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Analysis of Two Clonal Lines (Embryogenic and Non-Embryogenic) of *Agave fourcroydes* Using AFLP and MSAP

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Abstract

Somatic embryogenesis is a very efficient way to propagate economically important plants; however, not all genotypes within a species can be propagated using this method, as a combined effect of both genetic and epigenetic mechanisms may be involved in the response. The aim of the present study was to perform a comparative analysis of the genetic differences through amplified fragment length polymorphism (AFLP) and the epigenetic differences through methylation-sensitive amplified polymorphism (MSAP) of two *Agave fourcroydes* clonal lines, one highly embryogenic (K33) and the other non-embryogenic (K7). Genetic and epigenetic variabilities existed within each clonal line; however, the polymorphic profiles from the two marker systems allowed us to clearly distinguish the two clonal lines before somatic embryogenesis induction. During the induction, the changes detected were mainly 1) unmethylated fragments in the initial explants that were methylated during induction (methylation events) and 2) fragments with different methylation states in the initial explant that were unmethylated in some stages of the process (demethylation events). K33 showed greater dynamism in relation to methylation/demethylation events, while K7 presented the methylation events in a more constant range and at higher levels during all process.

Keywords

Agave, Clonal Line, Somatic Embryogenesis, DNA Methylation

1. Introduction

Somatic embryogenesis (SE) is a complex developmental process through which somatic plant cells, under certain *in vitro* culture conditions, present metabolic,

genetic, and epigenetic changes and cellular reprogramming that result in embryo production [1] [2]. This process involves culturing an explant (of appropriate tissue type and genotype) in a medium with a suitable combination of growth regulators [3].

The genotypic influence is a problem for the establishment of culture protocols because only some genotypes within a species are responsive to induction of SE. In addition to this genetically determined capability, variation may also be due to epigenetic changes, particularly in the pattern of chromatin condensation [3].

DNA methylation is an epigenetic mechanism that is crucial for diverse biological processes [4]. The relationship between DNA methylation and SE has been reported as the cause of the tissue specificity of explants [5] [6], the embryogenic capacity of cells in culture [7], changes that take place during cellular differentiation [8], changes in developmental state [9] [10] [11], cell culture aging [12] [13], the expression of embryogenesis-related genes [2] [14] [15] and the fidelity (trueness to type) of plants obtained by SE [13] [16] [17]. However, there are no reports on the effects of methylation and genotypes (embryogenic and non-embryogenic).

Amplified fragment length polymorphism (AFLP) and methylation-sensitive amplified polymorphism (MSAP) have similar experimental procedures; however, AFLP detects restriction site variations, and MSAP detects variations in cytosine methylation at CCGG sites [18]. AFLP is an efficient molecular marker that has been widely used to estimate genetic variability in plants [18] [19] [20] [21], and MSAP has been used as a genome-wide screening method to assess global DNA methylation [22] [23]. In plants, MSAP has been mostly used to identify differences in DNA methylation patterns [24] [25] [26] [27] [28] but has also been used to identify epigenetic variation in populations [29] [30] [31].

Agaves are plants used in the production of a wide variety of products, including liquors, fibers, cellulose and inulin [32]. Genotype-dependent responses have been observed in the SE of *A. fourcroydes*, where induction from stems of plantlets cultured *in vitro* has shown that the embryogenic capacity varies [33].

The current study aimed to compare two molecular marker systems, AFLP and MSAP, using two clonal lines, one with high embryogenic capacity (K33) and a non-embryogenic clonal line (K7), for SE induction. Furthermore, an analysis of methylation events during the SE induction of both clonal lines was performed.

2. Materials and Methods

2.1. *A. fourcroydes* Clonal Lines and Induction of SE

Shoots from *A. fourcroydes* clonal lines were used as the source of explant, obtained using the protocol of Robert *et al.* (2004). A clonal line includes all shoots derived from the same mother plant. The parental plants were young (30 - 50 cm

high) offshoots from selected elite plantation individuals in Telchac-Yucatán.

For this study, we chose a highly embryogenic clonal line (K33) that produces 100 embryos/explant and another non-embryogenic one (K7) that produces no embryos. Both clonal lines were grown in the same MSB maintenance medium [salts from Murashige and Skoog (MS) medium [35] with reduced nitrogen (10 mM KNO_3 and 5 mM NH_4NO_3) supplemented with 0.1 µM 2,4-D and 44.4 µM BA] before undergoing embryogenic induction.

For SE induction, the stems of shoots from both *A. fourcroydes* clonal lines were segmented into thin layers of approximately 0.5-mm thickness, placed on MS medium supplemented with 2.26 µM dicamba, 3% (*w/v*) sucrose, vitamin L2 [36] solidified with 0.3% (*w/v*) agar (Sigma-Aldrich G1910) and 0.3% (*w/v*) Phytagel™ (Sigma-Aldrich P8169) and cultured in the dark [33].

