropagated Musa AAA ‘Grande Naine’ was investigated. Explants were derived from either the young male floral apices or suckers, and shoot cultures induced from these explants were micropropagated for five subcultures. As controls for MSAP analysis (Xiong et al. 1999), equivalent leaf tissue was taken from ten conventionally propagated plants. Ten combinations of primers were used for AFLP analysis and eight primers for the MSAP analysis. No significant differences were found between either kind of explants using AFLP or in MSAP in leaf tissue of plants derived from conventional propagation. However, when compared to the explants, the micropropagated plants derived from them had significantly more DNA polymorphisms. In addition, we found that the explant source had a significant influence on the extent of AFLP DNA polymorphisms in regenerants. Inflorescence-derived regenerants gave the highest variation of 6.36% compared to sucker-derived regenerants which gave 3.96% polymorphisms.

A total of 107 (23%) out of 465 bands were found to be cytosine-methylated in micropropagated plants, whilst in conventionally propagated plants 18% of the bands were found to be cytosine-methylated. There was no significant difference in the extent of DNA methylation polymorphisms between inflorescence-derived micropropagated plants (3%) and sucker-derived plants (1.7%). Most of the polymorphic bands were of high molecular weight (above 700 bp) and were hyper-methylated. This was also the case for most of the hyper-methylated bands common to all micropropagated plants but which were not methylated in conventionally propagated plants. A correlation was found between some plants with AFLP polymorphisms and plants with methylation polymorphisms.

Thus the banana micropropagation process was found to generate significant genetic and possibly epigenetic changes in micropropagated ‘Grande Naine’ banana plants. The question as to whether the hypermethylation found in all regenerants is developmentally-related or a consequence of the tissue culture environment per se remains to be answered. The correlations found between AFLP and MSAP polymorphisms provide indirect evidence that hyper-methylation may induce base changes, perhaps by deamination (Kaeppler et al. 2000). All the regenerants are presently being grown to maturity in our experimental plantation in Yucatan so that a phenotypic characterization will be possible.

References

**Banana streak badnavirus sequences in Musa**

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Molecular and cytogenetic data show unequivocal evidence of the integration of banana streak badnavirus (BSV) sequences into the genome of Musa plantain Obino l’Ewai (AAB) and these sequences are essentially identical to that of an epimonal virus causing infection in Musa (Harper et al. 1999, Ndowora et al. 1999). There are two loci, differing in copy number of BSV sequence in Obino l’Ewai and, for at least one of them, the integrated sequence structure is rearranged with respect to the virus sequence. Significant BSV infections are detected in certain B genome containing Musa germplasm during meiosis or tissue culture and the circumstantial evidence points to epimodal BSV infection arising from the activation or mobilization of integrated BSV sequences. A model involving recombination has been proposed that links integrated sequence to the generation of replicative forms of the virus (Ndowora et al. 1999). This phenomenon has major implications for Musa pathology, improvement, germplasm movement and quarantine.

The BSV integration phenomenon has parallels in two other cases, the pararetroviruses Petunia vein-clearing virus (PVCV) (Richert-Pöggeler and Shepherd 1997) and Tobacco vein-clearing virus (TVCV) (Lockhart et al. 2000). Epimodal PVCV is found in Petunia hybrida and appears following environmental stress such as nutrient deficiency and episomal TVCV is found in the hybrid Nicotiana edwardsonii after changes in daylength. Integrad viral sequences essentially identical to the episomal virus sequences are found at high copy number in both the hybrid species. As is the case for Musa and BSV, the virus sequences are integrated into only one of the parental genomes of the hybrid, although epimodal virus is not detectable in that parent. This suggests that the other parental genome plays a part in the “activation” of the virus sequences in the hybrid.

Fragments of a tobacco pararetrovirus-like (TPV-L) sequence have been found in genomic DNA of Nicotiana sp. (Jakowitsch et al. 1999). We have shown that pararetrovirus sequences probably comprise an important and widespread component of plant genomes including Gymnosperms and Angiosperms. Their pres-
ence may have consequences for gene silencing and genome evolution. As yet there is no evidence that these sequences give rise to novel viral symptoms, as suggested for related integrated pararetroviral sequences.

We are examining the nature and genomic context of integrated BSV sequences in Obino l’Éwai and in other Musa. A moderately repeated sequence, which flanks the integrated BSV sequence in Obino l’Éwai (MusaOL) is concentrated with varying copy number near the centromeres of most chromosomes of both the A and B genome of Musa. The low numbers of BSV-related integrans per genome indicates that BSV integration occurred after the amplification and distribution of MusaOL sequences and hence is most likely a recent event.

References

Is banana streak virus strain OL the only active virus strain in the Musa genome?

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In 1999, there were severe outbreaks of banana streak virus (BSV) in plantings of the IRFA 909, 910 and 914 hybrids at separate locations in New South Wales and Queens-

land. These new hybrids, originating from the CIRAD Musa breeding programme, were under evaluation for resistance to Fusarium oxysporum f.sp. cubense for 12-18 months prior to symptom expression. These plants tested negative for BSV-Orme by immunocapture-(IC)-PCR. However, IRFA 909 and 910 did test positive for BSV-Goldfinger by IC-PCR. The badnavirus from IRFA 914 was unlike any previously examined. We have named this virus isolate BSV-IM. Using degenerate PCR primers, we amplified DNA of BSV-IM, and using sequence of the DNA fragment, designed virus-specific primers. Using this new PCR assay, we have shown that IRFA 909 and 910 were infected with both BSV-Goldfinger and BSV-IM. In repeated assays over time, IRFA 914 has only ever tested positive for BSV-IM, and not BSV-GF. We have also found BSV-IM infecting an IRFA 914 plant in New Caledonia.

We purified virus from IRFA 910, and have obtained DNA clones representing the whole genome of BSV-IM. We have completed sequencing this virus and initial sequence analyses suggest that the BSV-IM is a distinct virus species. When proteins encoded by ORFs I, II and III of BSV-OL (GenBank accession AJ002234) and BSV-IM were compared, the sequence identities were 60.5, 42.3 and 64.3%, respectively. We have considered the possibility that BSV-IM has arisen from integrated virus sequences. Our virus clones hybridized to EcoRI and HindIII digested DNA of two diploid B parents of the IRFA hybrid lines, but failed to hybridize to similarly digested DNA from cvs. Obino l’Éwai Calcutta 4 and several AAA cultivars. The virus clones also hybridized to uncut genomic DNA of both diploid B parents. Both diploid B parents have never shown symptoms of BSV infection, and have tested negative for BSV infection by immunosorbent electron microscopy of concentrated leaf extracts. The hybridization patterns observed are not consistent with those expected with episomal virus DNA. These results suggest that BSV-IM has arisen via activation of integrated sequences.

