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Genetic Analysis of Somatic Embryogenesis Derived Plants in Banana

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Abstract

Regenerated plants derived from somatic embryogenesis, using immature male flower technique of two banana Cavendish cultivars namely 'Grand Naine' and 'Williams', were used for somaclonal variation analysis. Thirty primer combinations of the amplified fragment length polymorphism marker system (AFLP) were used. A total of 1293 and 1302 bands were generated, of which 1275 (98.6%) and 1281 (98.4%) were monomorphic, and 18 (1.4%) and 21 (1.6%) bands were polymorphic in 'Grand Naine' and 'Williams', respectively. Both cluster analysis of unweighted pair-grouping method with arithmetic averages (UPGMA) and principal coordinate (PCO) analysis separated the two cultivars, and grouped each cultivar with its regenerated plants. AFLP analysis showed 8 and 16 bands specific to the regenerated plants of 'Grand Naine' and 'Williams', respectively, these were absent in their parents, in addition, we found evidence of the absence of 10 and 5 bands in the regenerated plants, respectively, which were presented exclusively in their parental plants. Such specific bands presented in the regenerated plants could be useful for further investigation on the genetic identification of somaclonal variation in banana. On the other hand, regenerated plants of both cultivars were transferred to the field; no gross phenotypic alteration has been detected until the beginning of the flowering period. However, further field evaluation of individual plants is required for the observation of possible phenotypic somaclonal variants that may show useful characters, e.g. resistance to biotic or abiotic stress as well as high yield and fruit quality.

Keywords: *Genetic variation, somatic embryogenesis, molecular marker, banana.*

Abbreviations: AFLP, amplified fragment length polymorphism; PCO, principal coordinate analysis; UPMGA, unweighted pair-grouping method with

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arithmetic averages.

Introduction:

Plants regenerated from somatic cell cultures may exhibit variation which may have a genetic or non-genetic basis. Genetic variation that occurs through plant tissue culture has been termed as 'somaclonal variation' (Larkin and Scowcroft, 1981). For obtaining true to type plants from a selected genotype, somaclonal variation is undesirable. On the other hand, somaclonal variation offers prospects for the recovery of useful mutants in tissue culture and for genetic improvement of banana (Sahijram *et al*, 2003).

Somaclonal variants derived from banana and plantain microprpoagation with different types of plant morphology and genetic variation have been widely reported (Sahijram *et al*, 2003; Giménez *et al*, 2005; Bairu et *al*, 2006; Mohamed, 2007 and Sheidai et *al*, 2008). Unlikely, few studies have been published on the occurrence of off-types banana plants produced through somatic embryogenesis (Strosse *et al*, 2003). In this regard, Côte *et al.*, (2000a) found that, a number of plants derived from somatic embryogenesis, in 'Grand Naine', were true to type and have agronomic characteristics comparable to *in vitro* plantlets derived via micropropagation. Similar findings were obtained with 'IRFA903' plants derived from seven months old cell suspensions (Côte *et al*, 2000b). Contrary to these results, Shchukin *et al*, (1997) found that 3.6% of somatic embryogenesis-derived regenerants of 'Grand Naine' were off-types.

Several molecular markers have been used for detecting somaclonal variation at the molecular level in banana and plantains including; randomly amplified polymorphic DNA (Bairu *et al*, 2006; Mohamed, 2007; Sheidai et *al*, 2008 and Vidal and Garcia, 2000), inter simple sequence repeats (Lakshmanan *et al*, 2007), sequence characterized amplified region (Suprasanna *et al*, 2008), representational difference analysis (Oh *et al*, 2007), selective amplification of microsatellite polymorphic loci (Giménez *et al*, 2005), amplified fragment length polymorphism (AFLP) and methylation-sensitive amplification polymorphism (Engelborghs *et al*, 1998; James *et al*, 2004; Engelborghs *et al*, 2004 and Bhatia *et al*, 2005).

The objective of this part of the present study was to detect the extent, if any, of somaclonal variation in plants derived from somatic embryogenesis, using immature male flowers method, of two banana cultivars namely 'Grand Naine' and 'Williams' (*Musa acuminata* Colla, AAA), using AFLP marker system.