2.2. DNA Extraction

The method reported by Echevarría-Machado *et al*. (2005) [37] was used for genomic DNA extraction. The concentration and purity of DNA were determined by spectrophotometry (Thermo Scientific NanoDrop™ 1000), and its integrity was verified by electrophoresis on a 1% agarose gel.

2.3. AFLP

In addition to 5 individual samples, a pool (bulk) of each clonal line was formed, for which equimolar concentrations of DNA extracted from 20 shoots (one 100-ng DNA mixture per shoot) were taken. The bulks were realized to reduce the variability between individuals.

AFLP was performed as described by Vos *et al*. (1995) with slight modifications. Approximately 300 ng of genomic DNA was digested with 2 U MseI and 2 U EcoRI restriction enzymes in a 20-µL reaction. The ligation reaction was performed with 20 µL of previously digested DNA, 1× ligase buffer, 5 pmol EcoRI adapters, 50 pmol MseI adapters, and 1 U T4 DNA ligase in a 30-µL reaction. The pre-amplification reaction was performed with 5 µL of the 1:5 (v:v) dilution of the ligation product, 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 pmol MseI primer, 1 pmol EcoRI primer, and 0.5 U *Taq* DNA polymerase in a 20-µL reaction. Selective amplification was performed in a 10-µL reaction with 5 µL of a 1:10 (v:v) dilution of the pre-amplification product, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10.67 mM MseI selective primer without label containing three user-selected nucleotides, 2.5 mM EcoRI selective primer (3) labeled with Well RED dye (D2, D3, D4) and containing three selective nucleotides (**Table 1**), and 0.5 U *Taq* DNA polymerase. PCR conditions were 5 min at 94˚C; 16 cycles of "touch-down" of 45 s at 94˚C, 45 s at 60˚C (0.5˚C decrease per cycle) and 30 s at 72˚C; 16 continuous cycles of 45 s at 94˚C, 45 s at 52˚C and 30 s at 72˚C; and 7 min at 72˚C.

Subsequently, 25 µL of sample loading solution (SLS, GenomeLab™ N˚ 608082) was taken, and to this solution was added 0.25 µL of STD 400 (DNA

Table 1. List of AFLP/MSAP primers and adapters used. DNA sequences are given in the 5' to 3' orientation.

Adapters/primers	Sequence (5'-3')
Adapters	
*Hpa*II/*Msp*I-adapter I	5'-CGACTCAGGACTCAT-3'
*Hpa*II/*Msp*I-adapter II	5'-GACGATGAGTCCTGAGT-3'
EcoRI-adapter I	5'-CTCGTAGACTGCGTACC-3'
EcoRI-adapter II	5'-AATTGGTACGCAGTCTAC-3'
Mse1-adapter I	5'-GACGATGAGTCCTGAG-3'
Mse1-adapter II	5'-TACTCAGGACTCAT-3'
Pre-amplification primers	
*Hpa*II/*Msp*I-pre	5'-GATGAGTCCTGAGTCGG-3'
Eco-pre	5'-GACTGCGTACCAATTCA-3'
Mes-pre	5'-GATGAGTCCTGAGTAAC-3'
Selective amplification primers	
***Hpa*II/*Msp*I primers**	
HM-CTA	5'-GATGAGTCCTGAGTCGGCTA-3'
HM-CAG	5'-GATGAGTCCTGAGTCGGCAG-3'
HM-CTT	5'-GATGAGTCCTGAGTCGGCTT-3'
***Eco*RI-primers**	
EcoR-ACG	5'-GACTGCGTACCAATTCACG-3'
EcoR-AAC	5'-GACTGCGTACCAATTCAAC-3'
EcoR-ACA	5'-GACTGCGTACCAATTCACA-3'
EcoR-ACT	5'-GACTGCGTACCAATTCACT-3'
EcoR-AAG	5'-GACTGCGTACCAATTCAAG-3'
EcoR-ACC	5'-GACTGCGTACCAATTCACC-3'
***MseI*-primers**	
MseI-CTA	5'-GATGAGTCCTGAGTAACTA-3'
MseI-CTT	5'-GATGAGTCCTGAGTAACTT-3'
MseI-CAG	5'-GATGAGTCCTGAGTAACAG-3'

Size Standard Kit-400 GenomeLab™ N˚ 608098) and 2 µL of diluted sample (2 µL of selective amplification product + 4 µL of formamide SLS) plus one drop of oil to prepare the plate that was run in the sequencer (Beckman CQ800).

2.4. MSAP

Samples were collected before the induction (day 0, prior to induction) and during induction (days 1, 3, 7, 15, 30, 45). Three replicates were made each day, each of which consisted of segmented stems from different individuals on the corresponding sampling day.

