Genomic changes associated with somaclonal variation in banana (*Musa* spp.)

- *Thomas J. Oh*[a,]
- *Margaret A. Cullis*[a],
- *Karl Kunert*[b],
- *Iris Engelborghs*[c],
- *Rony Swennen*[c]
- *Christopher A. Cullis*[a,d,]*

[a]NovoMark Technologies Inc., PO Box 22584, Beachwood, OH 44122-0854, USA
[b]Forestry and Agricultural Biotechnology Institute & Botany Department, **University of Pretoria**, Lunnon Road, Pretoria, South Africa
[c]Laboratory of Tropical Crop Improvement, Division of Crop Biotechnics, Faculty of Bioscience Engineering, Katholieke Universiteit Leuven, Kasteelpark Arenberg 13, B-3001 Leuven, Belgium
[d]Department of Biology, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106-7080, USA

*e-mail: cac5@case.edu
† Present address: Institute of Paper Science and Technology at Georgia Institute of Technology, 500 10th Street NW, Atlanta, GA 30332, USA

Received 8 August 2006; revised 19 October 2006

[Figures and Tables at the bottom of the document]

Abstract

The molecular basis of somaclonal variation is not precisely known, but both genetic and epigenetic mechanisms have been proposed. The available evidence points toward the

existence of labile portions of the genome that can be modulated when the cells undergo the stress of tissue culture. Therefore, the hypothesis that there are identifiable and predictable DNA markers for the early diagnosis of somaclonal variation has been tested. Representational difference analysis was used to isolate unique fragments of DNA (difference products) between visible culture-induced off-type and normal banana plants. Markers generated from six difference products differentiated between some of the off-type and normal pairs. The genomic region around one of these difference products has been extensively characterized and has a high degree of polymorphism, with variation in up to 10% of the nucleotides sequenced in the region. This same region has been shown to vary in other pairs of off-type and normal banana plants derived from tissue culture as well as in plants propagated commercially in vitro. The data are consistent with the hypothesis that there is at least one particularly labile portion of the genome that is especially susceptible to the stress imposed during tissue culture and that is associated with higher rearrangement and mutation rates than other portions of the genome. Consequently, the regions that are reported here have the potential to be used as early detection tools for identifying somaclonal variants.

Introduction

Somaclonal variation is the genetic variation that is observed when plants are regenerated from cultured somatic cells. During the micropropagation of valuable elite clones, this variation usually results in off-types that reduce the commercial value of resultant plants. Genetically enhanced (transgenic) plants also need to be carefully screened to avoid unwanted and unintended somaclonal variation. Various types of mutations have been described in somaclonal variants, including point mutations, gene duplication, chromosomal rearrangements, and chromosome number changes (Kaeppler et al. 2000, Peschke and Phillips 1992, Phillips et al. 1994). Transposable element movement and changes in DNA methylation (Koukalova et al. 2005, Kubis et al. 2003, Smulders et al. 1995), possibly through the function of small interfering RNA (Lippman et al. 2003), have also been implicated as potential mechanisms behind some somaclonal variation. The appearance of somaclonal variants may not be a process unique to in vitro propagation but may occur naturally in plant somatic and reproductive tissues (Cullis

2005). The trigger for all these types of changes can be described as genomic shock or plasticity, which occurs after the plant has exhausted its ordinary physiological responses to environmental stress (Cullis 1999). This genomic shock response may be a radical, but limited, genomic reorganization, which is an adaptive mechanism and can be activated under stress. The occurrence of hotspots of mutation and recurring menus of alternative alleles is consistent with this response being limited to a subfraction of the genome.
The sometimes massive but reproducible genetic changes observed with somaclonal variation offers the possibility for identifying specific DNA variants (be they structural or DNA modifications) that are associated with the release of this genetic variation. Therefore, they can be used to monitor the genome through the process of in vitro propagation. Such DNA markers could also be used to screen plants to determine the possibility of their being true-to-type.

The clonal propagation of horticultural species and crops, such as bananas and oil palm, is intended to produce chosen elite individuals in mass. Somaclonal variation is problematic under these circumstances, where even a low percentage of off-types is unacceptable for commercial use. A high percentage of off-types can be costly, as has been proven in both the banana and oil palm industries (Larkin 2004). In a similar vein, somaclonal variation can be problematic during the in vitro conservation of vegetatively multiplied crops and the genetic modification of crops where hundreds of individual transgenics would need to be exhaustively tested so that only proven elite individuals are chosen to become commercial releases. Therefore, the early detection of somaclonal variants is very useful in plant tissue culture and transformation studies.

A number of different molecular techniques are currently available to detect sequence variation between closely related genomes such as those between source plants and somaclones. These include random amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs) (Labra et al. 2001, Linacero et al. 2000). Both techniques are useful in comparing the DNA from any number of different samples for the differentiation of plants because of sequence variation by identifying random polymorphisms. However, these studies have not led to the development of

general markers applicable in the screening of micropropagated plants for somaclonal variation. Representational difference analysis (RDA) has been applied to detect variation in a limited number of plant species (Cullis and Kunert 2000, Donnison et al. 1996, Oh and Cullis 2003, Vorster et al. 2002, Zoldos et al. 2001). RDA technology has been used to detect genomic losses, rearrangements, amplifications, point mutations and pathogenic organisms between two genomes (Lisitsyn 1995, Lisitsyn et al. 1993, Michiels et al. 1998, Ushijima et al. 1997). The advantage of RDA is that a complexity of about 5×10^8 base pairs of DNA can be scanned in each subtraction, which is greater than can be achieved with other commonly used differentiation techniques, such as restriction fragment length polymorphisms, RAPDs, AFLPs or microsatellites.