We have also examined the possibility that other strains of BSV are also integrated into the Musa genome. Using probes to the complete genome of BSV-Mys (Geering et al. 2000), we observed complex hybridization patterns with EcoRI and HindIII digested DNA from three diploid B bananas, as well as from cvs. Obino l’Éwai (AAB group), Goldfinger (AAAB group) and Pisang Ceylan (AAB group), suggesting that sequence of BSV-Mys is integrated. Likewise, when probed with a 1.3 kb BSV-GF probe (Geering et al. 2000), a ca. 20 kb HindIII fragment was detected in DNA from two diploid B bananas, as well as from cvs. Obino l’Éwai, Goldfinger and Pisang Ceylan, suggesting that sequence of BSV-GF is also integrated. No hybridization was observed between either the BSV-Mys or BSV-GF probes and DNA from a range of AA and AAA cultivars, suggesting that the integrated DNA is linked to B genome of cultivated Musa.

Reference

Gene expression in transgenic plants

Agrobacterium-mediated transformation for the generation of transgenic banana (Musa spp.)

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A systematic evaluation of the successive steps in the natural Agrobacterium-plant interaction resulted in the elaboration of an efficient transformation protocol for banana. Chemotaxa and physical attachment of bacterial cells were observed in different cells and tissues of various banana cultivars (Pérez Hernández et al. 1999). Transient reporter gene expression was demonstrated in several tissues cocultivated with vir-induced Agrobacterium and the highest frequencies were found in embryogenic cell suspension cultures. Stable transformation was obtained after selection on genetinic- or Basta-containing medium. In total, more than 600 transgenic plants were regenerated in five independent experiments, and more than 90% of them expressed the introduced genes (gfp or gusA). Molecular characterization revealed a simple integration pattern in most transgenic plants. Transgenic plants containing the gene encoding the Ace-AMP1 antimicrobial peptide (Cammue et al. were screened with a leaf disc bioassay and candidate plants with increased fungus tolerance were identified (Pérez Hernández 2000).

References
A novel PCR-based method for the characterization of transgene insertion in transgenic plants

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An anchored-PCR (APCR) method was developed which allows a fast molecular characterization of transgenic plants generated via Agrobacterium-mediated transformation. Genomic DNA fragments obtained by digestion with restriction enzymes are specifically amplified with a T-DNA-specific primer in combination with an adaptor-specific primer. The incorporation of suppression PCR conditions (Siebert et al. 1995) resulted in a significant improvement and allowed the one-step amplification of specific APCR fragments. Southern hybridization of T-DNA border-specific probes to the APCR fragments revealed that they were indeed correctly amplified from the transgene(s). The APCR analysis of a tester set of 20 transgenic banana plants demonstrated that about 70% of them contained one or two transgene insertions, which compares favourably with the transgene insertion pattern in plants obtained via microprojectile bombardment (Becker et al. 2000). The technique also allowed the fine structure of the integrated transgene(s) to be revealed: correct as well as truncated insertions were observed, and plants containing vector backbone sequences could be identified. In addition, transgenic plants representing identical transformation events were easily recognized. Finally, nucleotide sequence analysis of cloned APCR fragments fully confirmed the above findings (Pérez Hernández 2000).

References


Virus and plant-derived promoters for transgene expression in banana

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Promoter regions derived from banana bunchy top virus (BBTV) satellite components (S1 and S2) and banana actin genes have been isolated and characterized in transgenic banana plants. The BBTV SI and S2 promoters directed vascular-associated reporter gene expression in both diots and monocots. In banana, the activity of these promoters was significantly increased by the inclusion of monocot-derived introns. Actin gene candidates and their associated 5' upstream sequences were isolated from a variety of plant sources, including banana, using a novel ligation-mediated PCR approach for amplifying flanking sequences. Expression levels and the tissue specificity of one particular banana actin gene (ACT1) were further characterized. Northern analysis suggested banana ACT1 is expressed in both reproductive and vegetative tissues. In transgenic banana plants, the ACT1 promoter directed strong reporter gene expression in both leaves and roots. Truncations of the ACT1 promoter indicated all the necessary regulatory elements required for high level (2-fold greater than CaMV 35S) near constitutive expression are located within 1.2 kb of the ACT1 ATG.

Better bananas - the biotech way

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At DNAP, our efforts in banana are focused on black Sigatoka resistance, with emphasis at the early stages of variety development on understanding expression characteristics of candidate gene expression signals. Using the chimeric uidA gene constructs to assess promoter function, we have been able to identify several promoters with relatively strong activity in leaf, fruit and root tissue. These activities seem to be maintained over several vegetative generations in the field. Two of these promoters have also been used in experiments to delay fruit ripening by inhibiting fruit-specific ethylene synthesis using sense suppression. Transgenic plants have been assessed in field trials in Costa Rica and southern Mexico and several lines have been shown to have significant delays in fruit ripening over multiple generations. A ~23 base RNA fragment diagnostic of the gene silencing phenomenon has been identified in these suppressed lines.

Transgenic lines expressing five putative disease resistance genes are currently undergoing field-testing in Costa Rica. Transformants expressing 11 more putative disease resistance genes or combinations of genes are at various stages of preparation. We are also using a leaf piece assay to assess some of our transgenics in house. The symptoms produced in this assay are similar to those seen in the field in terms of appearance, timing and cultivar specificity.

Biotechnological approaches for banana improvement

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Bananas and plantains are the fourth most important food crop and staple food crop for millions of people in the developing world. India is the largest producer of banana in the world. In this country, banana is the second most important fruit crop and is grown ~ 0.4 million ha with a production of 10 million tons. Conventional method of breeding is complicated due to the triploid nature and only a few diploid clones produce viable pollen. Improvement for disease resistance and productivity requires the use of biotechnological tools. Our group is engaged in tissue culture, somatic embryogenesis, synthetic seeds, in vitro mutagenesis and selection, DNA fingerprinting and Agrobacterium-mediated gene transfer. Thirty cultivars/wild species have been conserved and propagated in vitro. Tissue culture-raised plants planted at multilocations exhibited increased yield, early maturity and more uniform production cycle. In vitro cultures were gamma-irradiated and field evaluation of the irradiated population resulted in certain promising variants. Isolated variants and parent cultivars were analyzed in the field, and at molecular level using RAPDs. Protocols have been developed for somatic embryogenesis using shoot tip sections in cv. Rasthali (AAB) and male flower buds in cv. Shrimanti (AAA). Embryogenic cell cultures
have been successfully established and maintained by regular subcultures for the past two years (in Rasthali). High frequency conversion of somatic embryos to plants has been achieved and the somatic embryo-derived plants are being field-evaluated.

Agrobacterium-mediated transformation using embryogenic cell cultures of cv. Rasthali has been standardized and is now routinely used for gene transfer. Currently, we are working with an anti-microbial peptide, msi99 (a synthetic homologue of Magainin). Studies have shown that this peptide effectively inhibits the growth of Fusarium oxysporum, the causal agent of Fusarium wilt. Rasthali, a highly susceptible cultivar, has been transformed with msi99 and transgenic plants have been regenerated.