MSAP is a variant of AFLP that allows us to detect methylation patterns of genomic DNA using digestion enzymes that are sensitive to methylation. Sam-

ples were analyzed using the method reported by [39]. In this protocol, the frequent cutter MseI was replaced with two isoschizomers: HpaII and MspI. Conditions for digestion, ligation, pre-amplification, selective amplification and sample preparation for sequencing were the same as described in the AFLP protocol. Selective amplification included a total of five combinations (**Table 1**), from which the one with the greatest amount of polymorphism was selected. To reduce the possibility of artifacts, three replicates from different extractions were used for each combination.

2.5. Analysis of Data Obtained by AFLP and MSAP

Gene Marker™ software was used to obtain polymorphic profiles from both AFLP and MSAP. Automatic fragment detection criteria included fluorescence intensity greater than 50 units and a range of fragment sizes of 50 to 350 bp. Data were sorted into binary 0 - 1 matrices, where 0 means the absence and 1 the presence of the fragment.

In AFLP, fragment analysis consisted of a comparative study of the results, in which we determined the total number of fragments and the numbers of monomorphic and polymorphic fragments. The genetic similarity index was determined according Nei and Li [40].

In MSAP, the isoschizomers HpaII and MspI recognize the same tetranucleotide sequence (5'-CCGG-3') but differ in their sensitivity to the methylation status of the recognition site. This difference allows classification into four types [41]: Type I: when the resulting fragment came from digestion with both enzymes, it was unmethylated; type II: when the resulting fragment only came from digestion by EcoRI + MspI, it indicated a complete methylation profile or hemi-methylation of the internal cytosine ($C^{Me}CGG/C^{HMe}CGG$); type III: when the fragment only came from digestion of EcoRI + HpaII, it indicated a hemi-methylation profile of the external cytosine ($^{HMe}CCGG$); and type IV: when there was no cut by any of the enzymes, it indicated that no fragments were generated; this result may have been due to complete methylation of the external cytosine ($^{Me}CCGG$), complete methylation of both cytosines ($^{Me}C^{Me}CGG$), hemi-methylation of both cytosines ($^{HMe}C^{HMe}CGG$) or an actual absence of the fragment due to a polymorphism at the restriction site.

A site was considered a "polymorphic fragment" (PF) if there was at least one line in which the site was methylated and at least one line in which the site was not methylated. The numbers of specific fragments were also calculated across the clonal lines. Specific fragments are clonal line-specific and found only in a single clonal line.

2.6. Statistical Analysis

MSAP profiles were analyzed using the R environment (R Development Core Team, 2012) running package *msap* (v.1.1.6) [23]. This package uses four main steps: data input, fragment classification, data transformation and data analysis;

the Error Rate-based Threshold (ERT) is set as 5% by default. Briefly, the package uses a binary matrix indicating the presence (1) or absence (0) of EcoRI-MspI and EcoRI-HpaII fragments in the samples of each group. Then, the fragments are classified based on the four possible types described above [Msp 1/HPA1 (Type I), Msp 1/HPA0 (Type II), Msp 0/HPA1 (Type III) and Msp 0/HPA0 (Type IV)]. Posteriorly, it determines if each fragment is susceptible to methylation (MSL: Methylation Susceptible Loci) or if there is no evidence of methylation (NML: Non-Methylated Loci), following the procedure in Herrera & Bazaga [30]. The package uses a Euclidean distance matrix. The epigenetic variation is estimated using the Shannon diversity index. Within the MSL, the package provides a report of the related methylation levels for every group. The epigenetic differentiation is assessed by principal coordinate analysis (PCoA) and is tested using analyses of molecular variance (AMOVA; [42]) with 10,000 permutations. For the AFLP technique, the package skips all data transformation and classification of MSL/NML and goes directly to diversity/differentiation analysis.

3. Results

3.1. Genetic and Epigenetic Differences *within and* between Clonal Lines

To evaluate and characterize the genetic and epigenetic differences between two clonal lines (embryogenic and non-embryogenic) of *A. fourcroydes*, the AFLP and MSAP techniques were used. A polymorphism survey was performed within one clonal line (to assess the occurrence of variability) and among the clonal lines (to reveal differential epigenetic and genetic profiles).

A total of five AFLP primer combinations (Table 1) were used to generate AFLP profiles. The primer combinations that produced the greatest polymorphism were EcoRI-AAC/MseI-CTA, EcoRI-ACG/MseI-CAG and EcoRI-ACA/MseI-CTT. AFLP analysis revealed 258 total fragments distributed in the 12 samples of the two clonal lines. The numbers of polymorphic fragments were 142 (55.04%) for K33 and 167 (64.73%) for K7. The numbers of monomorphic fragments were 116 (44.96%) for K33 and 91 (35.27%) for K7. The numbers of specific fragments were 41 for K33 and 18 for K7 (Table 2). According to the Nei and Li coefficient, the similarity index varied from 0.72 to 0.91, with an average of 0.81 for the three primer combinations.

