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Genetic Resources

Analysis of a *Musa* spp. Somaclonal Variant Resistant to Yellow Sigatoka

MARÍA DEL CARMEN VIDAL and EVA DE GARCÍA*

Centro de Botánica Tropical, Facultad de Ciencias, Universidad Central de Venezuela Apto 47525, Caracas 1041-A, Venezuela

Abstract. Somaclonal variant CIEN BTA-03 resistant to yellow Sigatoka was obtained from a susceptible banana clone (Williams clone), by increasing the production of adventitious buds using 6-Belcilaminopurine at high concentrations. This somaclone has exhibited yellow Sigatoka resistance in the field for five consecutive years of asexual reproduction. We used RAPD markers to generate characteristic "fingerprints" for each probe, concluding that they are reliable tools for evaluating the genetic variability of *Musa* regenerants obtained by *in vitro* culture.

Key Words: banana clones, *Musa* spp., polymorphisms, RAPD, somaclonal variant, yellow Sigatoka

Abbreviations: RAPD, random amplified polymorphic DNA.

Introduction

Bananas and plantains are giant perennial herbs originated from intra- and interspecific crosses of *Musa acuminata* and *Musa balbisiana*. These crops are of vital importance to hundreds of millions of people in developing countries. Approximately 90% of the total production is used as food for domestic consumption. Sterility is the main handicap to banana breeding. The most important commercial varieties, i.e. the 'Cavendish' group of dessert banana, are entirely male and female sterile (Novak, 1992). The bananas are also susceptible to a wide range of plant pathogens including fungi, bacteria, viruses and nematode. Leaf spot disease is caused by many different fungal pathogens. One of the most relevant diseases is yellow Sigatoka caused by *Mycosphaerella musicola* and this limits their efficient production (Haddad and Meredith, 1977). The production of high-quality dessert bananas requires intensive spraying with costly chemical fungicides to protect against leaf spot diseases. Such treatments can only be applied in large plantations and are seriously criticized because of their increasingly harmful effects on the environment (Sági et al., 1995).

*Author for correspondence. e-mail: dsotelo@cantv.net, fax: 00 (58) 2-3735718, ph: 00 (58) 2-3731547.

Biotechnological methods can contribute significantly to the genetic improvement of the genus *Musa*. Somaclonal variation obtained by tissue culture techniques provides a rapid and reliable approach for plant improvement. In our laboratory, a somaclonal variant, CIEN BTA-03 resistant to yellow Sigatoka, was generated starting from the susceptible triploid clone of the 'Cavendish' group (Williams clone), by increasing the production of adventitious buds using relatively high concentrations of 6-Belcilaminopurine (Trujillo and García, 1996). This somaclone has tested resistant to yellow Sigatoka in the field for five consecutive years of asexual reproduction. The somaclonal variant shows morphological, anatomical and biochemical characteristics different from the Williams parental plant. These are higher length, reddish coloration in the pseudoshoot, quantitative differences in the number of stomata per square cm of the leaf surface, which is lower in both the upper and lower epidermis of the somaclone compared to the parental plant, and this leads to a thicker leaf blade (Hermoso et al., 1995). It is also important that the polyphenol content in the leaf tissue of somaclonal variant CIEN BTA-03 is higher than that of Williams (Noguera et al. 1995).

One difficulty in dealing with somaclonal variation is identification of any genetic variation with the regenerated plants. The RAPD technique, which uses single primers of arbitrary nucleotide sequence, allows random amplification of DNA sequences throughout the entire genome. Because RAPD polymorphisms result from either a nucleotide base change that alters the primer binding site, or from an insertion or deletion within the amplified region (Williams et al., 1993), polymorphisms usually result in the presence or absence of an amplification product from a single locus (Tingey et al., 1993). The products of these amplifications can be polymorphic and are useful as genetic markers (Hu and Quiros, 1991). In this report, we used the RAPD technique to characterize the somaclonal variant CIEN BTA-03 resistant to yellow Sigatoka and compared it with the maternal plant, nonresistant somaclones, and other banana resistant clones.

