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Polymorphism and methylation patterns in *Agave tequilana* Weber var. 'Azul' plants propagated asexually by three different methods

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ABSTRACT

Genetic variation in three forms of asexually propagated *Agave tequilana* Weber var. 'Azul' plants namely offsets, bulbils and *in vitro* cultured individuals was studied by AFLP analysis. Low levels of variation were observed between mother plants and offsets and a higher level between mother plant and bulbils. Families obtained from commercial plantations showed lower levels of variation in comparison to families grown as ornamentals. No variation was observed between the original explant and four generations of *in vitro* cultured plants. Epigenetic variation was also studied by analyzing changes in methylation patterns between mother plants and offspring in each form of asexual reproduction. Offsets and bulbils showed an overall decrease in methylation whereas *in vitro* cultured plants showed patterns specific to each generation: Generations 1 and 4 showed overall demethylation whereas Generations 2 and 3 showed increased methylation. Analysis of ESTs associated with transposable elements revealed higher proportions of ESTs from Ty1-copia-like, Gypsy and CACTA transposable elements in cDNA libraries obtained from pluripotent tissue suggesting a possible correlation between methylation patterns, expression of transposable element associated genes and somaclonal variation.

1. Introduction

Agave tequilana is a monocarpic semelparous plant species which in common with other members of the Agave genus is well adapted for growth in hot, arid conditions [1]. The plants flower after 5–8 years in the field and thousands of seeds can be produced [2], however in commercial plantations inflorescences are removed and flowering and seed production suppressed in order to preserve the accumulated carbohydrates which are exploited for the production of tequila [3]. Seeds may be produced by both self-fertilization and outcrossing although a large proportion are found to be unviable [2].

Throughout their life-cycle, *A. tequilana* plants produce offsets from rhizomes (around 1–2 per year) and traditionally plantations are grown or renewed using these asexually produced individuals [3]. Improvements in the efficiency of *in vitro* propagation of *A. tequilana* [4–9] however have recently made it feasible for plantations to be grown from materials obtained by tissue culture. Under natural conditions *A. tequilana* plants may also undergo a third form of asexual reproduction by the formation of bulbils on the inflorescence. When plants are permitted to flower but fertilization is unsuccessful, hundreds to thousands of small plantlets are formed vegetatively at the bracteoles below the flower buds [10]. These plantlets are formed from new meristems which initiate in these tissues, implying a developmental reprogramming from sexually reproductive to asexually reproductive growth [10].

Tequila production is under a "Controlled Denomination of Origin" where the majority of sugars used must be obtained from *A. tequilana* Weber, cultivar "Azul" or "Blue agave" grown in 5 designated geographical regions of Mexico. Around 100,000 hectares of Blue agave are grown and successive generations of asexual reproduction over many years in addition to the strict requirements for the denomination of origin have led to a very narrow germplasm base for this cultivar [11]. This system of monoculture could have serious consequences if adverse environmental conditions or pathogens such as bacteria or fungi prevail.

Several reports have shown that at least low levels of variability can be found in asexually propagated *A. tequilana* and other species such as *A. fourcroydes and A. angustifolia*, at the genetic level [11–17] and phenotypic variants are often observed in the field [3] and in plants obtained from *in vitro* culture [18,19]. In *A. fourcroydes* 20.67% of polymorphism was reported between mother plants and

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Table 1
Location and number of offspring analyzed for each *A. tequilana* mother plant.

Mother plants	Progeny analyzed	Location
MT1	5 offsets (H1A-H1E)	CINVESTAV, Irapuato, Gto. (Ornamental)
MT2	5 offsets (H2A-H2E)	CINVESTAV, Irapuato, Gto. (Ornamental)
MT3	5 offsets (H3A-H3E)	Ranch "El Coyote", Pénjamo, Gto. (Commercial plantation)
MT4	5 offsets (H4A-H4E)	Ranch "El Coyote", Pénjamo, Gto. (Commercial plantation)
MT5	4 offsets (H5A-H5D)	Exhacienda de Silva, Romita, Gto. (Commercial plantation))
MT6	5 offsets (H6A-H6E)	CINVESTAV, Irapuato, Gto. (Ornamental)
MT7	7 offsets (H7A-H7G)	CINVESTAV, Irapuato, Gto. (Ornamental)
MT8	7 offsets (H8A-H8G)	CINVESTAV, Irapuato, Gto. (Ornamental)
MT9	6 offsets (H9A-H9E), 13 bulbils (B9A-B9L, B9O)	Municipal Garden, Irapuato, Gto. (Ornamental)
MT10	Explants from 1 offset serially cultured *in vitro* for 4 generations (G1-G4) (48 individuals)	Juchipila, Zac. (Commercial plantation)

their respective progenies, suggesting that the plants may accumulate somatic mutations during their long life cycle. Some of these mutations may become fixed and inherited by the descendants [12]. Differences in levels of polymorphism between propagation methods has also been reported for Agave species with clear differences observed between *in vitro* propagated plants and offsets obtained from the field [19].