Intellectual property and genetically modified organisms

Keynote

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It is probably fair to say that patenting will now usually be in the mind of the genetics researcher for a number of reasons, including:
1. the changing nature of academic science, in particular the need for accountability in economic terms;
2. the nature of biotechnological research: expensive, time-consuming and easily copied; and
3. the increasing involvement of the private sector in the research phase.

The most influential international treaty on intellectual property (IP) rights is the Agreement on Trade Related Aspects of Intellectual Property Rights, or TRIPs, which is an annexure to the WTO Agreement. If a country wants to trade it has to have TRIPs-compatible IP law. Article 27 sets out the following patent law requirements:
• 27.1: Patents are mandatory for any inventions in all fields of technology. The elements of novelty, inventive step (non-obviousness) and industrial applicability (utility) must be satisfied.
• 27.2: Inventions may be excluded to prevent commercial exploitation of the invention to protect public order or morality, including protecting human, animal or plant life or health and avoiding serious prejudice to the environment.
• 27.3: Other inventions that may be excluded include: a) diagnostic, therapeutic and surgical methods for the treatment of humans or animals; b) plants and animals, but not microorganisms; c) biological processes for producing plants and animals, but not technical processes. Plant varieties must be protected in one way or another.

The most important virus disease affecting banana is the causal agent of Fusarium oxysporum f. sp. cubense (Foc). It is a major limiting factor to banana production in Australia. In the 1920s, bunchy top was the major limiting factor to banana production in Australia. The disease has since been controlled in Australia through the implementation of strict phytosanitary control measures backed up by strict government legislation. Our group has been characterizing this virus for the past 10 years in an effort to develop transgenic virus resistance and to further exploit the virus.

BBTV was initially thought to be caused by a luteovirus based on symptoms, persistent aphid transmission and dsRNA profiles. However, it is now known that BBTV is an isometric virus with a genome comprising at least six different components of circular single-stranded DNA (BBTV DNA-1 to DNA-6) ranging in size from 1018 to 1111 nucleotides. Each DNA component shares a common genome organization including (i) one major gene in the virion sense (except DNA-1 which contains two genes) with an associated polyadenylation signal, (ii) a conserved major common region (CR-M) and (iii) a potential TATA box located 3' of the stem-loop. The CR-M is located 5' of the CR-SL and comprises approximately 92 nt with at least 72% homology amongst the DNA components (except for DNA-1 which has a 26 nt deletion). The CR-M is believed to be involved in replication, where it is thought to act as a binding site for an endogenous ~80 nt DNA primer. The CR-SL comprises 69 nt with at least 62% homology between components. It incorporates a stem-loop structure which contains a 10 bp stem (14 nt conserved) and an 11 nt loop (9 nt conserved). Based on the sequence analysis of DNA-1, -3 and -5, there are two distinct groups of BBTV isolates, the South Pacific group (Australia, Burundi, Egypt, Fiji, India, Tonga and Samoa) and the Asian group (Philippines, Taiwan, Vietnam). These two groups differ by ~10% over the entire nucleotide sequence and by ~30% within the CR-M.

The major gene of DNA-1 contains motifs associated with rolling circle replication and dNTP binding and encodes a replication initiation (Rep) protein. This Rep protein has been shown to possess site-specific nickase and ligase activity (cleaves between nt 7 and 8 of the stem-loop). The function of the internal gene of DNA-1 is currently unknown. DNA-3 encodes the coat protein while the gene product of DNA-5 has been shown to possess retinoblastoma-binding activity and is thought to be a cell-cycle protein responsible for switching infected cells into S-phase to facilitate virus replication. DNA-4 and -6 appear to encode proteins associated with...
cell-to-cell movement and nuclear shuttling, respectively. The function of DNA-2 remains unclear.

BBTV has recently been classified in the genus Nanovirus – viruses with isometric virions which are phloem limited and possess a multicomponent, circular, single-stranded DNA genome. Other members of this genus include subterranean clover stunt virus (SCSV), faba bean necrotic yellows virus (FBNYV), milk vetch dwarf virus (MDV) and possible coconut foliar decay virus (CFDV).

BBTV DNA-1 to -6 are considered integral to the BBTV genome since these components are consistently associated with all BBTV infections worldwide. Several additional BBTV-associated DNA components have also been isolated from various BBTV infections. Like BBTV DNA-1, these additional components appear to encode Rep proteins. However, they differ from BBTV DNA-1 in several respects, including:

- genome organization – in general, the CR-M and CR-SL are absent, and the TATA box is located 5′ of the stem-loop; and
- they have a limited geographical distribution – they are almost exclusively restricted to the Asian group of BBTV.

We have been examining the replication of BBTV to determine (i) the integral components of the BBTV genome, (ii) which component encodes the ‘master’ Rep, and (iii) the role of the BBTV DNA-1 internal gene. These studies have involved the bombardment of Bluggoe embryogenic cell suspensions with cloned 1.1mers of the different BBTV DNA components either singly or in combination. DNA was extracted from the cells at 0, 4 and 8 days post-bombardment and analyzed with component-specific probes for replicative intermediates. These studies have shown that DNA-1 encodes the ‘master’ viral Rep protein and represents the minimal replicative unit of BBTV since this component, and not the additional Rep-encoding components, is capable of self-replication as well as directing the replication of the other integral BBTV genomic components. We also showed that the internal gene of DNA-1 is not essential for replication but enhances replication in cis (possibly analogous to the Ren protein of begomoviruses). Finally, we have identified potential Rep-binding sites (Iterons) on the BBTV genome which appear to be similar to those of the begomoviruses. The results of this study have suggested the possibility of two groups of nanoviruses: (i) BBTV – which infects monocots and contains an internal gene in the “master” Rep, and (ii) FBNYV, MDV and SCSV – all infect dicots and do not possess an internal gene in the “master” Rep.

The epidemiology of banana bunch top virus in Vietnam

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Banana bunch top virus (BBTV) causes the most serious viral disease of bananas worldwide. Banana bunch top disease almost destroyed the banana industry in Australia in the early 1920s, and similar epidemics have occurred in other countries throughout the world. BBTV was first identified in Vietnam in 1968, and is endemic throughout the country. However, the epidemiology of BBTV in Vietnam appears to be quite different to that observed in other countries, as it does not cause serious epidemics, and appears to move more slowly through a crop. BBTV is transmitted by the aphid Pentatoma nigrvensora, or through infected plant suckers and combs, and typically moves rapidly through a crop. However, in Vietnam it is not unusual to find older BBTV-infected plants adjacent to healthy plants, with banana aphids feeding on all plants. In addition, we have not observed typical BBTV symptoms on the local cultivar Chuoi tay. It is unknown whether Chuoi tay is a host for BBTV, or whether it is resistant to BBTV infection. To improve our understanding of BBTV epidemiology in Vietnam, we investigated a number of factors: (1) we investigated the level of sequence variability of DNA-1, the master rep-encoding component, and showed that sequence variability of BBTV in Vietnam is higher than previously recorded in Asia. We also observed that sequences separated into northern and southern Vietnamese isolates, depending on their origin in Vietnam; (2) we identified a new putative satellite DNA component endemic to Vietnam. Finally, we screened Chuoi tay plants from throughout Vietnam for BBTV, but did not detect virus in any plants using PCR and/or Southern hybridization. This suggests that Chuoi tay may be resistant to BBTV in Vietnam, which could be one of the factors influencing the epidemiology of banana bunch top disease in Vietnam.

