INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 14, No. 3, p. 65-71, 2019

RESEARCH PAPER OPEN ACCESS

Assessing Epigenetic Variation in Tissue Culture Based Plants of Sugarcane using High Performance Liquid Chromatography Approach

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Key words: DNA methylation, Methylcytosine, Cytosine, HPLC.

http://dx.doi.org/10.12692/ijb/14.3.65-71 Article published on March 15, 2019

Abstract

Tissue culture system is considered as a basis for genetic transformation studies in plants. Plant tissue culture regime could bring genetic and epigenetic variation in in vitro regenerated plants though. Cytosine methylation is an imperative factor that contributes in epigenetic variation. Therefore, in this study we investigated the epigenetic variation in in vitro regenerants of sugarcane genotypes, CPF-247 and CPF-248 by using high performance liquid chromatography (HPLC) technique. In this study, in vitro regenerated plants and field grown plants of both sugarcane genotypes were assessed and analyzed for the level of cytosine and methylcytosine. Thus calculated percent concentration of 5MeC for each sample were 0.0345% and 0.258% in field grown and in vitro regenerated plants of CPF-247 respectively as well as 0.0261% and 4.39% in the field grown and in vitro regenerated plants of CPF-248 respectively that showed an increase in methylcytosine concentration in *in vitro* regenerated plants than field grown plants. The results showed that level of methylcytosine was high in tissue culture based plants of both sugarcane genotypes. While in comparison between these genotypes revealed that degree of methylcytosine concentration is high in in vitro regenerated plants of CPF-248 than in vitro regenerated plants of CPF-247. These results indicated that dissected tissue culture with hormones may bring change in the level of DNA methylation and thereby it could be the reason of epigenetic variation, which may contribute in genotypic as well as phenotypic variations.

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Introduction

Epigenetic plays important role in gene regulation of plants, through modulation of DNA activity without altering the basic nucleotide structure (including DNA methylation and histone modification) (Chinnusamy *et al.,* 2009). It has been proved that epigenetic information is heritable between generations and provides memory of environmental stresses (Bond *et al.,* 2007; Grativol *et al.,* 2012).Recently, epigenetic modification mechanisms have been implicated for regulating transcription, replication, gene transposition and DNA repair. Small RNA directed modification in transcriptional and posttranscriptional control of gene expression has been proved important to chromatin modifications (Sahu *et al.,* 2013). These modifications are; DNA methylation, histone modification, chromatin reshaping and RNA interference etc (Bird *et al.*, 2007). Methylation, especially cytosine methylation at CpG, CpNpG and asymmetric CpHpH sites, can be induced by cis or trans acting DNA polymorphisms or by epigenetic phenomena (Lukens *et al.*, 2007; Miguel and Marum, 2011).

The important consideration in plant tissue culture studies are the maintenance of totipotent embryogenic callus, assessment of proper time for transformation of embryogenic callus, unsynchronized growth and development of somatic embryos and the occurrence of somaclonal variations with increasing age of callus (Heinz and Me, 1971) which result inplants of heterogeneous nature. The callus based tissue culture systemleads to genetic instability that brings somaclonal variation. That is why; it referred as tissue culture-induced variations (Kaeppler *et al.,* 2000).

Phenotypic and genetic variations have been greatly observed during plant tissue culture, which is more frequent than natural variation (Wang and Wang, 2012). Methylation induced variation during tissue culture has been considered as part of the molecular mechanism for somaclonal variation (Miguel *et al.*, 2011; Xu *et al.*, 2004). Steward *et al.* (2000) reported that heritable variation induced by DNA methylation during regeneration of maize tissue culture. Bardini *et al.* (2003) discovered extensive methylation changes in calluses of *Arabidopsis thaliana* exposed to kanamycin. Xu *et al.* (2004) observed methylation alterations during somatic embryogenesis in rose, and found the highest methylation level during differentiation of callus. Trejgell *et al.* (2009) reported different methylation in 18S rRNA and 25S RNA, when studying *in vitro* regeneration of *Carlina acaulis.*

Cytosine methylation level differs among species. For example, mammalian methylation is generally restricted to CpG sites, whereas plant has extensive asymmetric and CpNpG methylation. Plant methylated cytosine in genome may up to 30%, much higher than methylation in animals. Furthermore, cytosine methylation also differs among tissues and developmental stages of each plant species (Xu *et al.*, 2004) indicating DNA methylation is species and tissue specific, which varies during cellular differentiation and tissue development (Vanyushin, 2006).Therefore, this study was conducted to assess the epigenetic variation in term of cytosine methylation in tissue culture based *in vitro* regenerated plants of sugarcane using HPLC (High performance liquid chromatography) technique.