In banana, reduced height, thicker pseudostem and shorter but wider leaves are examples of the major visible differences between the naturally occurring dwarfs ‘Cachaco enano’, ‘Prata ana’ and ‘Figue Rose naine’ and their naturally occurring normal-sized variants ‘Cachaco’, ‘Prata’ and ‘Figue Rose’, respectively. In the case of ‘Cavendish’ banana a whole range of naturally occurring ‘Cavendish’ types exist gradually going from extra dwarf (‘Dwarf Parfitt’) to tall plants like the ‘Giant Cavendish’ (Daniells et al. 2001). This dwarf phenotype is often also obtained by in vitro culture (Damasco et al. 1998, Smith and Hamill 1993, Vuylsteke et al. 1991).

The overall aim of this study was to identify unique genomic DNA regions that could be developed into DNA markers for the diagnosis of somaclonal variation in banana. Several rounds of partial genomic DNA subtractions using RDA have been performed using the tall (off-type arising from tissue culture) and dwarf forms of ‘Curare enano’ (Fig. 1) to identify regions of the genome that differ in sequence or methylation status between these two types. DNA oligonucleotides designed from the sequences of the difference products have been used as primers in a polymerase chain reaction (PCR)-based system to compare pairs of normal and off-type banana plants. The genomic locus of one of these difference products appeared to be a hotspot (extremely labile) for variation arising during culture. This extended region has been shown to be useful as a diagnostic tool for somaclonal variation in banana.

Materials and methods

Plant material

Plant material was supplied by the International *Musa* germplasm collection of the International Network for the Improvement of Banana and Plantain (INIBAP) Transit Centre, where germplasm is stored since 1987–1992 at an ambient temperature of 16 ± 1°C and permanent illumination of 25 µmol m^{-2} s^{-1}. The medium is a Murashige and Skoog (1962) supplemented with 2.25 mg l^{-1} (10 µ*M*) 6-benzyladedinine and 0.175 mg l^{-1} (1 µ*M*) indole-3-acetic acid and solidified with 2–4 g l^{-1} Gelrite®. Under these conditions germplasm is subcultured on average once per year (Van den houwe et al. 1995, 2003), because growing conditions were set to slow down growth. However, under normal proliferating conditions (same as above but at 28 ± 2°C in a light cycle of 12–16 h of about 60 µE m^{-2} s^{-1}) plants are subcultured every 2 months.

Plant material was supplied by the International *Musa* germplasm collection of the INIBAP Transit Centre. This comprised in vitro plantlets of three different cultivars (Daniells et al. 2001) (‘Cachaco’, ABB, ITC.0643; ‘Figue Rose’, AAA, ITC.1159; and ‘Prata’, AAB, ITC.0207) in pairs of naturally occurring dwarf and normal-sized phenotypes, a pair of naturally occurring dwarf and in vitro-induced tall (normal) off-type of the cultivar ‘Curare enano’ (AAB, ITC.1165), and three Cavendish types, ‘Giant Cavendish’, ITC0346, ‘Dwarf Cavendish’, ITC0002, ‘Dwarf Parfitt’, ITC0548. Phenotypes, whether normal or variant, are transmitted without change to the next generation of plants in the field as banana plants produce ratoons (suckers) vegetatively and do not produce seeds. Naturally occurring accessions were found in farmers‘ fields, and the fact that they are now cultivated in many countries in the world without any phenotypic changes underlines their phenotypic stability.

The variant phenotype was created in vitro and is only phenotypically different from the other set of the pair by its plant size. This phenotype also remained stable during ratooning in the field or greenhouse. Other Cavendish plant materials were supplied by Du Roi Ltd, Tzaneen, South Africa.

DNA extraction

Total DNA was isolated from 300 mg of young leaf tissue from greenhouse or in vitro plantlets using the DNeasy DNA extraction kit (Qiagen, Hilden, Germany) with some modifications. Briefly, plant tissue was homogenized in a mortar with pestle and lysed as described by Dellaporta et al. (1983). The lysate was further purified on DNeasy columns according to the manufacturer's instructions. Typical yields were between 30 and 45 μg g^{-1} of leaf tissue. Four independent DNA extractions from the 'Curare enano' normal and dwarf individuals were analyzed.