Viruses and Musa germplasm


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Commercially important Musa spp. include edible bananas and plantains (mostly hybrids of M. acuminata and/or M. balbisiana) and the fibre crop Musa textilis. Six viruses have been characterized from Musa to date (Jones 2000), but additional, uncharacterized viruses have also been recognized.

Banana bunch top virus (BBTV) has 18-20 nm isometric virions and a multicomponent ssDNA genome. It is transmitted in the persistent manner by the banana aphid Pentatoma nigrvensora and has a scattered distribution in Africa and the Asia-Pacific region. Cucumber mosaic virus (CMV) has 29 nm isometric virions and a tripartite ssRNA genome. It is transmitted in the non-persistent manner by a number of aphid species, and has a widespread international distribution. Banana bract mosaic virus (BBBrMV) and Abaca mosaic virus (AbaMV) both have filamentous virions, a ssRNA genome and are transmitted in the non-persistent manner by a number of aphid species. AbaMV has only been recorded from the Philippines, while BBBrMV has a scattered distribution in the Asia-Pacific region. Banana streak virus (BSV) has bacilliform virions (30 x 130 nm) containing a dsDNA genome, and has a worldwide distribution.

The filamentous viruses of Banana mild mosaic virus (BanMMV) contain a ssRNA genome of 7353 nt, encoding five ORFs. Although related to carlaviruses, foveaviruses and potexviruses, the genome organization and phylogenetic relationships of BanMMV place it apart from all previously described virus taxa (Gambley and Thomas, in press). The virus occurs in a wide range of Musa genotypes and has a worldwide distribution. The virus often occurs as symptomless infections and mixed infections with other viruses, though its mode of transmission is not known. Its economic impact is unknown.

Serological and PCR-based diagnostic assays are available for all the characterized viruses of Musa, but BSV still presents challenges. With BSV, symptoms can be prominent, but occur sporadically. Considerable sequence diversity has been found in BSV, and five of these isolates (BSV-OL, BSV-Mys, BSV-GF, BSV-IM and BSV-Lac) are probably sufficiently distinct to be considered separate viruses (Geering et al. 2000, A.D.W. Geering, N.E. Olzsewski, B.E.L. Lockhart and J.E. Thomas, unpublished). Immunocapture (IC) assays are required to differentiate episomal and integrated BSV sequences. IC-PCR with microplate detection has been developed for all the characterized viruses of banana. A multiplex assay for BBMV, BBTV and CMV has been published (Sharman et al. 2000). Assays for BanMMV and all known strains of BSV (multiplex) have also been developed (M. Sharman, A.D.W. Geering, J.N. Parry and J.E. Thomas, unpublished). These assays are...
used in conjunction with ELISA and ISEM for routine virus indexing.

All viruses of Musa are transmitted through vegetative propagules, including in vitro plantlets, and this has implications for the health of planting material, the conduct of breeding and transformation programmes and the transfer of germplasm. Virus-free planting material is a major factor in field control of these pathogens and, additionally, several of these viruses have limited distributions. Few studies have been conducted on the transmission of banana viruses through tissue culture. Several studies have shown that, through normal subculturing, a proportion of virus-free meristems arise from initially BSV-infected clones. This process appears to be accelerated somewhat at elevated temperatures, and plants derived from these meristems remain virus-free (Thomas et al. 1995, and references therein). Recently, the reverse situation has occurred with BSV. Virus infections have been detected in progeny of hybrids from breeding programmes, where there was no evidence of virus infection in the parent lines. This has been shown to be due to «activation» or «release» of BSV sequences that are integrated into the Musa genome (Hu et al. 2000). Recent evidence suggests that several additional strains of BSV may be integrated into different components of the hybrid Musa genome (A.D.W. Geering, N.E. Olszewski, B.E.L. Lockhart and J.E. Thomas, unpublished).

The INIBAP Transit Centre at K.U. Leuven houses the world's largest in vitro Musa germplasm collection comprising over 1100 accessions. These accessions are being indexed for viruses at three international Virus Indexing Centres (CIRAD, Montpellier, PPRI, Pretoria, and QDPI, Brisbane), and only accessions testing negative for known viruses are released. BanMMV and BSV are the most frequently detected viruses, probably due to frequent latent infection, and the additional factor of BSV integration. BBT and BBMV have not been detected in the collection.

References


Elimination of banana and plantain (Musa spp.) viral diseases by cryopreservation

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Cryopreservation is becoming a routine in vitro technique which overcomes the serious limitations encountered by the traditional germplasm conservation strategies used in field, seed and in vitro culture collections. The conservation at ultra-low temperatures, usually at −196°C which is the temperature of liquid nitrogen, allows a long-term and contamination-free storage of plant genetic resources. Recently, Brison and collaborators (1997) have demonstrated that cryopreservation could be used, in addition to germplasm conservation, to eliminate viruses from in vitro plum shoots infected with plum pox virus with an eradication rate of up to 50%. The possibility of applying a short (few hours) cryopreservation treatment instead of a long (few weeks) heat treatment would be highly promising.

We previously reported on the successful cryopreservation of proliferating meristems of different accessions of banana, one of the most important staple food crops of the world (Panis et al. 2000). Bananas, which belong to the Musa genus, are found in about 120 countries, mainly tropical and subtropical, on five continents and provide subsistence to millions of people. However, banana plants are threatened by different biotic agents such as bacteria, fungi or viruses, like cucumber mosaic virus (CMV), banana bunchy top virus (BBTV), banana streak virus (BSV), banana bract mosaic virus (BBMV) and banana mild mosaic virus (BaMMV).

In the framework of an INIBAP project entitled “Development of in vitro culture techniques for the elimination of banana and plantain (Musa spp.) viral diseases”, we aimed to evaluate the effect of cryotherapy on the sanitary state of plant material in comparison with traditional methods such as meristem culture. For this purpose, cryopreservation was performed on meristematic clumps excised from highly proliferating meristem cultures by the vitrification procedure using the PVS-2 solution (Sakai et al. 1990).

Our results show that eradication rates after cryopreservation of highly proliferating meristems reach 39% (32 plants out of 83 tested plants) and 94% (31 plants out of 33 tested plants) for CMV and BSV respectively. In comparison, eradication rates obtained by culture of meristems excised from highly proliferating meristems reached 11% and 63% for CMV and BSV respectively.