Materials and methods

Preparation of experimental material

Sugarcane genotypes (CPF-247 and CPF-248) and their *in vitro* regenerated plantlets were experimental material. *In vitro* regenerated plantlets of CPF-247 was from 35 days old calli induced on callogenesis medium (2.75mg L^{-1} 2,4-D; 0.75 mg L^{-1} BAP and 0.25 mg L^{-1}Kinetin) and regenerated on regeneration medium (4.25mg L^{-1} BAP and 1.75 mg L^{-1}casein hydrolysate). Whereas, *in vitro* regenerated plantlets of CPF-248 was from 35 days old calli induced on callogenesis medium (7.75mg L^{-1} 2,4-D) and regenerated on regeneration medium (3.25mg L^{-1} BAP and 1.75 mg L^{-1} casein hydrolysate).

DNA isolation and hydrolysis

Genomic DNA of plant material was extracted using

established protocol. DNA hydrolysis was according to the protocol described by Gao *et al.* (2014). Therefore, DNA incubation in perchloric acid (70%) for 60 minutes at 100°Cwas done. pH was adjusted to 3-5 using 1 mol L^{-1} KOH and thereby $KClO_4$ precipitate was formed. Hydrolysate was collected by centrifugation (13200rpm for 5 minutes) and then was filtered through micropore membrane disc of size 0.45µm.

Detection of epigenetic variation using HPLC technique

For assessing DNA methylation level of *in vitro* regenerated plants in comparison with their respective parents, cytosine and methylcytosine concentrations were evaluated by using HPLC technique. For this purpose, cytosine and methylcytosine were used as standards and their standard curves were generated by using serial dilutions (1mg/ml, 0.2mg/ml, 0.04mg/ml and 0.08 mg/ml) made from 5mg/ml stock solutions of each cytosine and methylcytosine. For this, 5mg of each cytosine and methylcytosine were dissolved into 1 ml of mobile phase (10% methanol; 0.1 $molL^{-1}$ sodium pentane sulfonate; 0.2% triethylamine).

HPLC analysis

For HPLC analysis, filtered hydrolysate (20µl) of each sample was injected on HPLC C_{18}column allied to HPLC system. Analytes were determined using UV/Vis detector and output was monitored with Chromatographic separation (flow rate, 0.8ml/min and oven temperature, 40°C) was done with mobile phase and UV spectrum was recorded at 273 nm. Relative quantification was and the percentage of methylcytosine in each sample was calculated as formula given by Gao *et al.* (2014).

Results

DNA methylation is epigenetic factor, so in this study epigenetic variation was assessed based on change in DNA methylation level. For this analysis, *in vitro* regenerated plantlets of sugarcane genotypes that showed genetic variability in RAPD analysis (our unpublished data) were investigated for DNA methylation in comparison with their respective parent genotypes (CPF-247 and CPF-248field grown plants). Hence, to determine cytosine and methylcytosine concentrations in DNA hydrolysate of samples (*in vitro* regenerated plants and their parents) field grown plants of sugarcane genotype CPF-247 and CPF-248) standard curves of cytosine and methylcytosine (as standards) were developed. Thus, serial dilutions of standards were injected into HPLC system to get chromatogram, imparting peak area (Y) as presented in Table 1 for generating standard curves (Fig 1a & 1b).

Table 1. Peak areas of cytosine and methylcytosine concentrations.

Concentration	Peak area(cytosine concentrations)	Peak area(methylcytosine concentrations)
5000	500	38.6435
1000	95	4.499
200	21	2.916
40	19	2.131
8	9	1.692

Regression equation, y=a+bx (where, y, Peak area; x, Concentration; a, Intercept; b, slope) was used to determine cytosine and methylcytosine concentrations in each sample. Hence, the regression equations generated by software were, y=0.098x+5.767 (for cytosine) and y=131.87x-65.959 (for methylcytosine). Y values (peak area) of parent plant (field grown plants of sugarcane genotypes, CPF-247 and CPF-248) and their *in vitro* regenerated plantlets were obtained by injecting the hydrolysate of these samples in HPLC (Table 2 & 3).