Materials and Methods

Plant material

Six banana clones were selected that differ in susceptibility to yellow Sigatoka. Three of the clones are susceptible to the disease: the Williams clone of the 'Cavendish' group (donor plant) and two somaclones obtained by culture "in vitro" starting from the Williams clone (susceptible clones S1 and S2). Three of the clones are resistant to the disease: the somaclonal variant CIEN BTA-03 (resistant clone R1), the tetraploid (resistant clone R2) and the Topocho pelipita clones (resistant clone R3). The tetraploid and Topocho pelipita clones are naturally resistant to yellow Sigatoka.

DNA isolation

Plant DNA was extracted using a modified version of Dellaporta et al. (1983), increasing the concentration of β-mercaptoethanol. Quality of genomic DNA was examined by agarose (0.8%) gel electrophoresis, and the concentration and purity of DNA was determined in a Pharmacia Ultrospec III spectrophotometer. A set of

standard DNA stock solutions were prepared for PCR at a final concentration of 150 ng/μL and stored at –20ºC.

RAPD assays

DNA samples were isolated from the six banana clones described above. Twelve primers were used in this study. Ten primers were from Operon Technologies Inc.:

OPA Primers	**OPB Primers**
OPA 01 (CAGGCCCTTC)	OPB 01 (GTTTCGCTCC)
OPA 02 (TGCCGAGCTG)	OPB 05 (TGCGCCCTTC)
OPA 04 (AATCGGGCTG)	OPB 06 (TGCTCTGCCC)
OPA 12 (TCGGCGATAG)	OPB 07 (GGTGACGCAG)
OPA 17 (GACCGCTTGT)	
OPA 19 (CAAACGTCGG)	

The two Genosys (Houston, TX) primers used were:

pBS reverse sequence (GGAAACATATGACCATGA) and
Kpn-R (CCAAGTCGACATGGCACRTGTATACATAYGTAAC).

PCR was performed in a volume of 25 μL containing 67 mM tris-HCl (pH 8.8), 16.6 mM $(NH4)_2SO4$, 0.45%(v/v) Triton X-100, 200 μg/mL gelatin, 3 mM $MgCl_2$, 200 μM of each dNTPs, 0.2 μM primer, 150 ng DNA, and 2 units of *Taq* polymerase (Promega). The reaction mixture was overlaid with 60 μL of mineral oil. DNA amplification was performed in a PTC-100 Programmable Thermal Cycler (MJ Research, Inc.). The PCR program consisted of an initial denaturation for 1 min at 94ºC, then 40 cycles of 1 min at 94ºC, 1 min at 36ºC and 2 min at 72ºC. The mixture was stored at 4ºC until use. For each primer, tubes containing all the reaction components except for the DNA template were included as a control to check for contamination. The extracts of DNA were analyzed in a 0.8% agarose gel, using 4 μL of each DNA sample that was separated by electrophoresis. Amplification products were analyzed by electrophoresis in 1.5% agarose gels using tris-borate buffer, stained with ethidium bromide and photographed under UV light using a red filter (Sambrook et al., 1989). λ Phage DNA double digested with *Hin*d III and *Eco*R I endonucleases was used as a marker. Duplicate reactions were routinely performed to ensure reproducibility.

Results and Discussion

We have tested sensitivity of the RAPD technique for detecting polymorphism among the somaclonal variant CIEN BTA-03 (resistant to Yellow Sigatoka), the parental plant (Williams), two susceptible somaclones obtained by clonal propagation of Williams, and two naturally resistant clones to the disease. High molecular weight genomic DNAs were isolated from the leaf material following a modification of the method of Dellaporta et al. (1983). High levels of oxidation were obtained when using the recommended level of β-mercaptoethanol. With an increase to 1% β-mercaptoethanol, it was possible to fully control the oxidation of the extracts. The relation Å 260/280 oscillated around the 1.8 value, which