Representational difference analysis

The general outline for the RDA procedure described by Lisitsyn et al. (1993) and Vorster et al. (2002) was followed. Reciprocal subtractions were performed with the normal DNA being the tester and the dwarf DNA being the driver in one experiment and the dwarf DNA being the tester and the normal DNA being the driver in the other experiment. Amplicons (representations) were prepared by the digestion of 2 μg of DNA with 80 units of the restriction enzyme *Hpa*II, ligation of adapter sequences RHpa11 (5'-CGGTCGGTGAG -3') and RHpa24 (5'-AGCACTCTCCAGCCTCTCACCGAC-3') to the digested DNA and amplification of the ligation products by PCR to generate the first round of amplicons. All PCR amplifications were performed using a program with an initial denaturation of 1 min at 95°C, the appropriate number of cycles of 30 s at 94°C and 3 min at 72°C, a final extension for 10 min at 72°C and then an indefinite soak at 4°C. All amplicons were then digested with *Hpa*II to remove the RHpa24 adapter sequences from the ends of the amplicons. To add another set of adapters to the tester amplicons only, the *Hpa*II digest of tester amplicons was purified by using a low-melting-point agarose gel (Roche, Indianapolis, IN) and Qiagen-tip 20 (Qiagen) to eliminate digested adapter RHpa24. Then a different set of adapters, JHpa11 (5'-CGGTTTCATGG-3') and JHpa24 (5'-ACCGACGTCGACTATCCATGAAAC-3'), was ligated to the digested and purified tester amplicons before entering to the hybridization/amplification step (Lisitsyn et al. 1993). The adapters on the subtracted tester products were changed before each round of hybridization and amplification. The first hybridization reaction used 20 μg of driver DNA amplicons and 0.5 μg of tester

DNA amplicons (40:1 driver/tester ratio). The mixed amplicons were dried and redissolved in 4 µl of hybridization buffer [30 m*M* EPPS, (2-hydroxyethyl piperizine)-N'-(3-propene sulfonic acid), pH 8, and 3 m*M* EDTA] and overlayed with mineral oil. The DNA was denatured at 100°C for 10 min, 1 µl of sodium chloride (5 *M*) was added to a final concentration of 1 *M* and the reaction was incubated at 67°C for 16 h. The hybridization mixture was then diluted to 300 µl and an aliquot amplified. The first round of amplification following all hybridizations was for 10 cycles followed by digestion of the products with mung bean nuclease to remove single-stranded DNA. The nuclease-treated product was then amplified for an additional 20 cycles. From the resulting amplicons (first subtraction product) the adapters were removed by *Hpa*II digestion and a second pair of adapters with different sequences was ligated onto the amplicons used as tester DNA for the second hybridization (NHpa24 5'-AGGCAACTGTGCTATCCGAGGGAC-3'; NHpa11 5'-CGGTCCCTCGG-3'). The second hybridization used 20 µg of driver DNA amplicons and 0.01 µg of tester DNA amplicons (2000:1 driver/tester ratio) and following hybridization the products were amplified. Adapters were removed from the resulting amplicons and the subtraction products were filled in, blunt-end ligated into the pCRScript cloning vector (Stratagene) and transformed into *Escherichia coli* competent cells. Plasmid-containing colonies carrying an insert were selected for plasmid isolation and determination of the insert size and nucleotide sequence.

Primer design and testing

Pairs of primers were designed using the program, Primer3 (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primer pairs were used in a PCR using banana DNA as template at various annealing temperatures to optimize the PCR. The PCRs were carried out in 25-µl volumes containing 25 ng of total genomic DNA, 15 ng of primer, 200 µ*M* of each dNTP, 10 m*M* Tris–HCl (pH 8.3), 2 m*M* $MgCl_2$ and 0.5 units of *Taq* polymerase (Takara, Shiga, Japan). Amplification was performed using an MJRPTC60 system. The following standard amplification program was used: (1) 94°C for 1 min × 1 cycle; (2) 94°C for 1 min, 55°C or 60°C for 1 min depending on the primer pair, 72°C for 1 min × 35 cycles; (3) 72°C for 7 min × 1 cycle

followed by an optional soak period at 4°C. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

Flanking sequence isolation

A two-step PCR technique as outlined by Sorensen et al. (1993) was applied using each of four different flanking primers three of which (FP01, 02 and 04) are shown in Fig. 4 and two primers designed from the difference product sequence. These primers were used to amplify from the banana genome the 3'- and 5'-end DNA flanking sequences of one of the RDA subtraction products (Musomav2). For amplification of the two flanking sequences, 5'-end biotinylated primers complementary to sections of the RDA subtraction product were used in combination with a mixture of all four flanking primers to isolate a sequence complementary to the flanking regions of the RDA subtraction product. After PCR amplification, biotinylated DNA fragments were isolated by mixing 40 µl of the PCR mixture with 40 µl (200 µg) prewashed Dynabead M280-streptavidin as recommended by the supplier (Dynal Biotech, Oslo, Norway) and removing all biotinylated fragments from the mixture using a Dynal magnetic particle concentrator. Binding and washing steps were done in the presence of a buffer consisting of 10 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA and 2 *M* NaCl. After incubation for 15 min to remove the biotinylated DNA fragments with the particle concentrator from the mixture and washing the beads in buffer, the bead-bound DNA fragments were treated in 8 µl of 100 m*M* NaOH for 10 min to remove the beads and the biotin label. The resulting supernatant containing non-biotinylated DNA strands was then neutralized with 4 µl of 200 m*M* HCl and 1 µl of 1 *M* Tris–HCl (pH 8) diluted to 30 µl with distilled H_2O. A second PCR with 2 µl of this product was used to amplify the isolated flanking sequences with an internal (nested) primer. All PCRs were carried out using a standard PCR protocol with 42 cycles of amplification and primer annealing at 62°C. Amplified and agarose gel-purified DNA fragments were cloned into the vector pCRScript and sequenced.