Materials and methods:

Plant materials:

Plants were derived via somatic embryogenesis using the immature male flowers from the two Cavendish cultivars 'Grand Naine' and 'Williams' (Youssef *et al*, 2010a). Young cigar leaves from; 50 randomly selected regenerated plants and 20 mother plants from each cultivar were collected from the Instituto Nacional de Investigacion Forestales, Agricolas y Pecuaries (INIFAP) experimental research farm at Uxmal, Yucatán, México (Lat. 20° 24` 40.10`` N, Long. 89° 45` 24.90`` E, 8m altitude above sea level). Tissues were disinfected for one minute each step, with sodium hypochlorite 6% (v/v) and ethanol 70% (v/v), rinsed with distilled water, and excess of water removed with paper towel. Subsequently, leaf samples (100 mg) were weighted, wrapped in aluminium foil and frozen using liquid nitrogen and stored at -80°C until their use.

DNA extraction:

Total genomic DNA from all samples under study was extracted according to Dellaporta *et al.,* (1983) with some modifications. DNA concentration was determined using a spectrophotometer according to Stulnig and Amberger, (1994).

AFLP analysis:

AFLP analysis was performed according to Vos *et al.*, (1995) with some modifications. Two DNA-bulk samples from 20 mother plants of each Williams and Grand Naine genotypes, and two DNA-bulk samples from 50 regenerated plants from each cultivar, were used. Each bulk was made by mixing constant concentration of DNA from a mother or regenerated plants. Two hundred and fifty nano-grams of DNA from each bulk was digested with the restriction enzymes; *Eco*-R1 and *Mse*-1 (Invitrogen), followed by adaptor ligation using DNA-Ligase (Invitrogen) to generate template DNA for pre-amplification. PCR pre-amplification was carried out using AFLP primers each having one nucleotide. The PCR pre-amplified products were diluted to 1:25 in H_2O and used as templates for AFLP selective amplification using two AFLP primers each containing three selective nucleotides. Thirty primer combinations were used to detect the somaclonal variation in this study (Table 1). The final AFLP-PCR products were separated on a 6% sequencing gel (urea-PAGE) and visualised by staining with silver nitrate according to Bassam *et al.*, (1991) with some modifications.

Data analysis:

The polymorphic bands were scored independently as being either present (1) or absent (0) in each cultivar parental plants-bulk and regenerated plants-bulk. Only strong, reproducible and clearly distinguished bands were used for the analysis. A binary data matrix indicating the presence (1) or the absence (0) of bands was made from AFLP profiles. The percentage of polymorphism was calculated by dividing the number of polymorphic bands with the total number of regenerated bands.