A total of five MSAP primer combinations (Table 1) were deployed to generate MSAP profiles. The primer combinations that produced the greatest polymorphism were EcoRI-ACA/MH-CTA, EcoRI-ACA/MH-CAG and EcoRI-ACA/MH-CTT. MSAP analysis revealed 199 total fragments distributed in the 8 samples of the two clonal lines. The numbers of polymorphic fragments were 52 (26.13%) for K33 and 47 (23.62%) for K7. The numbers of monomorphic fragments were 147 (52%) for K33 and 152 (76.38%) for K7. The numbers of specific fragments were 29 for K33 and 34 for K7 (Table 2).

Table 2. Polymorphism based on AFLP and MSAP marker between two clonal lines, K33 and K7.

Marker	Clonal line	Samples	TF	MF	PF	%MF	%PF	CEF
AFLP	K33	6	258	116	142	44.96	55.04	41
	K7	6		91	167	35.27	64.73	18
MSAP	K33	4	199	147	52	73.87	26.13	29
	K7	4		152	47	76.38	23.62	34

TF: Total number of fragments; MF: Number of monomorphic fragments; PF: Number of polymorphic fragments; PP: Percentage polymorphism; CEF: Number of clone-especific fragments.

AMOVA revealed that the variances within clonal lines were greater than the variances between clonal lines for both AFLP and MSAP (Table 3). However, differences relating to genetic patterns (AFLP) were statistically significant between clonal lines (AMOVA; differentiation among clonal lines: Phi_ST = 0.2353, P = 0.0015). Differences relating to the genome-wide methylation patterns (MSAP) were also statistically significant between both lines (AMOVA; differentiation among clonal lines: Phi_ST = 0.2548, P = 0.0267).

PCoA of the AFLP and MSAP profiles was performed to visualize the clustering patterns of the clonal lines (Figure 1). PCoA revealed a clear separation between the two clonal lines for both AFLP (C1, 25% and C2, 12.4% of variance explained) and MSAP (C1, 25% and C2, 12.4% of variance explained).

3.2. *DNA Methylation* Patterns between Clonal Lines *before* SE *Induction*

The *msap* package identified a total of 186 MSL (Methylation Susceptible Loci) and 13 NML (Non-Methylated Loci). The frequencies (expressed as percentages) of the different states of methylation at the target sequences in K33 and K7 are displayed in Table 4. The methylation levels in MSL were 78.64% for K33 and 81.95% for K7.

Furthermore, the total fragments were classified into 16 combinations, "A to E" (Table 5), per their banding patterns to analyze the pairwise comparison of fragments between K33 and K7. The banding patterns were classified into two types: same (A1 to A4) and different (B1 to E3). Pairwise comparisons revealed that 89 fragments were grouped as same patterns (48.24% of all methylated sites) and 73 fragments were grouped as different patterns (51.76% of all methylated sites). Figure 2 illustrates some of the different patterns observed between the clonal lines.

3.3. DNA Methylation Patterns during SE Induction

During *SE induction* (1, 3, 8, 15, 30 and 45 days) (Figure 3(a)), the banding patterns of clonal lines were also observed. Patterns were grouped according to events that occurred during the entire process in relation to the initial pattern (day 0). These profiles were classified into three types of events as methylation (ME), demethylation (DM) and no change (NC). The results showed a particular

Table 3. Analysis of molecular variance results between clonal lines (K33 and K7) in reference to two techniques used.

Marker		df	SSD	MSD	Variance	Phi_ST	*P*-value
AFLP	Among clonal lines	1	75.75	75.75	8.189	0.2353	0.0015
	Within clonal lines	10	266.2	26.62	26.62		
	Total	11	341.9	31.08			
MSAP	Among clonal lines	1	32.18	32.18	4.647	0.2548	0.0267
	Within clonal lines	6	81.56	13.59	13.59		
	Total	7	113.7	16.25			

df: degrees of freedom, SSD: Sum of Squares Differences, MSD: Mean Squares Deviation, *P*-values were derived from a random permutation test with 10,000 permutations.

Table 4. Report of methylation levels in each clonal line. Percentages are referred to the total number of polymorphic loci after the error rate filtering.

Band pattern		State	Embryogenic clonal line	Non-embryogenic clonal line
*Msp*I	*Hpa*II		K33	K7
1	1	Unmethylated (Type I)	21.36%	18.05%
1	0	Internal cytosine methylation (Type II)	17.55%	22.02%
0	1	Hemimethylated (Type III)	16.39%	17.88%
0	0	Full methylation or absence of target (Type IV)	44.70%	42.05%

"0": fragment absence; "1": fragment presence.

dynamic, especially in the K33 clonal line (**Figure 3(b)**). In contrast, K7 had less significant changes throughout the process (**Figure 3(c)**).