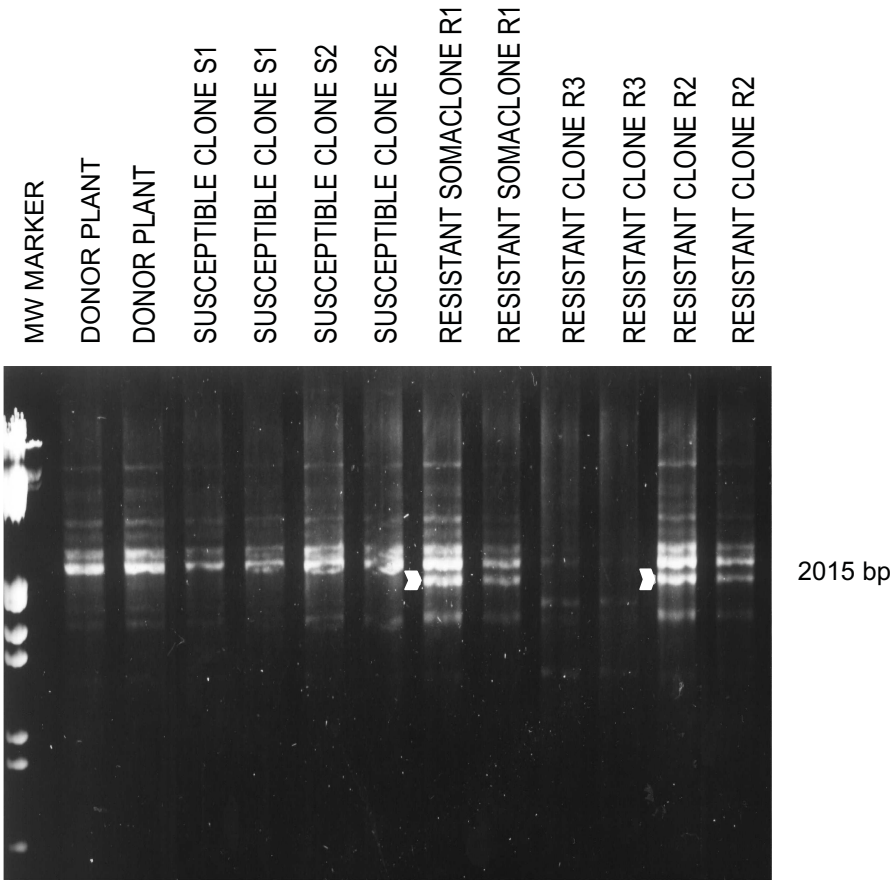

Figure 1. RAPD analysis of polymorphisms of banana donor plant and its somaclones using primer pBS reverse sequence. Electrophoresis of amplification products was in 1.5% agarose gel using tris-borate buffer. The molecular weight marker was from bacteriophage λ DNA that was double-digested with *Hin*d III and *Eco*R I. Arrows indicate polymorphic DNA bands.

indicates low protein contamination in the DNA preparations. DNA analysis of the samples showed little degradation during extraction, and that the molecular size of all DNA samples was approximately 21000 bp (results not shown). This proved that, using this extraction process, DNA purity is acceptable for PCR amplification.

In order to test the applicability of RAPD for generating molecular markers in banana, 12 different primers were used in the six DNA samples. Amplification products were obtained with the 12 primers tested. Not all of the amplification products were able to genetically differentiate the banana clones (results not shown). Both Genosys primers produced polymorphism in RAPD patterns among the analyzed DNA probes. Of the ten Operon primers only two, OPA-04 and OPB-01, were useful for differentiating the banana clones.