Table (1): Sequences of thirty AFLP primer combinations

No.	Code	Sequence (5`-3`)	No.	Code	Sequence (5`-3`)
1	Eco-1 Mse-1	GACTGCGTACCAATTCAAC GATGAGTCCTGAGTAACAA	16	Eco-4 Mse-1	GACTGCGTACCAATTCACG GATGAGTCCTGAGTAACAA
2	Eco-1 Mse-3	GACTGCGTACCAATTCAAC GATGAGTCCTGAGTAACAG	17	Eco-4 Mse-3	GACTGCGTACCAATTCACG GATGAGTCCTGAGTAACAG
3	Eco-1 Mse-15	GACTGCGTACCAATTCAAC GATGAGTCCTGAGTAACTC	18	Eco-4 Mse-15	GACTGCGTACCAATTCACG GATGAGTCCTGAGTAACTC
4	Eco-1 Mse-16	GACTGCGTACCAATTCAAC GATGAGTCCTGAGTAACTT	19	Eco-4 Mse-16	GACTGCGTACCAATTCACG GATGAGTCCTGAGTAACTT
5	Eco-1 Mse-14	GACTGCGTACCAATTCAAC GATGAGTCCTGAGTAACTG	20	Eco-4 Mse-14	GACTGCGTACCAATTCACG GATGAGTCCTGAGTAACTG
6	Eco-2 Mse-1	GACTGCGTACCAATTCAAG GATGAGTCCTGAGTAACAA	21	Eco-5 Mse-1	GACTGCGTACCAATTCACT GATGAGTCCTGAGTAACAA
7	Eco-2 Mse-3	GACTGCGTACCAATTCAAG GATGAGTCCTGAGTAACAG	22	Eco-5 Mse-3	GACTGCGTACCAATTCACT GATGAGTCCTGAGTAACAG
8	Eco-2 Mse-15	GACTGCGTACCAATTCAAG GATGAGTCCTGAGTAACTC	23	Eco-5 Mse-15	GACTGCGTACCAATTCACT GATGAGTCCTGAGTAACTC
9	Eco-2 Mse-16	GACTGCGTACCAATTCAAG GATGAGTCCTGAGTAACTT	24	Eco-5 Mse-16	GACTGCGTACCAATTCACT GATGAGTCCTGAGTAACTT
10	Eco-2 Mse-14	GACTGCGTACCAATTCAAG GATGAGTCCTGAGTAACTG	25	Eco-5 Mse-14	GACTGCGTACCAATTCACT GATGAGTCCTGAGTAACTG
11	Eco-3 Mse-1	GACTGCGTACCAATTCACA GATGAGTCCTGAGTAACAA	26	Eco-8 Mse-1	GACTGCGTACCAATTCACC GATGAGTCCTGAGTAACAA
12	Eco-3 Mse-3	GACTGCGTACCAATTCACA GATGAGTCCTGAGTAACAG	27	Eco-8 Mse-3	GACTGCGTACCAATTCACC GATGAGTCCTGAGTAACAG
13	Eco-3 Mse-15	GACTGCGTACCAATTCACA GATGAGTCCTGAGTAACTC	28	Eco-8 Mse-15	GACTGCGTACCAATTCACC GATGAGTCCTGAGTAACTC
14	Eco-3 Mse-16	GACTGCGTACCAATTCACA GATGAGTCCTGAGTAACTT	29	Eco-8 Mse-16	GACTGCGTACCAATTCACC GATGAGTCCTGAGTAACTT
15	Eco-3 Mse-14	GACTGCGTACCAATTCACA GATGAGTCCTGAGTAACTG	30	Eco-8 Mse-14	GACTGCGTACCAATTCACC GATGAGTCCTGAGTAACTG

The software NTSYSpc ver. 2.20s (Applied Biostatistics Inc.) was used to calculate the genetic similarities using Jaccard's coefficient (Jaccard, 1908) of similarity. Cluster analysis was carried out on similarity estimates using the unweighted pair-group method with arithmetic averages (UPGMA). Genetic distances were calculated as [(1- Jaccard's similarity) x 100]. One thousand repetition counts were used to generate the bootstrapping using Free Tree program. A 3D Scatter plot of the principal coordinate analysis (PCO) was also carried out, using NTSYSpc program.

Results:

Regenerated plants from somatic embryogenesis, using immature male flowers method, of two banana cultivars namely 'Grand Naine' and 'Williams' were used in this study to detect the somaclonal variation using AFLP molecular marker technique. AFLP primer sets (Table 1) generated a range of 31 to 62 bands with an average of 46 bands per primer combination. A total of 1293 and 1302 bands were scored for Grand Naine and Williams, respectively (Table 2).

Out of the 30 AFLP primer sets used in this study, 14 primers (46.7%) showed polymorphism, of which 8 primers (i.e. 2, 5, 8, 12, 14, 18, 20, 27) were polymorphic for Grand Naine and generated 18 (1.39%) polymorphic bands, while 12 primers (i.e. 2, 3, 4, 6, 8, 12, 13, 14, 16, 18, 20, 22) were polymorphic for Williams, and generated 21 (1.61%) polymorphic bands (Table 2). The number of polymorphic bands ranged from 1 to 5 and 1 to 3 bands in Grand Naine and Williams, and the percentage of polymorphism ranged from 1.89 to 10.20% and 1.72 to 8.57%, respectively (Table 2).

In addition, regenerated plants of Grand Naine and Williams represented 8 and 16 additive bands, respectively, which were specific and not found in their parents' bulk profile, meanwhile 10 and 5 bands, existed only in the parents' bulk profile (Table 2 and Fig. 1). The maximum number of specific bands for regenerated plants of Grand Naine and Williams was five and three, respectively. These were generated by Eco-ACA/Mse-CTT, and by both of Eco-ACC/Mse-CTT and Eco-AAG/Mse-CAA primer combinations, respectively (Table 2).