Methylation events (ME) at CCGG sites were greater in K7 and stayed constant throughout the process, in the range of 48.8% - 56.2% (**Figure 3(c)**); in K33, they varied between 18.6% and 50% (**Figure 3(b)**). In K33, the highest peak in the ME was 50% at day 8, which corresponds to the beginning of callus formation (**Figure 3(a)**). From day 15, which corresponds to the formation of embryogenic masses, the ME gradually decreased to 33.4%. On day 30, the formation of globular embryos is evident, and at day 45, other embryo stages are also found on the explant (**Figure 3(a)**). On both days, the ME were 31.3% for day 30 and 23.7% for day 45.

Demethylation events (DE) at CCGG sites were higher in K33, ranging from 13% to 32.1% during the process; in K7, a narrower range of 8% to 13.6% was observed (**Figure 3**). In K33, the highest peak in the DE was 32.1%, which corresponds to explant swelling (day 3). On day 8, which corresponds to callus formation, the DE were the lowest at only 13%. Posteriorly, the DE gradually increased as the embryos developed (**Figure 3(b)**).

The events in which no changes were observed during the process (NC) ranged from 37% to 49.4% in K33 (**Figure 3(b)**); in K7, these events presented a more constant range of 35.2% - 38.3% (**Figure 3(c)**).

Table 5. DNA methylation pattern at CCGG sites between two clonal lines (embryogenic and non-embryogenic) of *Agave fourcroydes* before induction of somatic embryogenesis.

Methylathion	Type	Banding Pattern				Number of bands	Frequency %
		Embryogenic clonal line (K33)		Non-embryogenic clonal line (K7)			
		*Msp*I	*Hpa*II	*Msp*I	*Hpa*II		
Same pattern	A1	1	1	1	1	20	10.05
	A2	1	0	1	0	10	5.03
	A3	0	1	0	1	8	4.02
	A4	0	0	0	0	58	29.15
	Total A					**96**	**48.24**
Different pattern	B1	1	0	1	1	8	4.02
	B2			0	0	19	9.55
	B3			0	1	1	0.50
	Total B					**28**	**14.07**
	C1	0	1	1	1	5	2.51
	C2			0	0	13	6.53
	C3			1	0	5	2.51
	Total C					**23**	**11.55**
	D1	1	1	0	0	10	5.03
	D2			1	0	4	2.01
	D3			0	1	9	4.52
	Total D					**23**	**11.56**
	E1	0	0	1	1	6	3.02
	E2			1	0	19	9.55
	E3			0	1	4	2.01
	Total E					**29**	**14.58**
	Total					**103**	**51.76**

"0": fragment absence; "1": fragment presence.

4. Discussion

4.1. Genetic and Epigenetic Variation within and between the Clonal Lines

In this work, we observed that epigenetic and genetic variability exists within and between the clonal lines of *A. fourcroydes* (Table 2 and Table 3).

It is possible that the variability observed within clonal lines may be due to tissue culture conditions. It has been reported that the stress occasioned by these conditions may produce gene mutations, epigenetic changes, and activation of transposable elements, leading to somaclonal variation [43] [44] [45] [46] [47].

In agaves, genetic fidelity in plants generated by *in vitro* culture has been studied in *A. fourcroydes* [48] and *A. tequilana* [49] [50]. Using AFLP as a molecular marker, González *et al.* (2003) detected 19.9% polymorphisms when comparing mothers and their somatic embryogenesis-derived daughter plants of *A.*