Results

RDA difference products

Twenty clones varying in length from 48 to 297 bp were isolated from the subtraction between the normal (tester) and dwarf (driver) phenotypes of 'Curare enano' and sequenced. Six of these clones (Musomav1–6) were characterized and their sequences have been deposited in GenBank with accession numbers (DQ842002, DQ842001, DQ842003, DQ842004, DQ842005, and DQ842006). No difference products were obtained when the reciprocal subtraction (dwarf tester and normal driver) was performed. The sequences were analyzed with both BLASTN and BLASTX to determine any related sequences in the non-redundant (nr) databases of GenBank. None of the sequences had significant homology ($E < 10^{-10}$) with BLASTN searches. Musomav1 had two non-overlapping regions of homology with the *Musa acuminata* expansin1 (EXP1) gene and the *Arabidopsis thaliana* transketolase AT3G60750 gene. Equivalent homologies were also found with the BLASTX search. The BLASTX searches identified more potential homologies in addition to those seen with Musomav1. Musomav5 and 6 both had potential homologies with kinase genes while Musomav3 had similarity to a putative disease resistance protein. Musomav2 and 4 had no significant similarities with either BLASTN or BLASTX searches.

Primers were designed from these six sequences (Table 1) and the result of PCRs using these primers with the 'Curare enano' normal and dwarf DNAs used in the subtraction is given in Fig. 2. Two of the primer sets, one consisting of primers 1 and 2 and the other of primers 3 and 4 showed differences between the two starting DNAs (indicated by arrowheads in Fig. 2). The primer sets (1 and 2, 3 and 4) revealed a polymorphism between normal and dwarf with a band being produced in the normal that was absent from the dwarf. With primers 1 and 2 a complex of differences were observed between the starting materials, while the PCR product with primers 3 and 4 was a single band in the normal DNA and no band with the dwarf DNA. This presence with one DNA but not with the other is consistent with a structural change in the DNA rather than with a modification, such as methylation, of the DNA. Five of the six pairs of primers were used to amplify DNA from the other sets of normal and dwarf banana DNAs (Fig. 3). All these

sets of primers differentiated between the normal and dwarf phenotypes of each cultivar. Primers 3 and 4 were most useful in distinguishing between the normal and dwarf phenotypes (Fig. 2B), only failing to distinguish between the 'Cachaco' normal and dwarf. Although there are differences between the lines, none of the other markers distinguish between the plants based solely on the height phenotype. Because primers 3 and 4 did not amplify in five of the six dwarf phenotypes but did amplify in all the normal phenotypes, this particular subtraction product was subjected to additional characterization.

Isolation of flanking sequences around difference product Musomav2

The flanking regions of this difference product were isolated resulting in the region depicted in Fig. 4. The whole region was sequenced (GenBank accession number DQ842001). The complete sequence was again characterized using BLASTN and BLASTX to determine any related sequences in the nr databases of GenBank with no significant similarities being reported. The positions of additional primers that were designed are indicated in Fig. 4. These additional primers were used to amplify from 'Curare enano' normal and dwarf DNAs (Fig. 5) and some were also used to amplify from the banana dwarf and normal pairs. The variation using these additional primers demonstrated that many primers can be designed from this region to distinguish between the normal and dwarf phenotypes. These observations strongly suggest that this whole region of the genome is highly variable. The complete pairwise sequence comparison for all five sequences is shown in Fig. 6 with the positions of gaps in the sequence noted. The three individuals (5, 6 and 11) derived from culture do show minor sequence differences, which would be consistent with the region being modified during in vitro propagation of banana even when obvious phenotypic variation is absent.

Methylation differences

Because the basis for somaclonal variation has been proposed to be epigenetic and the result of DNA or chromatin modification, tests on the methylation status of various regions around Musomav2 were carried out. The primers 45 and 4, 45 and i4, and r5 and r6 (from the flanking regions of Musomav2) were used to amplify DNA from normal and

dwarf ‘Curare enano’ prior to and after digestion with the isoschizomers *Msp*I and *Hpa*II (Fig. 7) which are differentially affected by the methylation of their common recognition site. The results show that the amplification from dwarf with primers 45 and i4 is essentially unaffected by either digestion, indicating that both the C residues in the restriction sites are methylated. However, the amplification from the normal is reduced in both cases, indicating that neither of the C residues in most of the restriction sites are methylated. The level of amplification from the normal DNA is much greater than that seen with the dwarf DNA prior to digestion. The differential amplification with primers 45 and i4 is not because of lower DNA concentration but because of the efficiency of amplification supported. The equivalent amplification from both DNA samples with primers r5 and r6 (Fig. 7C) supports this conclusion. When primers r5 and r6 are used, the level of amplification from both samples is equivalent. Both are greatly reduced by *Hpa*II digestion. No amplification was observed from the normal DNA after *Msp*I digestion, although the level of amplification seen with the dwarf DNA sample is similar to that observed after *Hpa*II digestion. This result is consistent with methylation differences between normal and dwarf phenotypes of ‘Curare enano’ occurring within this region in addition to the actual sequence variation already identified.

Testing of Primers 3 and 4 for the detection of in vitro-associated variation

Primers 3 and 4 were used in PCRs with the DNA isolated from 300 individual plants supplied by a commercial in vitro propagation facility. The result was that 14 of the 300 showed no amplification with the primer pair 3 and 4. However, all DNA samples showed the expected amplification with the primer pair r5 and r6. The frequency of observed non-amplification (4.7%) was approximately equal to that expected for the rate of dwarf types observed in this batch of plants. However, the plants have not been followed to determine whether or not this primer pair exclusively identified dwarf types, a range of other off-type phenotypes or apparently normal plants.