On the other hand, the UPGMA dendrogram showed the relationship between each donor parent and its regenerated plants, in which the similarity between the parents and their regenerated plants were 98.6 and 98.4% , with genetic distance of 1.4 and 1.6% in Grand Naine and Williams, respectively (Fig. 2 and Table 3). Moreover, scatter plot of PCO analysis demonstrated the association of these parents with their regenerated plants, in which the two cultivars were separated from each other and each cultivar was placed near to its regenerated plants (Fig. 3).

Regenerated plants of the two cultivars were transferred to the field for phenotypic evaluation and comparison to their parental plants. No gross phenotypic alteration, e.g. mosaics, variegation, dropping leaves and dwarfs, etc., has been detected, in both cultivars, until the beginning of the flowering period.

Table (2): Levels of polymorphism between parents and their regenerated plants, and a survey of unique bands in two banana cultivars as revealed by AFLP analysis

Primers[a]		Grand Naine				Williams			
		TNB	NPB	+/- REG	%P	TNB	NPB	+/- REG	%P
1	Eco-1/Mse-1	41	0		0.00	42	0		0.00
2	Eco-1/Mse-3	52	1	-1	1.92	52	1	-1	1.92
3	Eco-1/Mse-15	57	0		0.00	58	1	+1	1.72
4	Eco-1/Mse-16	31	0		0.00	35	3	+3	8.57
5	Eco-1/Mse-14	56	3	+1, -2	5.36	58	0		0.00
6	Eco-2/Mse-1	44	0		0.00	44	3	+3	6.82
7	Eco-2/Mse-3	45	0		0.00	45	0		0.00
8	Eco-2/Mse-15	42	2	-2	4.76	42	1	+1	2.38
9	Eco-2/Mse-16	40	0		0.00	39	0		0.00
10	Eco-2/Mse-14	38	0		0.00	38	0		0.00
11	Eco-3/Mse-1	43	0		0.00	43	0		0.00
12	Eco-3/Mse-3	46	3	+2, -1	6.52	46	1	+1	2.17
13	Eco-3/Mse-15	44	0		0.00	45	1	+1	2.22
14	Eco-3/Mse-16	49	5	+5	10.20	49	2	+2	4.08
15	Eco-3/Mse-14	31	0		0.00	31	0		0.00
16	Eco-4/Mse-1	38	0		0.00	37	3	+1, -2	8.11
17	Eco-4/Mse-3	45	0		0.00	47	0		0.00
18	Eco-4/Mse-15	32	2	-2	6.25	31	1	-1	3.23
19	Eco-4/Mse-16	62	0		0.00	62	0		0.00
20	Eco-4/Mse-14	53	1	-1	1.89	52	3	+2, -1	5.77
21	Eco-5/Mse-1	50	0		0.00	50	0		0.00
22	Eco-5/Mse-3	35	0		0.00	36	1	+1	2.78
23	Eco-5/Mse-15	45	0		0.00	45	0		0.00
24	Eco-5/Mse-16	38	0		0.00	38	0		0.00
25	Eco-5/Mse-14	50	0		0.00	51	0		0.00
26	Eco-8/Mse-1	40	0		0.00	40	0		0.00
27	Eco-8/Mse-3	39	1	-1	2.56	39	0		0.00
28	Eco-8/Mse-15	37	0		0.00	37	0		0.00
29	Eco-8/Mse-16	34	0		0.00	34	0		0.00
30	Eco-8/Mse-14	36	0		0.00	36	0		0.00
Total		1293	18	+8, -10	1.39	1302	21	+16, -5	1.61

[a] Primer numbers relate to table 4.1, **TNB**: total number of bands, **NPB**: number of polymorphic bands, **+/-REG**: addition or missing of specific bands in regenerated plants, **%P**: percentage of polymorphism.