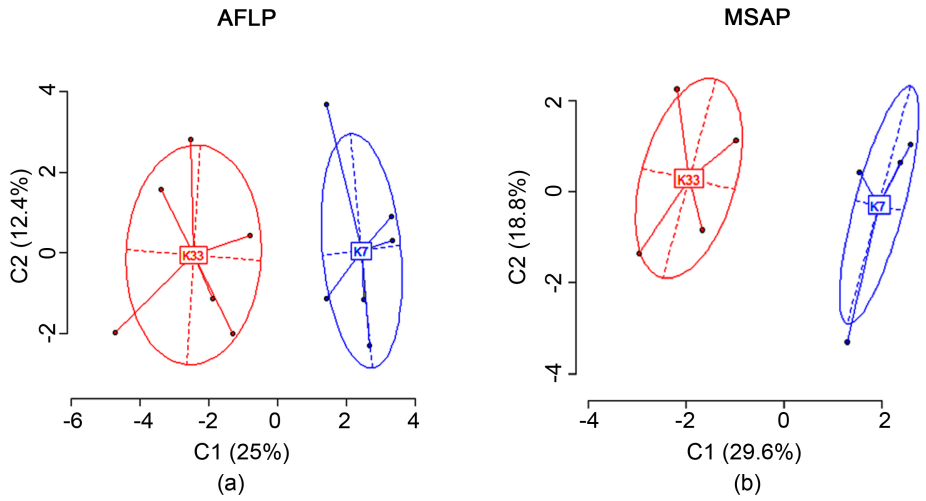

Figure 1. Principal coordinate analysis (PCoA) results for genetic (AFLP, panel (a)) and epigenetic (MSAP, panel (b)) differences between the two clonal lines, K33 and K7. The first two coordinates (C1 and C2) are shown, with the explained variance percentages in brackets. Different points represent individual samples. Labels indicate the centroids for the point cloud in each clonal line. Ellipses denote the average dispersion associated with each value. The long axis shows the direction of the maximum dispersion, while the short axis shows the direction of minimum dispersion.

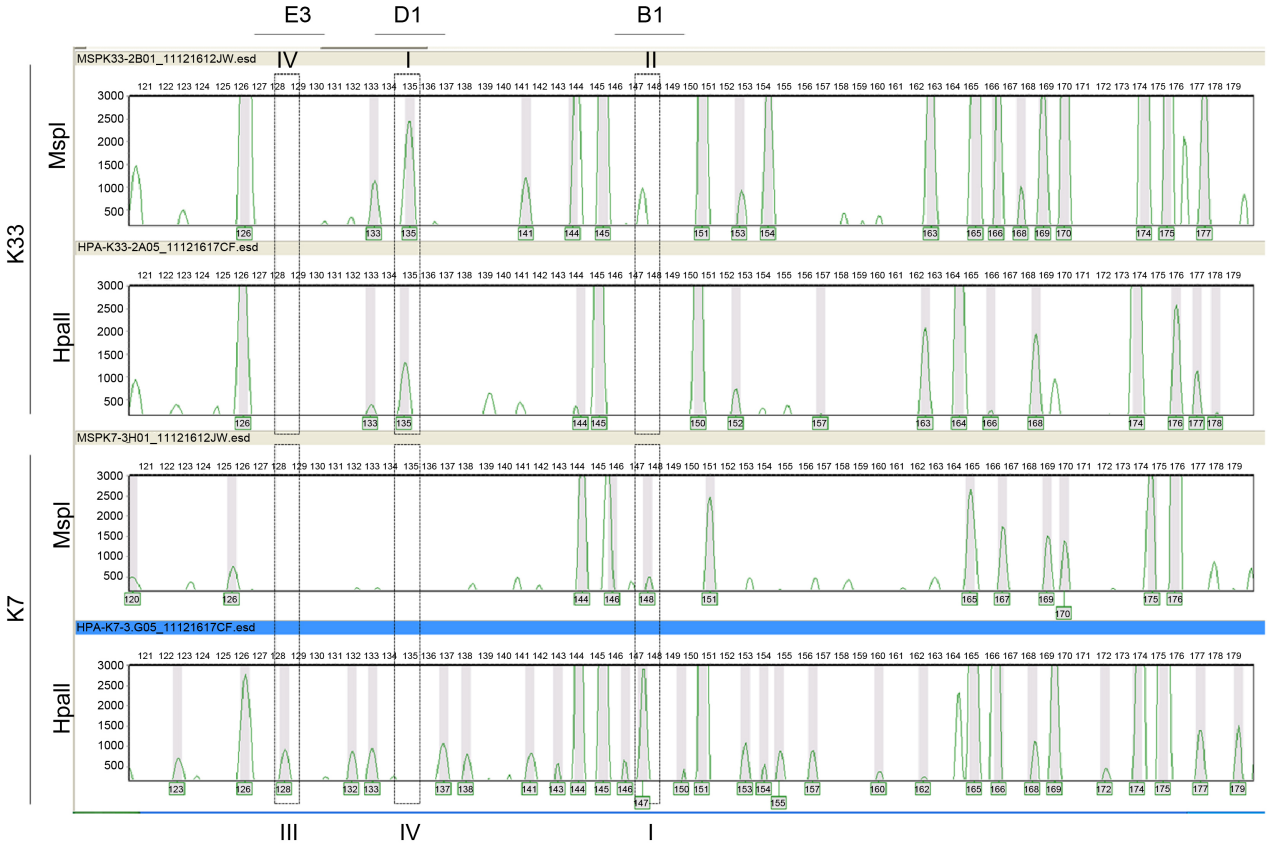

Figure 2. Representation of an electropherogram from MSAP showing unique methylation sites in each clonal line. I: unmethylated, II: $^{HMe}CG/^{Me}CG$, III: ^{HMe}CCG, IV: $^{Me}CCGG/^{HMe}C^{HMe}CGG/\ ^{Me}C^{Me}CGG$/mutation. E3, D1 and B1: methylation patterns according to Table 5.