Discussion

RDA has proved to be useful in identifying regions of the banana genome that vary between different phenotypes. The observation that difference products were isolated

when the normal phenotype was used as tester, but not when the dwarf phenotype was used as tester, is consistent with earlier observations using two other in vitro-derived off-types (Cullis and Kunert 2000). This evidence is consistent with the idea that all the fragments present in the off-types are also present in the normal plants, but not vice versa. A possible explanation is that the banana varieties that were tested were triploid and not all three copies of a particular sequence were modified in the somaclones.

The findings in this study are consistent with the notion that there is a labile fraction of the genome that is modified during the generation of somaclonal variation in banana. The difference products reported here have all the characteristics of representatives of this labile region of the genome, although not all are diagnostic of either the normal or dwarf phenotypes. Thus, primer pair 5 and 6 amplifies a band in ‘Cachaco’ dwarf but not in ‘Cachaco’ normal, while the reverse is true for the other pairs of normal and dwarf lines. It should also be noted that ‘Cachaco’ dwarf and normal is the only pair that is not distinguishable using the primer pair 3 and 4 (from Musomav2) and may have a genetic basis different from those of the other pairs of normal and dwarf phenotypes. The observation that many of the independent banana variants can be distinguished with the six sequences described here supports the notion of a limited set of loci being modified during the generation of variation during in vitro propagation. The characterization of the extended region around one of the difference products (Musomav2) has been used to design sets of primers that differentiate between the two starting phenotypes (normal and dwarf) as well as between other pairs of normal and dwarf banana plants arising from culture. The region contains a large number of mutations that arise frequently during in vitro propagation supported by the observation of sequence differences among individuals from in vitro propagation. This high rate of variation also makes the region a candidate for identifying single-nucleotide polymorphisms as possible markers for variation arising through in vitro propagation of bananas. Musomav2 appears to contain both sequence variation and variations in the methylation status. Therefore, both sequence and methylation differences occur in somaclonal variants in banana.

It is important to note that in the pair of normal and dwarf ‘Curare enano‘ forms, the normal (tall) individual arose from culture. However, the sequence of this region is most closely related to the sequence derived from other tall but not dwarf forms. Therefore, it

appears that the change that had occurred in culture was a reversion to the previous sequence. This type of reversion has similarities with the reversion of *Arabidopsis* hothead mutations (Lolle et al. 2005), although more data are needed before this comparison can be validated. Another possibility is that the initial explant was chimeric and the normal form was a selection from this chimera in the culture.

The primers 3 and 4 from Musomav2 have been tested to determine if they can be used to distinguish individuals from a commercial in vitro propagation facility. A small number of the plants could be distinguished by failing to amplify a product with these two primers. A larger number of individuals need to be tested, and the altered individuals grown up and their phenotype determined to confirm the frequency of association between this marker and a specific phenotype. However, the preliminary evidence indicates that primers 3 and 4 can be used as a diagnostic for identifying off-types in banana plants produced through in vitro propagation. The sequences reported here and the primers developed from them (particularly primers 3 and 4) have the potential to be developed into a robust diagnostic DNA marker for somaclonal variation.

Edited by H. G. Nam

References

Cullis, CA (1999) Environmental stress – a generator of adaptive variation? In: Lerner HR (ed) *Plant Adaptations to Stress Environments*. Marcel Dekker, New York, pp 149–160

Cullis, CA (2005) Mechanisms and control of rapid genomic changes in flax. *Ann Bot (Lond)* **95**: 201–206

Cullis CA, Kunert KJ (2000) Isolation of tissue culture-induced polymorphisms in bananas by representational difference analysis. *Acta Hortic* **530**: 421–428

Damasco OP, Adkins SW, Godwin ID, Smith MK, Drew RA (1998) Use of a SCAR-based marker for the early detection of dwarf off-types in micropropagated Cavendish bananas. *Acta Hortic* **461**: 157–164

Daniells J, Jenny C, Karamura D, Tomekpe K (2001) *Musa*logue: a catalogue of *Musa* germplasm. In: Arnaud E, Sharrock S (compiled by these persons) Diversity in the Genus *Musa*. INIBAP, Montpellier, France, 213 pp. Available at http://www.inibap.org/pdf/IN010189_en.pdf

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. *Plant Mol Biol Rep* **1**: 19–21

Donnison IS, Siroky J, Vyskot B, Saedler H, Grant SR (1996) Isolation of y chromosome-specific sequences from *Silene latifolia* and mapping of male sex-determining genes using representational difference analysis. *Genetics* **144**: 1893–1901

Kaeppler SM, Kaeppler HF, Rhee S (2000) Epigenetic aspects of somaclonal variation in plants. *Plant Mol Biol* **43**: 179–188

Koukalova B, Fojtova M, Lim YK, Fulnecek J, Leitch AR, Kovarik A (2005) Dedifferentiation of tobacco cells is associated with ribosomal RNA gene hypomethylation, increased transcription, and chromatin alterations. *Plant Physiol* **139**: 275–286

Kubis SE, Castilho AMMF, Vershinin AV, Heslop-Harrison JS (2003) Retroelements, transposons and methylation status in the genome of oil palm (Elaeis guineensis) and the relationship to somaclonal variation. *Plant Mol Biol* **52**: 69–79