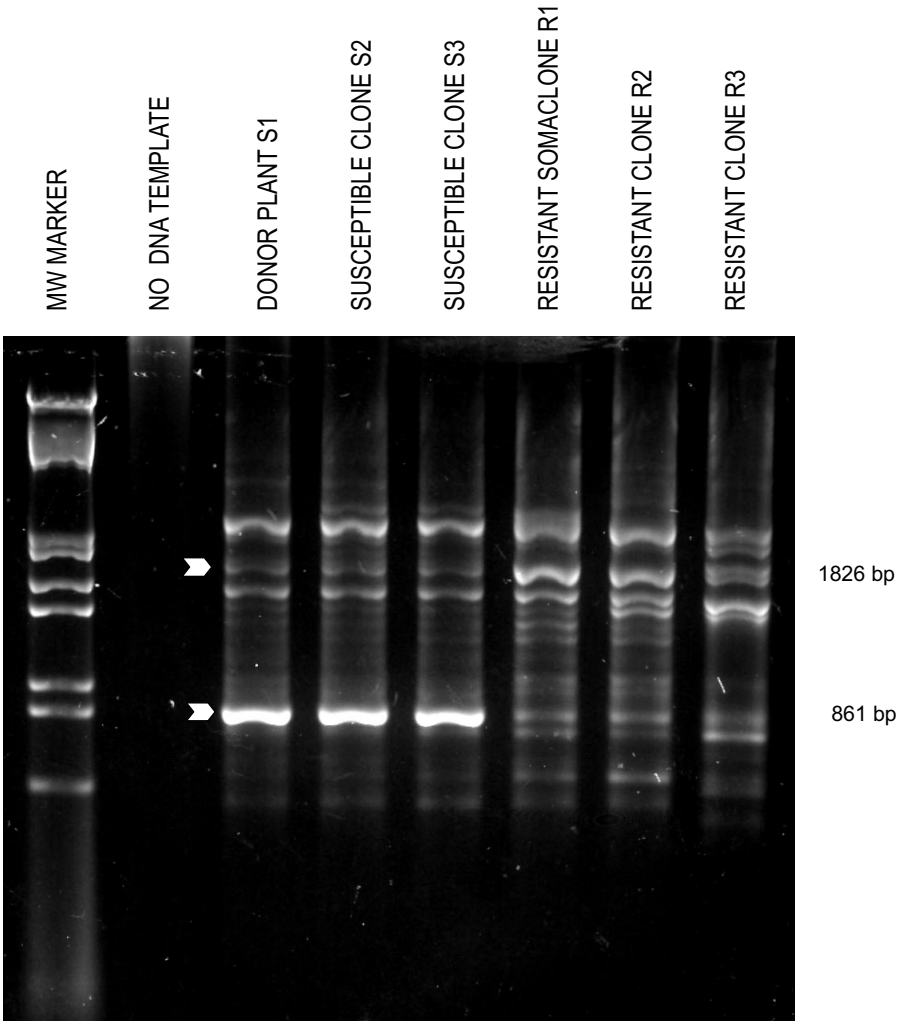

Figure 2. RAPD analysis of polymorphisms of banana donor plant and its somaclones using primer Kpn-R. Experimental conditions as in Figure 1.

The RAPD patterns of primer pBS rev. sec. (Figure 1) exhibited a 2,015 bp band in two clones resistant to yellow Sigatoka: Somaclonal variant CIEN BTA-03 (R1) and the Topocho pelipita clone (R3). Amplification patterns are shown twice in order to evaluate if the RAPD patterns can be reproduced. The results indicate that highly reproducible RAPD patterns can be obtained when applying this DNA extraction protocol to banana leaves and using the established amplification and incubation conditions.

With Kpn-R primer (Figure 2), a density polymorphism was obtained that can be used to differentiate the banana clones susceptible and resistant to yellow Sigatoka. The 861 bp band is more intense in the original plant and susceptible