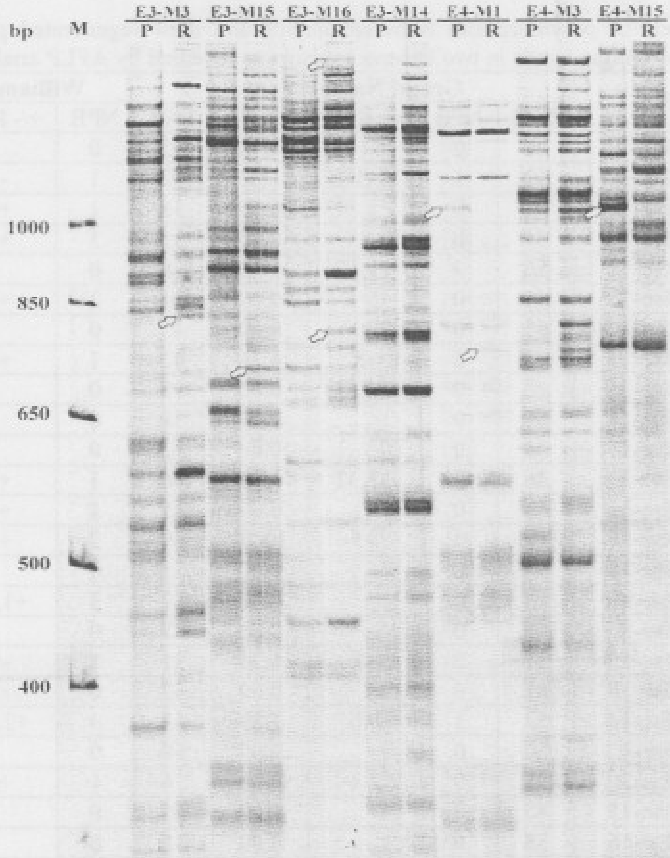

Fig. 1: Unique bands (*arrows*), specific for Williams parental plants (P) and their regenerated plants (R), generated by some AFLP primer combinations.

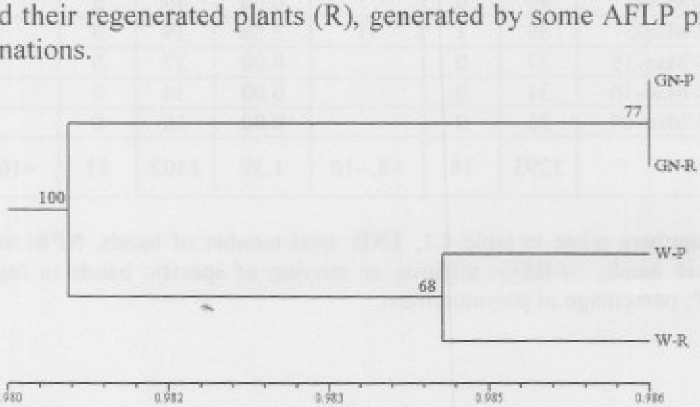

Fig. 2: AFLP-UPGMA dendrogram of the donor parents of Grand Naine (GN-P) and Williams (W-P) and their regenerated plants (GN-R and W-R) based on the data of all tested primers, *numbers* indicate the bootstrapping.

Table (3): Jaccard's similarity coefficient matrix (*above diagonal*) and genetic distance (*below diagonal*) between the donor parents and their regenerated plants calculated form data of all tested primers

	GE-P	GE-R	W-P	W-R
GE-P	-	98.6	98.4	98.0
GE-R	1.4	-	97.8	98.1
W-P	1.6	2.2	-	98.4
W-R	2.0	1.9	1.6	-

GE-P: Grand Enain Parental plants, GE-R: Grand Enain Regenerated plants, W-P: Williams Parental plants, W-R: Williams Regenerated plants.