Labra M, Savini C, Bracale M, Pelucchi N, Colombo L, Bardini M, Sala F (2001) Genomic changes in transgenic rice (*Oryza sativa* L.) plants produced by infecting calli with *Agrobacterium tumefaciens*. *Plant Cell Rep* **20**: 325–330

Larkin P (2004) Somaclonal Variation: Origins and Causes. *Encyclopedia of Crop and Plant Science*. Marcel Dekker,
New York, pp 1158–1161

Linacero R, Alves EF, Vazquez AM (2000) Hot spots of DNA instability revealed through the study of somaclonal variation in rye. *Theor Appl Genet* **100**: 506–511

Lippman Z, May B, Yordan C, Singer T, Martienssen R (2003) Distinct mechanisms determine transposon inheritance and methylation via small interfering RNA and histone modification. *PLoS Biol* **1**: 420–428

Lisitsyn N (1995) Representational difference analysis: finding the differences between genomes. *Trends Genet* **11**: 303–307

Lisitsyn N, Lisitsyn N, Wigler M (1993) Cloning the differences between two complex genomes. *Science* **259**: 946–951

Lolle SJ, Victor JL, Young JM, Pruitt RE (2005) Genome-wide non-Mendelian inheritance of extra genomic information in Arabidopsis. *Nature* **434**: 505–509

Michiels L, Van Leuven F, Van Den Oord JJ, De Wolf-Peeters C, Delabie J (1998) Representational difference analysis using minute quantities of DNA. *Nucleic Acids Res* **26**: 3608–3610

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol Plant* **15**: 473–497

Oh TJ, Cullis CA (2003) Labile DNA sequences in flax identified by combined sample representational difference analysis (csRDA). *Plant Mol Biol* **52**: 527–536

Peschke VM, Phillips RL (1992) Genetic implications of somaclonal variation in plants. *Adv Genet* **30**: 41–75

Phillips RL, Kaepler SM, Olhoft P (1994) Genetic instability of plant tissue cultures: breakdown of normal controls. *Proc Natl Acad Sci U S A* **91**: 5222–5226

Smith M, Hamill S (1993) Early detection of dwarf off-types from micropropagated Cavendish bananas. *Aust J Exp Agric* **33**: 639–644

Smulders MJM, Rus-Kortekaas W, Vosman B (1995) Tissue culture induced DNA methylation polymorphisms in repetitive DNA of tomato calli and regenerated plants. *Theor Appl Genet* **91**: 1257–1264

Sorensen AB, Duch M, Jorgensen P, Pedersen FS (1993) Amplification and sequence analysis of DNA flanking integrated proviruses by a simple two-step polymerase chain reaction method. *Am Soc Microbiol* **67**: 7118–7124

•

Ushijima T, Morimura K, Hosoya Y, Okonogi H, Tatematsu M, Sugimura T, Nagao M (1997) Establishment of methylation-sensitive representational difference analysis and isolation of hypo- and hypermethylated genomic fragments in mouse liver tumors. *Proc Natl Acad Sci U S A* **94**: 2284–2289

Van den houwe I, De Smet K, Tezenas de Montcel H, Swennen R (1995) Variability in storage potential of banana shoot cultures under medium term storage conditions. *Plant Cell Tissue Organ Cult* **42**: 269–274.

Van den houwe I, Lepoivre P, Swennen R, Frison E, Sharrock S (2003) The world banana heritage conserved in Belgium for the benefit of small-scale farmers in the Tropics. *Plant Genet Resour Newsl* **135**: 18–23

Vorster BJ, Kunert KJ, Cullis CA (2002) Use of representational difference analysis for the characterization of sequence differences between date palm varieties. *Plant Cell Rep* **21**: 271–275

Vuylsteke D, Swennen R, De Langhe E (1991) Somaclonal variation in plantains (*Musa* spp. AAB group) derived from shoot-tip culture. *Fruits* **46**: 429–439

Zoldos V, Siljak-Yakovlev S, Papes D, Sarr A, Panaud O (2001) Representational difference analysis reveals genomic differences between *Q. robur* and *Q. suber*: implications for the study of genome evolution in the genus *Quercus*. *Mol Genet*

Figures and Tables

Fig. 1. Individuals of the two starting clones of ’Curare enano‘ dwarf and normal at the same scale.

Fig. 2. Gel electrophoresis of PCR products with various sets of primers designed from the difference products. Lanes 2, 4, 6, 8, 10 and 12 – DNA from normal ‘Curare enano’; Lanes 3, 5, 7, 9, 11 and 13 – DNA from dwarf ‘Curare enano’. Lane 1, Molecular Weight Marker VI (MWM VI, Roche); lanes 2 and 3, primers 1 and 2; lanes 4 and 5, primers 3 and 4; lanes 6 and 7, primers 5 and 6; lanes 8 and 9, primers 7 and 8; lanes 10 and 11, primers 9 and 10; lanes 12 and 13, primers 11 and 12. Arrowheads indicate the size of the expected fragment.