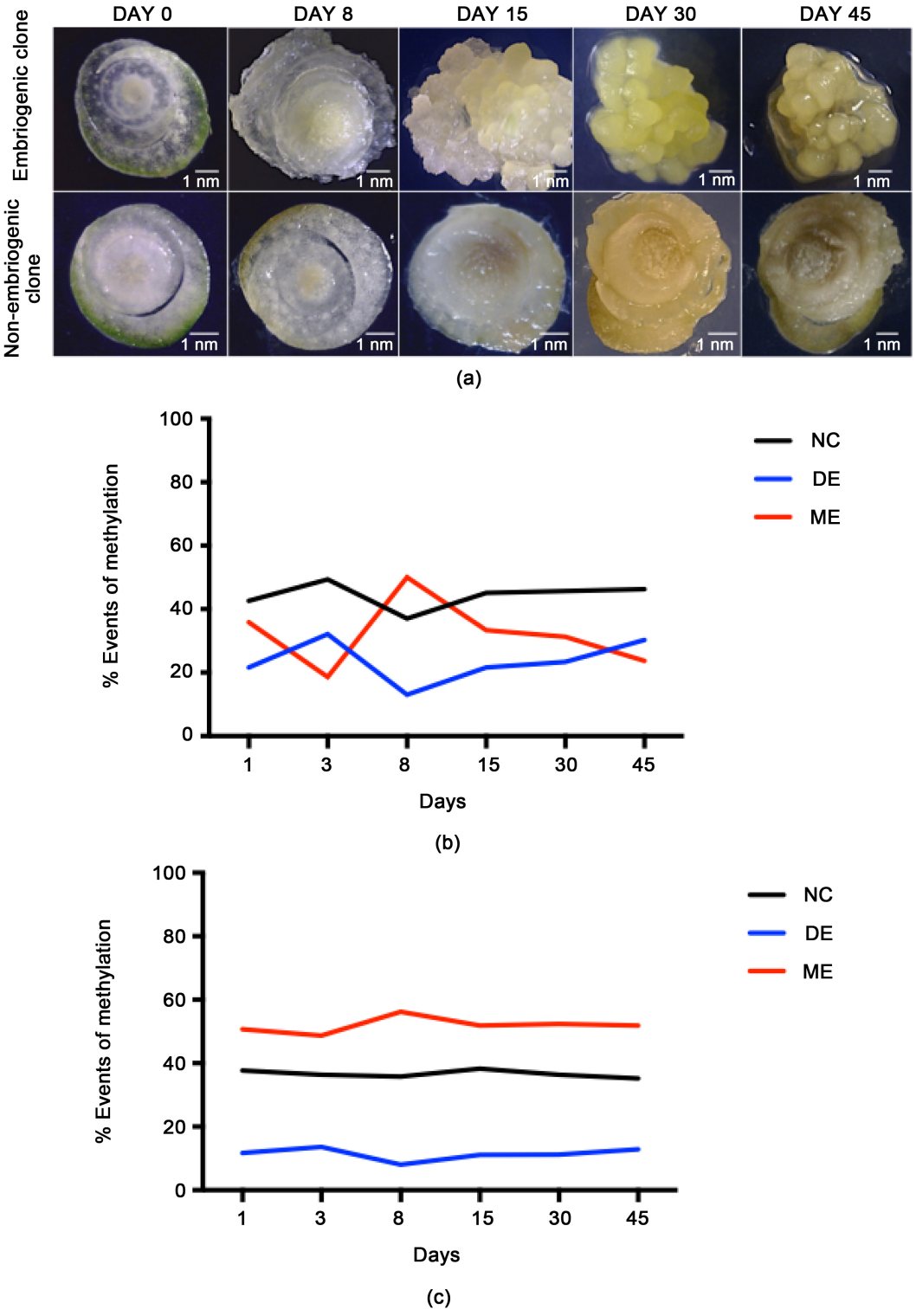

Figure 3. (a) Responses to induction of somatic embryogenesis of an embryogenic clone (top) and a non-embryogenic clone (bottom) of *Agave fourcroydes*; (b) Percentage of methylation events in an embryogenic clone (K33); (c) Percentage of methylation events in a non-embryogenic clone (K7).

fourcroydes, a value that is much lower than those found in this work (55.04% for K33 and 64.73% for K7). In *A. tequilana*, using the same molecular marker,

Diaz *et al*. (2013) detected no genetic variation (0 polymorphic bands) between the original explant and four generations of *in vitro* cultured plants. However, Torres-Morán *et al*. (2010), using ISTR as a marker, observed that genetic variability existed in plants generated by *in vitro* culture methods (somatic embryogenesis and axillary buds) for *A. tequilana*.