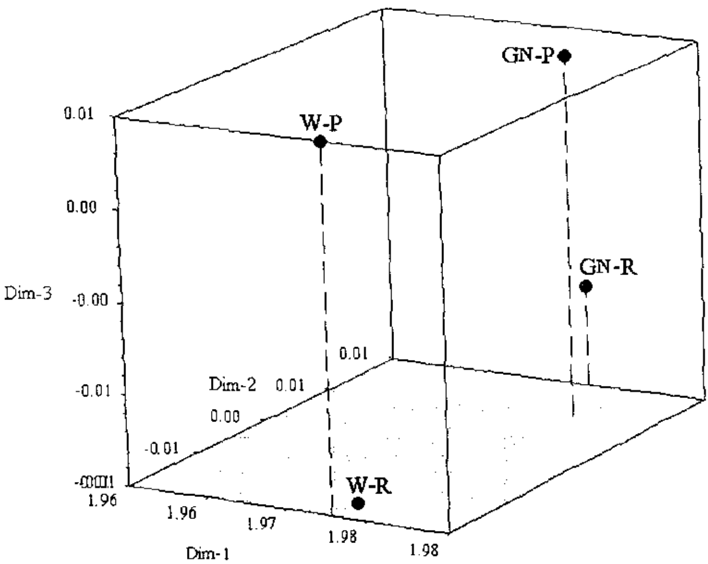

Fig. 3: Scatter plot showing the relationships amongst banana donor parents of Grand Naine (GN-P) and Williams (W-P) and their regenerated plants (GN-R and W-R) based on principal coordinate analysis using AFLP.

Discussion:

Somaclonal variation has been classified to either that which arises from pre-existing variation in the explant or the variation which may be induced by tissue culture conditions (Skirvin *et al*, 1994). Additionally, there are several factors affecting somaclonal variation, including; genotype, explant source, *in vitro* period, number of subcultures and cultivation conditions in which the culture is established (Bordallo *et al,* 2004). Variation may occur in chromosomes (structure or number), DNA rearrangement, or point mutations.

In this study, plants derived from somatic embryogenesis were used for detection of somaclonal variation. Only a small number of studies have been published on the occurrence of off-types banana plants produced through somatic embryogenesis in comparison to other tissue culture techniques (Strosse *et al,* 2003). In this regard, Shchukin *et al.,* (1997) found that, the rate of somaclonal variation in plants produced via somatic embryogenesis was less than that in shoot-tip-propagated plants, using 'Grand Naine'. Furthermore, Cabrera-García *et al.,* (2009) evaluated Cavendish plants regenerated from proliferating inflorescence-derived embryogenic suspension cultures, and reported that no off-types were observed among the embryogenesis-derived plants during either the *in vitro* phase or the acclimatization period in the nursery.

The percentage of polymorphism detected between regenerated plants and their parents, in this study, was significantly less than previous reports. For example, Bairu *et al.,* (2006) reported 55% of RAPD-polymorphism in Cavendish banana plants derived from the tenth subculture of micropropagation. While, Mohamed, (2007) found that the polymorphism percentage in plants of sixth subculture of Williams, using RAPD markers, ranged from 9.1 to 100%. In addition, Sheidai *et al.,* (2008) analyzed the somaclonal variation in the first, third, fifth, seventh and ninth subcultures of meristem tip cultures of *M. acuminata* and they found in total 51.40% of polymorphism detected by RAPD. On the other hand, our results here are different from those of Abu Harrirah and Khalid, (2006) who used male inflorescences of *M. acuminata* cv. Berangan (AAA) for direct regeneration (*via* organogenesis) and found no genetic variations among regenerated plants in comparison to their mother plants, using RAPD molecular markers. This difference could be attributed to the use of different growth regulators, since they have used Benzylaminopurine for direct regeneration and we used 2, 4-dichlorophenoxyacetic acid for embryogenic callus induction.

Although somaclonal variation is undesirable in the context of micropropagation, but it can be used to get an advantage for genetic improvement of banana (Sahijram *et al,* 2003). In this regard, several useful somaclonal variants for various attributes have been identified, e.g. TC1-229, semi-dwarf and resistant to *Fusarium* wilt, derived from Cavendish banana (Tang *et al,* 2000), Tai-Chiao No.1 and Formosana, which are reported to be tolerant to *Fusarium* wilt Tropical Race 1V, and were derived from Giant Cavendish (Hwang, 2002). However, the majority of somaclonal variants are undesirable such as the mosaic type heterogeneity in Cavendish banana (Reuveni and Israeli, 1990).