Ultrastructural study of highly proliferating meristems performed after a 1-week in vitro culture following cryopreservation showed that cryotherapy acts as a micro-scalpel. Small areas of living cells located in the meristematic dome and at the base of the primordia survive the cryopreservation procedure, while more differentiated cells, distant to the apical dome are killed. Associated with an uneven distribution of viral particles in the meristem, this could explain the efficiency of cryopreservation. The specific localization of viral particles within the meristem is now under investigation. We hope to gain a better understanding of the variations in observed eradication rates according to the virus and according to the therapy.

References


A DNA-based diagnostic test for ‘tropical’ race 4 of Fusarium wilt of banana

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Fusarium wilt of banana is a significant problem to the Australian banana industry. The
for the presence of fungus which causes the disease, *F. oxysporum* f.sp. *cubense* (*Foc*), is a highly diverse pathogen. At present, only a limited portion of the global diversity of *Foc* has been found in Australia. Thirty-three different vegetative compatibility groups (VCGs) and genotypes of *Foc* have been identified globally, of which nine occur in Australia. Nearly all of the diversity within *Foc* has been identified in Asia, and our proximity to Southeast Asia presents considerable risk of introduction of new strains of *Foc*, and in particular further introductions of the Cavendish-competent strain ‘tropical’ race 4. ‘Tropical’ race 4 is widespread throughout Indonesia and Malaysia, and has recently been detected in Irian Jaya. Several outbreaks of the ‘tropical’ race 4 strain of *Fusarium* will have already occurred in the Northern Territory, and so far these have been contained by quarantine measures. This strain of *Fusarium* will pose a threat to the major Cavendish production areas in north Queensland, which are presently free from all Cavendish-competent strains of the pathogen.

We are currently developing a DNA-based diagnostic test that is specific for the ‘tropical’ race 4 strain of *Foc*. We have thoroughly analyzed genetic diversity within *Foc* from the genus to the strain-specific taxon levels using total genomic fingerprinting methods such as DNA Amplification Fingerprinting (DAF) and other PCR-based methods such as restriction fragment length polymorphism (RFLP) and sequence analysis of the ribosomal (r) DNA. We have identified DNA sequence information that is unique to the ‘tropical’ race 4 strain of *Foc* and designed PCR primers that specifically amplify DNA only from the ‘tropical’ race 4 strain. Database searches of DNA sequence information published in Genbank have indicated that there are no matches for these primers with any other organism, but we are currently completing the laboratory screening of the specificity of these primers. We will then adapt our laboratory PCR conditions for amplification of *Foc* DNA directly from infected plants and infested soil. The diagnostic test will then require validation and field-testing, prior to release to industry and/or commercial laboratories.

We are also developing a DNA-based identification system that will allow the accurate characterization of all the strains of *Foc* that occur in Australia. This diagnostic system will allow the detection and identification of *Foc* directly from banana planting material and soil. This system will be useful for screening fields for the presence of *Foc* races prior to planting, screening rhizomes or suckers used for planting material, identifying isolates of *Foc* from infected plant tissue or infested soil, and will also be a useful research tool to study the biology and ecology of *Foc*.

**Isolation of potential disease resistance genes from banana**

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We are also developing a DNA-based diagnostic test that is specific for the ‘tropical’ race 4 strain of *Foc* and designed PCR primers that specifically amplify DNA only from the ‘tropical’ race 4 strain of *Fusarium* will have already occurred in the Northern Territory, and so far these have been contained by quarantine measures. This strain of *Fusarium* will pose a threat to the major Cavendish production areas in north Queensland, which are presently free from all Cavendish-competent strains of the pathogen.

Bananas are susceptible to a wide range of diseases, of which *Fusarium* wilt and black and yellow Sigatoka are among the most devastating. Although most commercially grown dessert bananas are susceptible to these fungal pathogens, resistance has been identified in wild banana cultivars. A novel approach to identify the resistance genes (R genes) which confer these resistance traits is to amplify genomic banana DNA using degenerate primers designed to class 3 R genes. This approach has been used successfully on lettuce, soybean, rice and maize but to date no banana R gene candidates (RGCs) have been published.

We have used degenerate primers to amplify five independent RGC sequences from banana, all of which show homology to previously characterized R genes. The five sequences were isolated from both resistant and susceptible cultivars and were present in low copy numbers. In addition, all five sequences were amplified from RNA, indicating that they were transcribed. When the DNA and RNA sequences from resistant and susceptible cultivars were compared, variability was observed between the five RGC sequences (<53% homology) and within each RGC (97–100% homology). Amplification of RGC flanking sequences revealed a 5' leucine zipper (LZ) domain and a 3' leucine rich repeat (LRR) domain, which is consistent with class 3 R genes.

**Banana streak virus promoters are highly active in transgenic banana and other monocot and dicot plants**

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Genetic engineering of plants has proven to be a useful method for the introduction of new desirable traits that are reflected in altered phenotypes, for example enhanced disease resistance. Regulatory sequences or promoters are required to drive efficient expression of the introduced gene in transgenic plants. Viral promoters, such as the 35S promoter of cauliflower mosaic virus, CaMV (Kay et al. 1987), have been frequently used for constitutive expression of transgenes in many crops. To obtain strong promoters that are suitable for high-level gene expression in transgenic banana, we have analyzed three novel promoter sequences from Australian banana streak badnavirus (BSV) isolates. These were evaluated in different transient and stable transformation assays using reporter genes encoding the green fluorescent protein (GFP) and B-glucuronidase (GUS) reporter enzymes (Schenk et al. 2001). In these experiments, 1322 bp (Cv), 2105 bp (My) and 1297 bp (Go) DNA fragments surrounding the transcription initiation site of the Cavendish, Mysore and Goldfinger BSV isolates (Geering et al. 2000) were analyzed for transcription-promoting activity.

Using transient expression assays, the Cv, My and Go fragments were all shown to have promoter activity in a wide range of plant species including monocots (banana, maize, barley, millet, sorghum), dicots (tobacco, canola, sunflower, *Nicotiana benthamiana*, tipu tree), gymnosperm (*Pinus radiata*) and fern (*Nephrolepis cordifolia*) (table 1).

GUS reporter enzyme activity was analyzed in transgenic *in vitro*-grown banana plants (cultivar Three Hand Planty) transformed with the Cv or My promoter constructs. Longitudinal and cross sections of roots, corms, pseudostems and leaves revealed blue staining in all cell types analyzed (for colour photos, visit http://www.uq.edu.au/~uqtreman). The strongest expression was observed in the corm and the vascular tissue. In roots, a high staining intensity was observed in vascular tissue and emerging side roots. Quantitative GUS activity levels for plants containing the My promoter constructs were higher in leaf, root and corm tissue compared to plants harboring maize ubiquitin promoter constructs (Table 1). In glasshouse grown banana plants, the My promoter showed higher activities than the maize ubiquitin and cauliflower mosaic virus 35S promoters (Table 1). The Cv promoter showed activities that were similar to (root and corm) or higher than (leaf) those of the maize ubiquitin promoter in *in vitro*-grown banana plants, but which were significantly reduced in larger glasshouse-grown plants (Table 1). This may be related to silencing associated with the integrated BSV sequence (Ndowora et al. 1999, Harper et al. 1999) in Three Hand Planty (AAB
Table 1. Overview of BSV Cv, My and Go promoter activities compared to the CaMV 35S and maize ubiquitin promoters in different plant species. Values representing the highest expressing plant: GUS enzymatic activity (MU) in nmol MU/h/mg protein and GFP accumulation in mg GFP/mg protein.