Agave somaclonal variants could be useful sources for the improvement of agronomically important traits as is the case for other crops such as coffee, sugarcane, rice and strawberry [20–24]. In other plant species where changes underlying somaclonal variation have been studied in detail effects of point mutations have been observed as have epigenetic changes in methylation patterns [25,26]. The latter has also been related to the reactivation of transposons which in turn may lead to phenotypic modifications in tissue cultured materials in some crops [27,28]. If the current trend of exploiting *in vitro* propagated materials for commercial cultivation of *A. tequilana* is maintained or expanded, the challenge will be to limit the levels of somaclonal variation in plants propagated for this purpose. On the other hand given the complexity and time necessary for traditional breeding strategies in this species, it is essential to identify sources of variation which could provide the basis for selection of genotypes with agronomically important traits ranging from higher carbohydrate content to disease resistance.

Based on these observations and in order to obtain more detailed information on the levels of genetic and epigenetic variation in asexually propagated *A. tequilana* plants, the objectives of this work were to compare the levels of polymorphism and changes in methylation patterns observed under three asexual propagation methods: offsets, bulbils and *in vitro* propagated materials and to explore the possibility that transposon associated gene expression may be related to the genetic and epigenetic changes observed.

2. Materials and methods

2.1. Plant material

Samples of *A. tequilana* Weber var. Azul were obtained from different sources as shown in Table 1: (1) eight different mother plants with 4–7offsets each, (2) a mother plant with six offsets and 13 bulbils, (3) plantlets obtained from a single explant by two different methods and cultured *in vitro* for four generations (Table 1).

2.2. In vitro culture

Explants for *in vitro* culture were obtained from meristem culture based on an unpublished protocol developed by Nava-Cedillo. Briefly: offsets were carefully washed and disinfected before excision of the apical meristem. From the meristem two different forms of explants were obtained: (A) the shoot apical meristem (SAM) tip and (B) Sections obtained from the region immediately below the SAM (Shown in Supplementary Fig. 1). The explants were cultured in Ericksson-Linsmaier multiplication medium with 0.6 mg/l indolacetic acid (IAA) and 9 mg/l benzylaminopurine (BAP) as growth regulators. (Supplementary Fig. 1) Shoots were separated from the original explants after 2 or 3 weeks. The first shoots to appear were denominated first generation (G1) and the most vigorous were grown until rooted in medium without growth regulators where they were maintained for 4 or 5 weeks. Rooted plants were then selected for preparation of explants and multiplication to form the second generation (G2). The third and fourth generations (G3, G4) were produced in the same fashion.

2.3. DNA extraction

Genomic DNA was extracted from all samples using a modified CTAB-chloroform method described by Edwards (2000) [29] using 1 g of young leaf tissue. Modifications to the method were reported by Gil-Vega et al. [11]. Aliquots of 2 μL were used for quantification by Nanodrop and integrity was verified in 1% ethidium bromide stained agarose gels by comparison with a 100 ng/μL control DNA sample.

2.4. AFLP analysis

AFLP analysis was carried out as described by Vos et al. [30] using six -eight oligonucleotide combinations of fluorescently labeled EcoR1 + NNN and unmarked MseI + NNNN (Table 2). Banding patterns were visualized on a LI-COR 4200 sequencer and analyzed using SAGA^{MX} AFLP® Quantar software with manual revision. Three–five repetitions were carried out to ensure accuracy of determination of polymorphisms. NTSYSpc 2.1 software (Exeter software, N.Y.) was used to analyze binary encoded banding data using the Jaccard [31] and Nei & Li [32] methods and UPGMA to produce dendrograms. Robustness of topology was assessed by Bootstrap analysis [33] on 1000 re-sampled data sets. Levels of

Table 2
Primer combinations used for AFLP and MSAP analysis.

Primers combination	Unmarker primer	Fluorescently labeled primer
1-AFLP	MseI + AGTC	EcoRI + AAC (700 nm)
2-AFLP		EcoRI + ACT (800 nm)
3-AFLP		EcoRI + AGA (700 nm)
4-AFLP		EcoRI + ACG (800 nm)
5-AFLP		EcoRI + ACA (700 nm)
6-AFLP		EcoRI + AGC (800 nm)
7-AFLP		EcoRI + AAG (700 nm)
8-AFLP		EcoRI + AGG (800 nm)
1-MSAP	HpaII + ACT	EcoRI + AGA (700 nm)
2-MSAP		EcoRI + ACG (800 nm)
3-MSAP	HpaII + ACC	EcoRI + ACC (700 nm)
4-MSAP		EcoRI + ACG (800 nm)
5-MSAP	HpaII + ACG	EcoRI + AGA (700 nm)
6-MSAP	HpaII + ACA	EcoRI + AGC (800 nm)