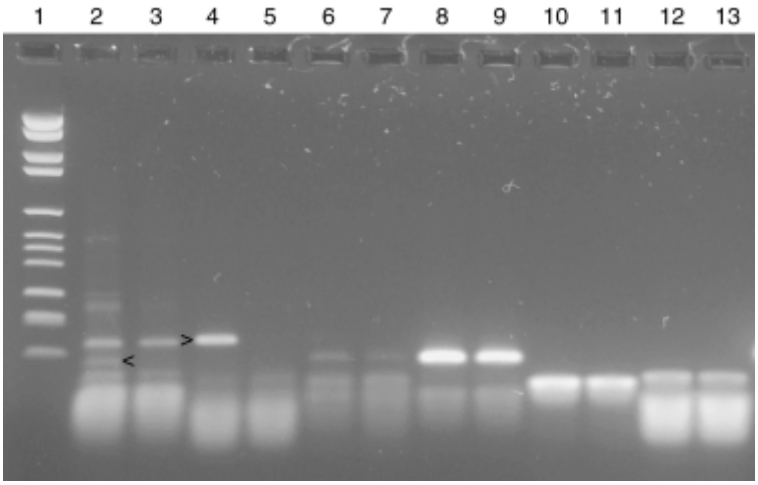


Fig. 3. Gel electrophoresis of PCR products with various sets of primers designed from the difference products used in PCR amplifications with nine banana samples. Lane 1, Molecular Weight Marker VI (MWM VI, Roche); lane 2, 'Dwarf Parfitt'; lane 3, 'Dwarf Cavendish'; lane 4, 'Giant Cavendish'; lane 5, 'Prata' (normal); lane 6, 'Prata ana' (dwarf); lane 7, 'Figue Rose' (normal); lane 8, 'Figue Rose naine' (dwarf); lane 9, 'Cachaco' (normal); lane 10, 'Cachaco enano' (dwarf). (A) Primers 1 and 2, (B) primers 3 and 4, (C) primers 5 and 6, (D) primers 9 and 10, and (E) primers 11 and 12.

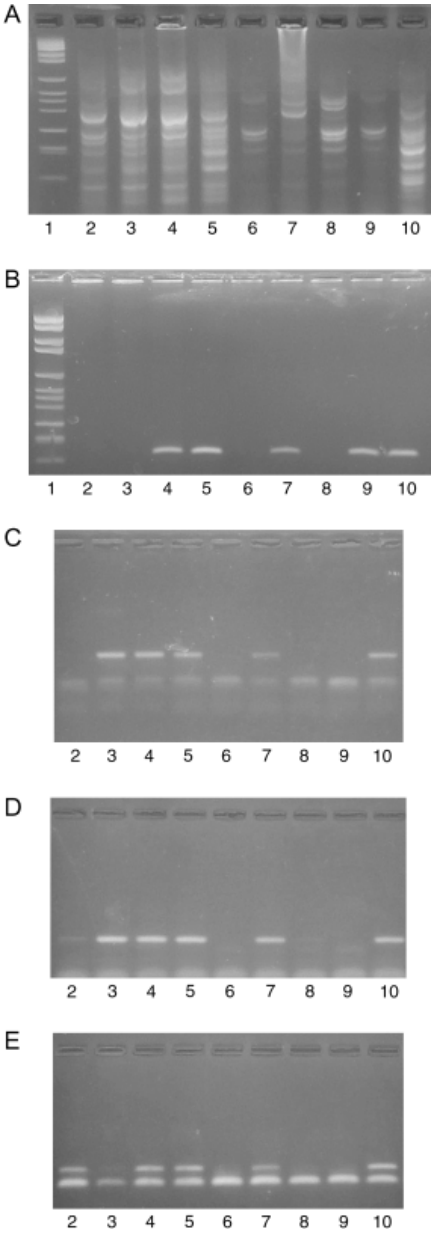


Fig. 4. Isolation and organization of the extended labile genomic region around Musomav2 indicating the positions of the various primers used for amplification (not to scale to facilitate the indication of the primer positions). The region between primers 3 and 4 (filled region) indicates the position of the original difference product Musomav2.

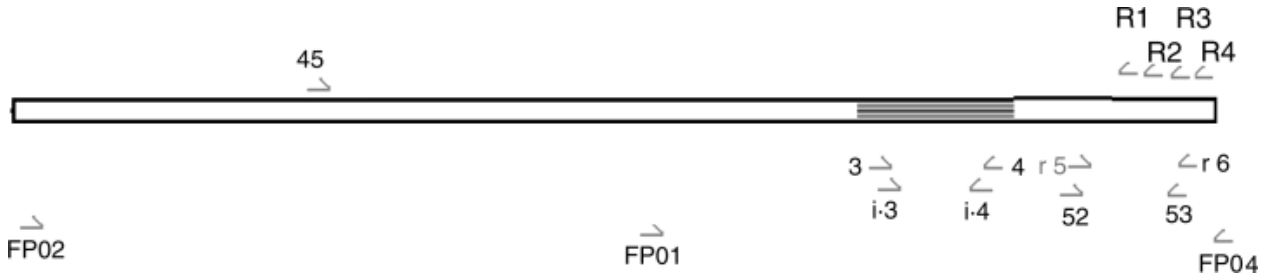


Fig. 5. PCR products from 'Curare enano' normal and dwarf samples using primers 52 and 53 (lanes 2 and 3), 45 and i4 (lanes 4 and 5), 3 and 4 (lanes 6 and 7), i3 and R1 (lanes 8 and 9), i3 and R2 (lanes 10 and 11), i3 and R3 (lanes 12 and 13), i3 and R4 (lanes 14 and 15) (as indicated in Fig. 4). Lane 1, Molecular Weight Marker VI (MWM VI, Roche).