<table>
<thead>
<tr>
<th>Transgenic plants</th>
<th>Cv</th>
<th>My</th>
<th>Go</th>
<th>CaMV 35S</th>
<th>Maize ubiquitin</th>
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</thead>
<tbody>
<tr>
<td>Banana (leaf in vitro)</td>
<td>1076 MU</td>
<td>6299 MU</td>
<td>nt</td>
<td>214 MU</td>
<td>214 MU</td>
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<tr>
<td>Banana (root+corm in vitro)</td>
<td>2502 MU</td>
<td>10650 MU</td>
<td>nt</td>
<td>2571 MU</td>
<td>2571 MU</td>
</tr>
<tr>
<td>Banana (leaf glasshouse)</td>
<td>0 MU</td>
<td>1658 MU</td>
<td>430 MU</td>
<td>418 MU</td>
<td>nt</td>
</tr>
<tr>
<td>Sugarcane (leaf glasshouse)</td>
<td>13.1 GFP</td>
<td>&lt; 0.05 GFP</td>
<td>nt</td>
<td>11.6 GFP</td>
<td>nt</td>
</tr>
<tr>
<td>Sugarcane (stem glasshouse)</td>
<td>5.57 GFP</td>
<td>nt</td>
<td>nt</td>
<td>0.80 GFP</td>
<td>nt</td>
</tr>
<tr>
<td>Tobacco (leaf in vitro)</td>
<td>0.68 GFP</td>
<td>1.35 GFP</td>
<td>nt</td>
<td>1.68 GFP</td>
<td>nt</td>
</tr>
<tr>
<td>Tobacco (leaf glasshouse)</td>
<td>&lt; 0.06 GFP</td>
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<td>nt</td>
<td>0.29 GFP</td>
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Transplant assays

| Maize (sweet corn)                         | +++         | +++         | +++         | +++          | +++            |
| Barley                                     | +++         | ++          | nt          | +            | nt             |
| Banana                                    | +++         | +++         | nt          | nt           | nt             |
| Mfet                                       | +++         | +++         | nt          | nt           | +++            |
| Sorghum                                    | +++         | +++         | nt          | +            | +++            |
| Canola                                     | ++          | ++          | nt          | +++          | nt             |
| Tobacco                                    | ++          | ++          | nt          | +++          | nt             |
| Sunflower                                  | ++          | ++          | nt          | +++          | nt             |
| N. benthamiana                             | ++          | ++          | nt          | +++          | nt             |
| Tipu tree                                  | +++         | +++         | nt          | +++          | nt             |
| Pine tree                                  | ++          | ++          | nt          | +            | nt             |
| Faintbore fern                             | ++          | ++          | nt          | ++           | nt             |

rt = not tested, +++ = strong expression, ++ = moderate to strong expression, + = moderate to weak expression.

References


“CIEN BTA-03”, a new somaclonal variant resistant to yellow Sigatoka: biochemical, genetic and molecular characterization and agronomic studies

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In 1996, Trujillo and de Garcia obtained a somaclonal variant resistant to yellow Sigatoka by induction of adventitious shoots from the triploid clone Williams, subgroup Cavendish, locally named ‘Brasilero’, which is susceptible to the disease (Trujillo and de Garcia 1996, Trujillo et al. 1999). This somaclonal variant is not only resistant to the disease, but also displays a series of morphological and anatomical characteristics that distinguish it from triploid clones: a) leaf blade 1.4 times thicker than that of clone Williams (Hermoso et al. 1997, Trujillo et al. 1997); b) lower number of stomata per mm2 in both upper and lower epidermis (Hermoso et al. 1997, Trujillo et al. 1997); and c) higher phenol content. This clone was called CIEN BTA-03 (Figure 1).

The aim of this work is to report the data of the biochemical, genetic and molecular characterization of CIEN BTA-03, as well as referring to the evaluation of the resistant behaviour of the variant in the field.

The biochemical studies based on the analysis of the proteins by electrophoresis in denaturing acrylamide SDS-PAGE gels, stained with coomassie blue and scanned in an Imaging Densitometer model GS-690 (Bio-Rad) demonstrated the presence of two polypeptides (14 and 17 kDa) in the Williams clone that are neither observed in the CIEN BTA-03 clone, nor in the Fragro 7 (AAAA), both resistant to yellow Sigatoka (Giménez 1998).

Cytogenetic analysis showed that both clones presented mosaic tissues, but with a different chromosome number distribution; 22% of the cells of clone Williams have more than 33 chromosomes and 78% have less than 33 chromosomes. On the contrary, 65% of the cells in the resistant somaclonal variant CIEN BTA-03 have more than 33 chromosomes and 35% have less than 33 (Giménez 1998; Giménez et al. 2000).

The flow cytometry analysis demonstrated that somaclone CIEN BTA-03 presents a DNA content similar to or higher than that of clone Fagro 7 (Figure 2). The values obtained in the banana/rice mean ratio (B/R index)
Cluster analysis was done using the data obtained by random amplified polymorphic DNA (RAPD) markers for CIEN BTA-03 and 16 different genotypes of Musa spp. (Giménez 1998, Giménez et al. 2000, Vidal and de García 2000). Fifty-six polymorphic bands were used for the cluster analyses using Ward’s Unweighted Pair-Group Average (UPGA), and Weighted Pair-Group Average (WPGA) to calculate City-Block (Manhattan) distances. The dendrograms generated by the different methods were identical and showed that CIEN BTA-03 grouped with FHIA-02 (AAAB) and is not closely related to the Cavendish subgroup, to which the parent cultivar William belongs (AAA) (Giménez 1998, Giménez et al. 2000).

Field evaluation of the resistant character of CIEN BTA-03 (García et al. 2000) shows that this somaclone can be grouped with the cultivar Yangambi km5, based on its resistance to yellow Sigatoka (Figure 3). This somaclone has proved to be also resistant to black Sigatoka (Figure 4).

The efficiency and productivity indexes of CIEN BTA-03 were compared with the FHIA-01, FHIA-02 and FHIA-03 indexes (García et al. 2000). CIEN BTA-03 indexes are very similar in value to FHIA-02 and FHIA-03 indexes (Table 1).

We concluded that we have a new clone resistant to yellow Sigatoka, with a high probability of being also resistant to black Sigatoka, with good agronomic characteristics. It produces a bunch of 34.53 kg and has a productivity index of 0.28 kg per day.