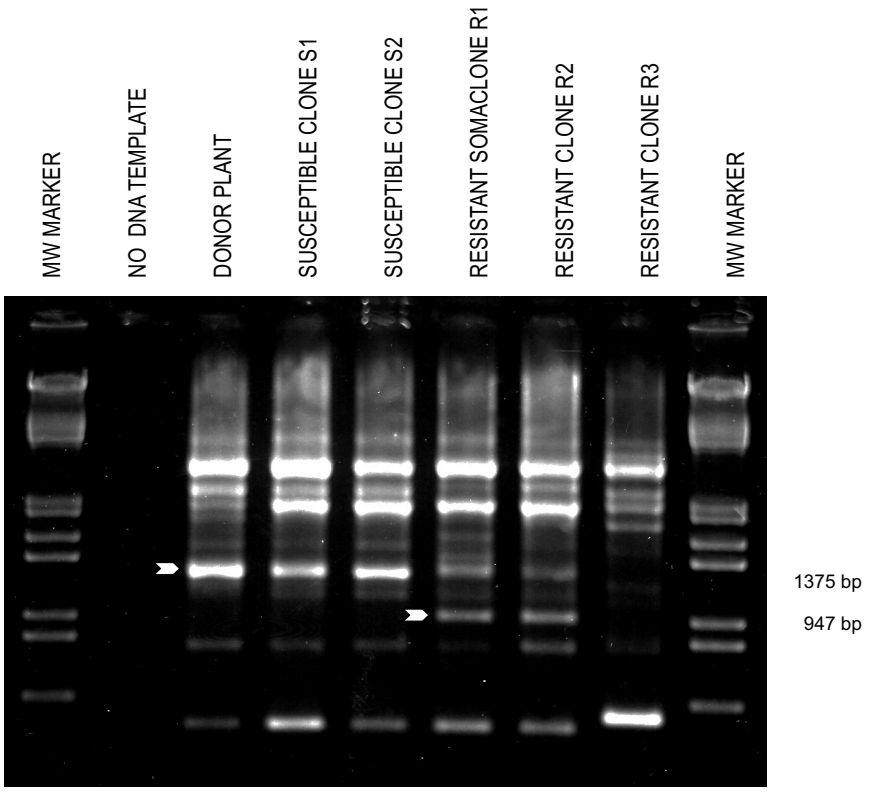

Figure 3. RAPD analysis of polymorphisms of banana donor plant and its somaclones using primer OPB-01. Experimental conditions as in Figure 1.

somaclones. Also, another polymorphism was apparent, the 1,826 bp band is more intense in the somaclonal variant CIEN BTA-03 (R1) and in the tetraploid clone (R2).

Amplification products from OPB-01 (Figure 3) revealed a 947 bp band that can be used to characterize two resistant clones: Somaclonal variant CIEN BTA-03 (R1) and the tetraploid clone (R2). We also noted a density polymorphism that characterized two susceptible clones (the 1,375 bp band is more intense in the original plant and its somaclones).

The RAPD patterns obtained with OPA-04 (Figure 4) showed 764 bp bands in the three clones susceptible to yellow Sigatoka. The somaclonal variant CIEN BTA-03 (R1) can be identified by the presence of a 591 bp band. There is also a density polymorphism: the 1,053 and 1,020 bp bands are more intense in somaclonal variant CIEN BTA-03.

Successful use of RAPD markers requires knowledge of the factors that influence PCR amplification of DNA. Williams et al. (1993) pointed out that the G+C content of the primer and its length highly influences the amplification products obtained by RAPD analysis. In our experiments, we found that a 10 bp