The differences in genetic stability observed in this work and previous reports may be due to various factors, such as the type of species, molecular marker, propagation path, explant, culture medium, growth regulators and time of culturing in *in vitro* conditions. In this study, before undergoing embryogenic induction, both clonal lines were kept in maintenance medium with 2,4-D and BA for 15 subculture cycles (1 month of subculture). It has been reported that the duration, the number of subculture cycles and exposition to plant growth regulators may cause somaclonal variation [46] [51] [52] [53].

Epigenetic variability has been little studied in *in vitro*-cultured plants of *Agave*. Díaz-Martínez *et al*. (2012) studied methylation patterns between the original explant and four generations of *in vitro*-cultured plants of *A. tequilana*, noting that each generation showed specific patterns. In other species, it has been observed that micropropagated plants present epigenetic variations in relation to the parental plant [16] [45] [54].

In spite of the genetic and epigenetic variability within clonal lines, both were separated into two groups using both techniques (**Figure 1**). This separation is possibly due to the conservation of some markers (genetic and/or epigenetic) of the parental plant. In a previous study with *A. fourcroydes*, it was observed that each mother plant and its somatic embryogenesis-derived daughter plants clustered, indicating the conservation of superior characteristics in the micropropagated daughter plants [48].

4.2. DNA Methylation Patterns before SE Induction

In this study, MSAP revealed that clonal lines K33 and K7, which have high or null embryogenic potential, respectively, showed epigenetic differences (DNA methylation at CCGG sites) before undergoing embryogenic induction. Even when using the same explant (stem shoots), the clonal lines each had fragments with specific methylation states and shared only 54.94% of fragments with the same methylation pattern (**Table 5**). It has been reported that different plant genotypes present differences in their DNA methylation patterns [18] [55] [56] and between tissues in an individual [57].

Cells seem to have their own epigenetic signature inserted into the genotype [58]. However, during *in vitro* culture, some cells are subject to epigenetic reprogramming that results in the elimination of existing epigenetic markers in the nuclei, followed by establishment of a series of different markers [59].

Although the same tissue was used as the explant for SE induction, the responses were very different in the two clonal lines (**Figure 3**); perhaps the cells involved in embryo formation present an epigenetic status that was different in

the stem of the embryogenic clonal line compared to that in the stem of the non-embryogenic clonal line. The role of methylation in embryogenic capacity has been reported in *Brassica oleracea*, in which two types of explants that share the same genome, hypocotyl (more embryogenic) and cotyledon (less embryogenic), show differences in both the DNA methylation levels and the methylation patterns of CCGG sites [6]. In *Pinus*, the differentiation of needle explants and its relationship with organogenic capability has been associated with increases in heterochromatin-related epigenetic markers, including high DNA methylation, low acetylated histone H4 levels, and the presence of histone H3 methylated at Lys9 [60].

4.3. Dynamics of DNA Methylation Patterns during SE Induction

The stress generated by *in vitro* culture conditions, especially the exposure to the high concentration of auxin used for SE induction, can lead to the activation of the embryogenic program [1] [2]. In this study, when exposed to growth regulators (in this case, dicamba), K33 began the embryogenic process; however, these same conditions did not induce embryogenesis in similar explants of K7, which possibly implies a different capacity of genetic-epigenetic response, as indicated by the fact that before being induced, both clones showed different genetic and epigenetic profiles.

The embryogenic clonal line (K33) showed more dynamic changes in methylation-demethylation events (at CCGG sites) compared to the non-embryogenic clonal line (K7) (**Figure 3(a)**). This plasticity (methylation/demethylation) is related to the regulation of several genes involved in SE [2] [61] and to embryo development [11] [62].

Demethylation events at CCGG sites were observed on day 3 (swelling of explants) and during the formation of somatic embryos (days 15, 30 and 45). It has been observed that the decrease in DNA methylation is related to the developmental process [7] [63] [64] [65] [66].

In the non-embryogenic clonal line (K7), the methylation events at CCGG sites were more frequent and remained constant throughout the process (**Figure 3(b)**), which might be the cause of the inhibition of developmental gene expression in the embryogenic process. In *Eleutherococcus senticosus*, it has been observed that the non-embryogenic callus presented a higher level of methylation compared with the embryogenic callus [9].

Different studies show that epigenetic changes might be related to totipotency acquisition, cellular reprogramming, induction of SE, and embryo development [2] [15] [61]. In this work, we observed that both clonal lines showed different genetic and epigenetic patterns before beginning the induction process (even when using the same explant). Furthermore, when they were subjected to the induction process, the embryogenic line presented greater dynamism in terms of methylation-demethylation events (at CCGG sites), whereas the non-embryogenic line presented high numbers of methylation events throughout the process.

From these results, we hypothesize that, in addition to genetic differences, methylation polymorphisms between clonal lines (K33 and K7) could be responsible for their different responses to SE induction factors.

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