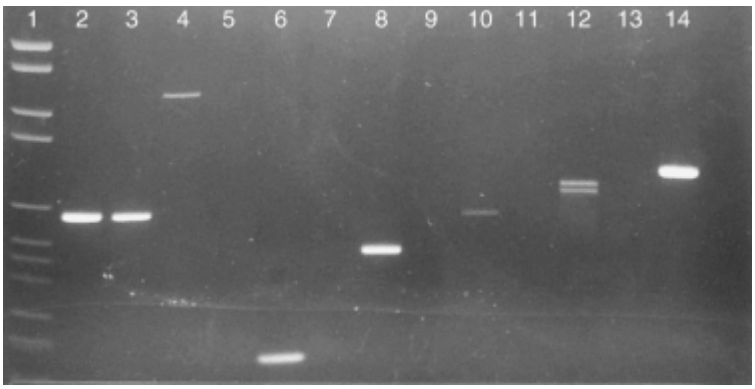


Fig. 6. Pairwise comparisons of the region bounded by primers 4 and 45 from 'Curare enano' normal (N), dwarf (D) and three normal 'Cavendish' individuals (5, 6, 11) from in vitro propagation indicating the positions of the gaps. The alignments were made using Align 2 sequences in BLAST.

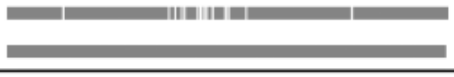
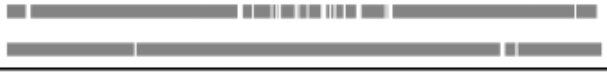
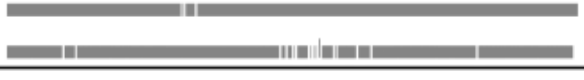
Clone 1 size (bp)	Clone 2 size (bp)	Identity	Gap	Figure - Gaps in clone 1 - Gaps in clone 2
N4 (1355)	D (1333)	1195/1312 (91%)	21/1310	
N (1357)	5 (1371)	1337/1371 (97%)	14/1371	
N (1357)	6 (1369)	1337/1370 (97%)	14/1370	
N (1357)	11 (1358)	1314/1360 (96%)	5/1360	
D (1333)	5 (1371)	1183/1325 (89%)	35/1325	
D (1333)	6 (1369)	1182/1323 (89%)	33/1323	
D (1333)	11 (1358)	1159/1313 (88%)	24/1313	
5 (1371)	6 (1369)	1326/1377 (96%)	14/1377	
5 (1371)	11 (1358)	1301/1374 (94%)	19/1374	
6 (1369)	11 (1358)	1306/1372 (95%)	17/1372	

Fig. 7. Amplification of ‘Curare enano’ normal (N) and dwarf (D) DNAs before (N and D) and after *Hpa*II digestion (NH and DH) or *Msp*I digestion (NM and DM). The nil lane was the PCR with no added DNA. (A) Primers 45 and i4, (B) primers 45 and 4, and (C) primers r5 and r6.

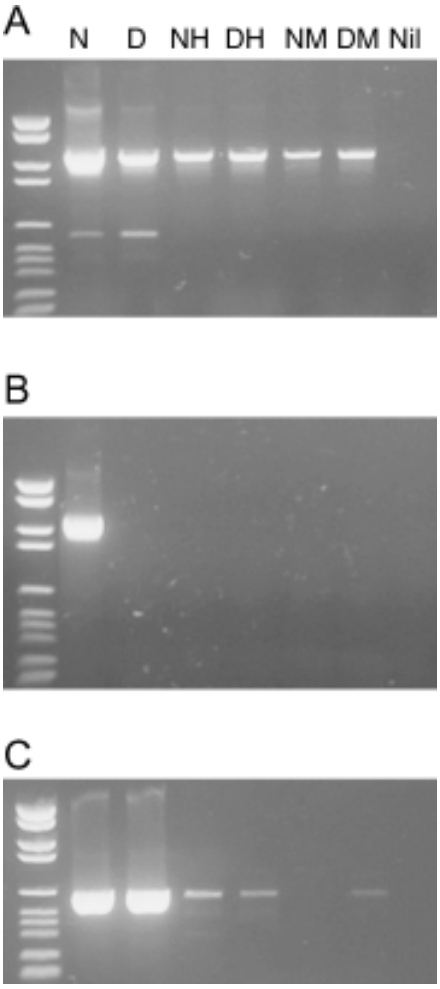


Table 1. Primer sequences from differential products

Source DNA	Primer number	Primer sequence (5' – 3')
Musomav1	1	AGG CAC CAC TGC GGG TCG T
Musomav1	2	TGC TGG GAG TTG TTC GTG GA
Musomav2	3	AGG GAA AGG CAG CTA GAG AAA
Musomav2	4	TTC GCT CGT TGT AGT GAT TTC
Musomav3	5	TCA CGT ACT CTG CTG TGG AAA
Musomav3	6	TGC AAA CTG GGT CCT GAG TT
Musomav4	7	TCA CCT ACG AAA CAA ACC ACA
Musomav4	8	AGA ACG AGA AAA GGG CAG AA
Musomav5	9	TGG CTT TCT CGT GCA GGT A
Musomav5	10	ACT CGC TTG GTA CAC CAG GAT
Musomav6	11	GTC GTC GGG ATC TGC ACC AG
Musomav6	12	AAG CAA TTC GTG GCG GAG GTT