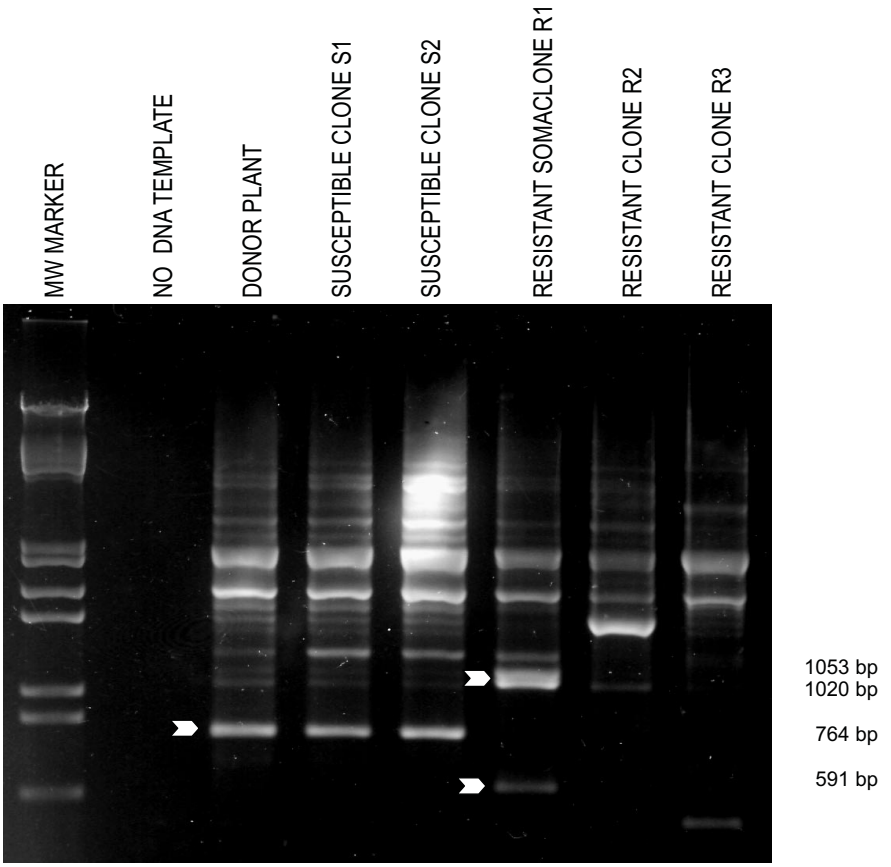

Figure 4. RAPD analysis of polymorphisms of banana donor plant and its somaclones using primer OPA-04. Experimental conditions as in Figure 1.

primer with a G+C content of 60 or 70%, as well as longer primers with a 40% G+C content (pBS rev. sec. and Kpn-R) allow amplification of different *Musa* clones and permit polymorphic RAPD patterns. Welsh and McClelland (1990), using pBS rev. sec., obtained RAPD polymorphisms and were able to distinguish three rice variants. However, Oropeza et al. (1995) were only able to obtain amplification products using 10 bp primers with a G+C content of 60 or 70%. Genosys primers (pBS rev. sec. and Kpn-R) did not develop RAPD patterns, even with different annealing temperatures in the PCR reaction.

Somaclonal variant CIEN BTA-03 is resistant to yellow Sigatoka. It also shows morphological and anatomic changes that persist during clonal propogation, which indicates a genetic basis for these changes. RAPD polymorphisms originate from DNA base changes, base substitutions, and deletions or insertions occuring within a primer binding site. In our work, RAPD analysis using arbitrary oligonucleotide primers efficiently differentiated banana clones by their genetic changes due to somaclonal variation. The level of polymorphism between the clones we tested indicates that it should be possible to use a small number of

appropriated primers to differentiate between clones of banana. In a few cases, RAPD technology has been used to analyse plants regenerated from *in vitro* cultures either for the detection of somaclonal variants or analysis of the genetic stability. RAPD technology has been used to identify the level polymorphisms of somaclonal variants of *Triticum* spp. (Brown, 1991 and Brown et al., 1993), *Saccharum* spp. (Oropeza et al., 1995; Taylor et al., 1995a and Taylor et al., 1995b), *Picea glauca* (Isabel et al., 1995), sugar beet (Munthali et al., 1996), and *Asparagus officinalis* L. (Dan and Stephens, 1997). Also, once an elite clone with desirable properties has been isolated, it is important to monitor its identity and genetic stability during micropropagation. RAPD technology has also been used to analyze genetic stability of micropropagated clones of *Festuca pratensis* (Vallés et al., 1993) and *Achillea* spp. (Wallner et al., 1996).

We have used RAPD markers to generate a characteristic "fingerprint" for each probe and conclude that it is a reliable tool for evaluating genetic variability in the *Musa* regenerants obtained by "in vitro" culture. RAPD is also useful for distinguishing the different genotypes of *Musa* under study. Our results show that the induction of adventitious buds using high concentration of cytokinin can produce a genetic variation among regenerants that can easily be detected by RAPD markers.

The marker bands found in the resistant clones can be used to study the molecular basis for resistance in clone CIEN BTA-03 to yellow Sigatoka. For example, OPB-01 shows a 947 bp band in somaclonal variant CIEN BTA-03 and in the tetraploid clone. Also, pBS rev. sec. shows a 2,015 bp band in both clones. Sequencing of these bands can help to establish the mechanism responsible for the resistance to yellow Sigatoka in this somaclonal variant.

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