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References


Biodiversity and evolution

Characterization of Musa germplasm held at INIBAP genebank using STMS-PCR markers

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The International Musa Germplasm Collection maintained by INIBAP and hosted by the Catholic University of Leuven (KUL), holds more than 1100 accessions. The objective of the genebank is to conserve Musa diversity for the benefit of the international community and to distribute Musa species and cultivars for the purpose of research and development.

The objective of this project is to obtain a molecular characterization of this germplasm in order to facilitate the classification and management of the genebank. Every year since 1998, about 200 individuals have been characterized at CIRAD-FLHOR in Guadeloupe with molecular markers. Among the different methods available, the choice of sequence tagged microsatellite site markers (STMS) is justified by numerous advantages: these PCR highly polymorph codominant markers useable on in vitro plantlets are available, and the patterns can be interpreted in terms of genotypes, thus allowing the detection of species-specific alleles or identification of similarities. STMS polymorphism was assayed by non radioactive urea-polyacrylamide gel electrophoresis, a simple transferable method less costly than most other molecular techniques (Lagoda et al. 1998a). Patterns and migration procedures on small and large gels were developed and applied depending on the differentiation required among clones. The 10 STMS markers used have a high discrimination potential and are localized independently on the different linkage groups (Lagoda et al. 1998b). At least 18 alleles have been identified for each STMS. Some alleles specific to the schizocarpa, balbisiana and Australimusa genomes were identified which allow the identification of interspecific clones. Most clones revealed different patterns except for clones within subgroups, such as Cavendish. The classification of clones was checked. Over 464 clones were studied, 34 classification errors were identified, classification of 23 clones was completed and 31 non-classified clones were assigned to a group and when possible to a subgroup.

These data help to complete the germplasm morphological database (INIBAP-MGIS), together with the data on ploidy level analysis through flow cytometry (see Dolezel et al., above) and eventually the data on chromosome genomic characterization through GISH (genomic in situ hybridization) (D’Hont et al. 2000).

References


Molecular studies on Musa acuminata ssp. malaccensis and selected local Malaysian species

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Banana is the second largest fruit crop in Peninsular Malaysia and contributes more than RM20 million in export earnings (Jamaluddin 1998). However, widespread disease problems remain a major constraint to the industry, and require that intensive efforts be made to introduce new resistant cultivars.

The banana programme at the University of Malaya and Universiti Putra Malaysia has recently established a molecular breeding group, which will focus on local indigenous species with a major emphasis on the wild banana Musa acuminata ssp. malaccensis. The programme currently includes an expressed sequence tag (EST) project, STMS analysis, retrotransposon analysis, analysis of potential disease resistance genes and taxonomical studies based on flow cytometry and cytology.

A CDNA library, constructed in a phage vector Ittir1ex2, has been established for EST analysis of Musa acuminata ssp. malaccensis genes. Clones from the library are being randomly sequenced and analyzed as part of a long-term banana genomics project. Similarly searches against known sequences deposited in the databases have already revealed identities with genes of known function and with other EST clones. All the sequences obtained will be used to generate a Musa EST database to be used for the further understanding and potential exploitation of banana genes.

Retrotransposon analysis has identified Ty 1-copia-like elements in 10 varieties of banana. A database search showed nucleotide identities ranging between 85-97% and predicted aminocacid identities of between 57-82% when compared to known RT genes of Ty 1-copia-like retrotransposons. The sequences were subdivided into eight distinct groups similar to the Ty 1-copia retrotransposons found in other plant species such as Tto1 in Nicotiana tabacum (Hirochika and Hirochika 1993). Ty 3-gypsy-like retrotransposons have also been isolated with identities ranging from...
55-80% when compared to similar elements in the database. The ubiquity and heterogeneity of the Ty 1-copia like and Ty 3-gypsy-like retrotransposons make them a suitable marker for the determination of biodiversity of banana species in Malaysia.

In a separate project, flow cytometry (Dolezel et al. 1991) was used to study ploidy and nuclear genome size variation in Musa species indigenous to Malaysia, i.e. Musa acuminate subspecies, Musa balbisiana, Musa violascens and Musa textilis. No variation was observed in ploidy level, whereas a large amount of variation in the genome size was observed among the different Musa species analyzed. Less variability was observed at the intraspecific level within the species Musa acuminate. Statistical and cluster analysis of data on genome size related in a grouping agreed well with the generally accepted taxonomic classification of Musa.

Studies on disease resistance focus on resistance of local wild bananas to Fusarium oxysporum, the major pathogen of banana in Malaysia. The ultimate aim will be the introgression of resistance genes from the wild species into cultivated varieties using approaches integrating genomics and marker-assisted selection.

The overall integrated approach of the programme, with close links to transformation and breeding groups in the country, hopes to contribute to Musa improvement programmes both locally and globally.

References


Genetic characterization of Brazilian commercial triploid and tetraploid cultivars and wild diploid genotypes using microsatellites

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In Brazil, banana cultivars from subgroups “Pome” and “Silk” (AAB) are widely cultivated, mostly by small growers. The breeding programme of EMBRAPA Mandicano Fruticultura, Cruz das Almas, Bahia, Brazil, has developed tetraploid hybrids based on a limited number of triploid commercial selections and wild diploids. Identical cultivars with distinct names (synonymous) and distinct genotypes with similar names (homonymous) might be a common phenomenon, and somatic mutations tend to accumulate in banana. The objectives of this work were to characterize 33 triploid commercial cultivars and tetraploid hybrids, plus 49 wild diploid genotypes from EMBRAPA’s breeding programme using microsatellite markers. Primers were purchased from Research Genetics Inc. (Huntsville, AL, USA), and amplified fragments were scored on denaturing polyacrylamide gels stained with silver nitrate. Based on cluster analysis, triploid and tetraploid cultivars grouped according to genome composition (presence of B genome) and to subgroup classification. No difference was detected among cultivars from subgroups “Cavendish” and “Pome”. Cultivars with erroneous subgroup classification were identified. Tetraploid selections from the same cross were not identical, and presented expected similarity with maternal triploids. Diploids were highly diverse, with the main parental diploid lines employed to develop tetraploid hybrids being very distinct. Some primers amplified more than one locus suggesting that loci duplication might be common in banana, as previously described in the literature. Genetic distances may be used for selecting future crosses.

Studies of Mycosphaerella fijiensis populations structure and of partial resistance of bananas

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The ascomycete fungus Mycosphaerella fijiensis (anamorph Paracercospora fijiensis) causes black leaf streak disease (BLSD), the most destructive leaf spot disease of bananas (Jones 2000). Knowledge of the extent and distribution of variability within M. fijiensis is necessary for breeding and management of BLSD resistance. A study of the genetic structure of M. fijiensis populations on a global scale showed that individual populations can maintain a high level of genetic diversity and that recombinant plays an important role in this pathogen (Carlier et al. 1996). Thus partial but supposed durable resistance should be preferentially used in breeding programmes. The main objectives of this work were to describe the genetic structure of M. fijiensis populations at continental and local scales and to evaluate the efficiency and the durability of partial resistance.