Our AFLP results showed the presence and absence of unique bands specific for parental plants as well as regenerated plants in each cultivar. The presence of these specific bands in the parental plants and loss of them in the regenerated plants indicates the loss or alteration of certain loci during tissue culture due to somaclonal variation, while the occurrence of specific bands in the regenerated plants and their absence in mother plants may indicate the occurrence of genetic changes leading to formation of new binding sites in these plants (Sheidai *et al*, 2008).

When there is no observation of phenotypic alteration in plants derived through tissue culture, genetic variation could be detected by molecular markers (Rani *et al*, 1995 and Youssef *et al*, 2010b). Our results can be compared with those of Côte *et al.*, (2000a,b) who found that, in 'Grand Naine' and 'IRFA903', a number of plants derived from four or seven months old embryogenic cell suspensions, respectively, were observed phenotypically to be true to type and have agronomic characteristics comparable to *in vitro* plantlets. However, our AFLP analysis was successful in detecting the genetic variation between regenerated plants and their mother plants. This phenomenon could be present because these genetic variations may occur in noncoding regions in the genome. Additionally, since the banana cultivars used in this study are triploid 'AAA' and have three copies of each chromosome, the genetic alteration might be redundant.

In conclusion, the AFLP marker technique was shown to be a good tool for detection of genetic variation in somatic embryogenesis-derived plants. However, further field evaluation of individual plants is required for the observation of possible phenotypic somaclonal variants that may show useful characters, e.g. resistance to biotic or abiotic stress as well as high yield production and fruit quality. Furthermore, such specific bands presented in the regenerated plants may be of importance in understanding the genetic basis of somaclonal variation in *Musa*.

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التحليل الوراثى لنباتات الموز الناتجة من زراعة الأجنة الخضرية

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تم فى هذا البحث استخدام نباتات ناتجة من زراعة الأنسجة باستخدام الأجنة الخضرية في صنفين من الموز الكافنديش (جراند ناين وويليامز) وذلك للكشف عن الإختلافات الوراثية الناتجة من زراعة الأنسجة. تم استخدام ثلاثين توليفة بادئ خاصة بالواسم الجزيئي AFLP وأظهرت النتائج تكوّن عدد 1293 حزمة و1302 حزمة كان من بينها 1275 (بنسبة 98.6٪) و 1281 حزمة (بنسبة 98.4٪) متماثلة بينما تكوّن عدد 18 حزمة (بنسبة 1.4٪) و21 حزمة (بنسبة 1.6٪) مختلفة في الصنفين جراند ناين وويليامز بالترتيب. وأظهر كلا من التحليل العنقودي UPGMA والتحليل المحوري الرئيسي PCO إنفصال الصنفين المستخدمين عن بعضهما وربط كل صنف بالنباتات الناتجة منه. كما نتج من تحليل الـ AFLP عدد 8 حزم و 16 حزمة وحيدة وخاصة بالنباتات الناتجة من زراعة الأنسجة فى كلا من جراند ناين وويليامز بالترتيب، بينما غابت هذه الحزم فى النباتات الأم. بالإضافة لذلك، كان هناك غياب لعدد 10 حزم و 5 حزم فى النباتات الناتجة من زراعة الأنسجة بينما ظهرت هذه الحزم فقط في النباتات الأم لكلا الصنفين بالترتيب. ومن الممكن أن تمثل هذه الحزم الوحيدة والخاصة بالنباتات الناتجة من زراعة الأنسجة أهمية فى التعريف الوراثي للإختلافات الوراثية الناتجة من زراعة الأنسجة في الموز. تم نقل النباتات الناتجة من زراعة الأنسجة لكل من الصنفين إلى الحقل بنجاح ولم يتم ملاحظة وجود أي إختلافات في الشكل الظاهري حتى بداية الإزهار. وبالرغم من ذلك، فإنه يلزم عمل تقييم لهذه النباتات بصورة فردية في الحقل لملاحظة أي نبات مختلف والذي من الممكن أن يُظهر صفات مفيدة مثل مقاومة الظروف الحيوية وغير الحيوية أو الإنتاجية العالية وجودة الثمار.

كلمات البحث: الاختلافات الوراثية – تكوين الأجنة الخضرية – الواسمات الجزيئية – الموز.