To study the population structure of a single pathogenic species, we have first to distinguish this species from close relatives and to determine its distribution. Such a survey conducted in South and Southeast Asia, led to the discovery of the previously undescribed fungus, Mycosphaerella euumusa (anamorph Septoria euumusa, Carlier et al. 2000). From a taxonomic and phylogenetic study of the ribosomal DNA, we showed that at least nine species, belonging to Mycosphaerella or related anamorph genera, can be isolated from banana leaves (Carlier et al. unpublished). Considering the presence of all these species, the primers defined in the ITS region (Johanson and Jegger 1993) are not strictly specific to either M. fijiensis or M. muscicola. These results show that a good knowledge of the fungal species complex is necessary to develop diagnostic tools. From the phylogenetic study we developed another tool based on a restriction assay of the ITS region and began looking for new specific primers. Such tools should be useful to determine the distribution and the importance of the different species.

The population structure of M. fijiensis at continental and local scales was analyzed from samples collected in Latin American, Caribbean and African countries using eight cleaved amplified polymorphic sequences or CAPS, as molecular markers (Zapater et al. unpublished). Within local populations, we found that most of the genetic variability is distributed on a small scale corresponding to the plant scale. In Latin America and the Caribbean zone, the genetic diversity of M. fijiensis in Honduras and Costa Rica are relatively high compared to the populations elsewhere, suggesting that the pathogen first entered the zone here. Within both Latin America/Caribbean and African areas, a high level of genetic differentiation was detected between most of the populations analyzed indicating that gene flow is limited (Rivas et al. and Carlier et al. unpublished). It is likely that the disease, therefore, has spread in the regions through infected plants and/or through restricted dispersion of ascospores. Continued research at a country level will help to specify the relative importance of both means of transmission. Aggressiveness variability was evaluated in two samples collected in Cameroon and the Philippines by inoculation on five partially resistant cultivars using a leaf
New cytological methods to study old problems in Musa L.

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Musa breeding is hampered by a number of constraints including a lack of knowledge of chromosome structure, ploidy and causes of sterility. There are no established karyotypes in Musa because of its poorly staining small, uniform chromosomes and difficulties in obtaining good spreads. Defining correct ploidy levels and establishing techniques for causes of sterility are necessary in Musa breeding. This study describes (i) the use of silver nitrate as a staining agent for Musa chromosomes, (ii) a new procedure to examine meiotic chromosomes in Musa, (iii) ploidy variation in Musa germplasm, and (iv) pollen tube growth in Musa. Acetocarmine, the most common stain used in Musa cytology, is effective for condensed chromosomes such as those in metaphase, but is ineffective for prophase chromosomes. Silver nitrate is shown to be a useful alternative stain for Musa chromosomes. An improved method to examine meiosis in Musa is described. The procedure involves dissection of microsporocytes from the anthers, centrifugation to obtain a large number of microsporocytes, digestion with enzymes and treatment of cells with ethanol-acetic acid. Although Giemsa and Leishman's stains for each of the stages was present, silver nitrate was most effective for the less contracted prophase chromosomes. This technique will be useful to develop pachytene karyotypes, characterize new hybrids, and identify nuclear restitution mechanisms (FDR or SDR). Ploidy and genome composition in some of our Musa germplasm showed differences from that of existing data showing the need to better characterize existing germplasm. Finally, a method to observe pollen tube growth in the styles of Musa hybrids will be described.

Biochemistry and fruit ripening

Synthetic seeds in banana: a novel propagation and delivery system

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Edible bananas are vegetatively propagated by suckers as viable seeds are not usually produced. New and effective means of propagating bananas would be advantageous over the conventional use of sucker material, for germplasm maintenance, exchange and also transportation. In vitro culture of vegetative meristems or floral apices is the most promising method for mass propagation. The production of synthetic seeds by encapsulating somatic embryos and vegetative propagules is rapidly becoming an applied technique with potential for mass propagation of elite plant species. Synthetic seed technology will have a significant impact on crop production, in both vegetatively propagated and seed-propagated crops. For the vegetatively propagated plants, synthetic seeds would allow direct planting of clonal varieties and may provide a means for maintenance of elite germplasm.

Synthetic seeds have been prepared by encapsulating shoot tips and somatic embryos and their conversion into plantlets has been studied. Shoot tips of cv. Basrai encapsulated in sodium alginate containing different gel matrices regenerated in vitro on various substrates. Use of Whites medium resulted in high conversion of encapsulated shoot tips into plantlets. Somatic embryos derived from embryogenic cell cultures of cv. Rasthali were also employed for the preparation of synthetic seeds. The encapsulated embryos converted into plants with varying frequencies on different gel matrices and substrates. Plantlets developed from synthetic seeds have been successfully transferred to soil. Synthetic seeds offer a useful tool since they can be handled like seeds and can be useful for storage, delivery and transport of banana germplasm.

Evaluation of regeneration and transformation systems in Musa acuminata var. Pisang Mas (AA) and Pisang Berangan (AAA)

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Fusarium wilt of banana (Panama disease) is native to peninsular Malaysia and has been recorded as a serious threat to the local industry (Thompson and Johnston 1953). However, attempts at improvement by conventional methods have been impeded by the infertile nature of cultivated bananas. For this reason, we are developing tissue culture and transformation protocols for use on our local banana varieties, Musa acuminata var. Pisang Mas (AA) and Pisang Berangan (AAA) in our laboratory. Regeneration methods have been attempted from single and naked meristems (scalls), meristematic globules and embryogenic calli. Embryogenic calli were derived from...
from meristems (Novak et al. 1989) and male inflorescences (Escalant et al.). The highest number of plants regenerated are from scalps. We are now planting these regenerated plants in the field to test for somaclonal variation. We have observed that the regeneration frequency is higher in Pisang Berangan (AAA) than in Pisang Mas (AA). Cell suspensions of both varieties are also being established. Cell suspensions from male inflorescences developed at a faster rate than those from apical meristems.

Plant transformation using both biolistic and Agrobacterium methods were attempted. Scalps and embryogenic calli were shown to be most responsive in the transformation experiments. Histochemical assays were used to optimize transformation parameters and identify suitable explants. Cell suspensions of both varieties will be used for transformation in future.

We are also isolating the antifungal gene from wild banana, Musa acuminata ssp. malaccensis. According to published data, this species is known to be resistant to Fusarium wilt races 1 and 4 (Vakili 1965).

Innovations for the commercial production of tissue culture plants are also being developed. We have developed a chamber which we call a «steriponics chamber» which merges the principles of tissue culture and aeroponics. The advantages of this chamber include faster plant production, minimum contamination risk and less dependency on labour. This chamber could also be used for physiological and pathogen assessment experiments.

A data tracking system has also been developed to monitor plant production using a bar coding system. The use of this system would allow monitoring of virus-indexed plants and quality control, and provide the necessary production data.

References