Thereby, putting the value of peak area in lieu of 'y', concentration (x) of each sample is obtained.

By using calibration curves of both standards, comparison of peak areas at similar retention times was made for relative quantification. Percentage of 5MeC in each sample was calculated using the formula given by Gao *et al.* (2014); **"***Concentration of 5MeC/ (concentration of 5MeC + cytosine)".*

Table 2. Peak areas for cytosine and concentration of cytosine.

Samples	Peak area (Y) for cytosine	Cytosine concentration
Field grown plant of CPF-247	159	1563.60 µg/ml
in vitro regenerated plantlet of CPF-247	280	2798.29 µg/ml
Field grown plant of CPF-248	232.8680	2317.35 µg/ml
in vitro regenerated plantlet of CPF-248	6.9040	11.602 µg/ml

Genotype CPF-247

In field grown plant Concentration of 5methyl cytosine (5MeC) = 0.0345%

[*Calculation:*0.541/0.541+1563.60= 0.000345 µg/ml = 0.0345%]

In in vitro regenerated plantlet Concentration of 5methyl cytosine (5MeC) = 0.258%

[*Calculation:*7.241/7.241+2798.29= 0.00258 µg/ml = 0.258%].

Genotype CPF-248

In field grown plant Concentration of 5methyl cytosine (5MeC) = 0.0261%

[*Calculation:* 0.605/ 0.605+2317.35 = 0.000261 µg/ml = 0.0261%]

In in vitro regenerated plantlet Concentration of 5methyl cytosine (5MeC) = 4.39%

[*Calculation:*0.533/0.533+11.602 = 0.0439 µg/ml = 4.39%].

HPLC analysis of genotypes, CPF-247 and CPF-248 (field grown plant) and their *in vitro* regenerated plant showed difference in DNA methylation level. According to results, 0.258% and 0.0345% cytosine methylation was estimated in in vitro regenerated plantlet and field grown plant of CPF-247 respectively. Whereas, 4.39% and 0.0261% DNA methylation was detected in case of in vitro regenerated plantlet and field grown plant of CPF-248 respectively. There is an increase in DNA methylation level in case of in vitro regenerated plantlets of both genotypes. In this study, in vitro regenerated and field grown plants of sugarcane genotype CPF-247 and CPF-248 were assessed and analyzed for the level of cytosine and methylcytosine.

The results showed that level of methylcytosine was less in field-grown plant while tissue culture brought increase in methylcytosine level. Hence it could be predicted that is tissue culture regime, cytosine of in vitro regenerated plants got methylated which led to decrease in cytosine concentration.

Table 3. Peak areas for methylcytosine and concentration of methylcytosine.

Samples	Peak area (Y) for methylcytosine	Methylcytosine concentration
Field grown plant of CPF-247	5.484	0.541 µg/ml
in vitro regenerated plantlet of CPF-247	888.929	7.241 µg/ml
Field grown plant of CPF-248	13.8480	0.605 µg/ml
in vitro regenerated plantlet of CPF-248	4.4335	0.533 µg/ml

Discussion

Results of this study indicated that dissected tissue culture with hormones may bring change in the level of DNA methylation and thereby it could be the reason of epigenetic variation in sugarcane. Rival *et al.* (2013) while working on oil palm tissue culture obtained similar results. They explored the correlation between exposure time of tissue under *in vitro* regime and epigenetic stability. They reported hypermethylation of DNA of *in vitro* regenerated

plants with long time on tissue culture media. Kaeppler *et al.,* (2000) also documented cytogenetic instability in somaclonal variants due to epigenetic variation in genome o in vitro proliferated sapling. It is well known transgenic plants usually present different epigenetic phenomenon compared to the wild genotypes, and DNA methylation is an important variation during tissue culture (Neelakandan and Wang, 2012). Variation of DNA methylation during tissue culture of *B. napus*, and reported the influence of hormone to methylation levels.

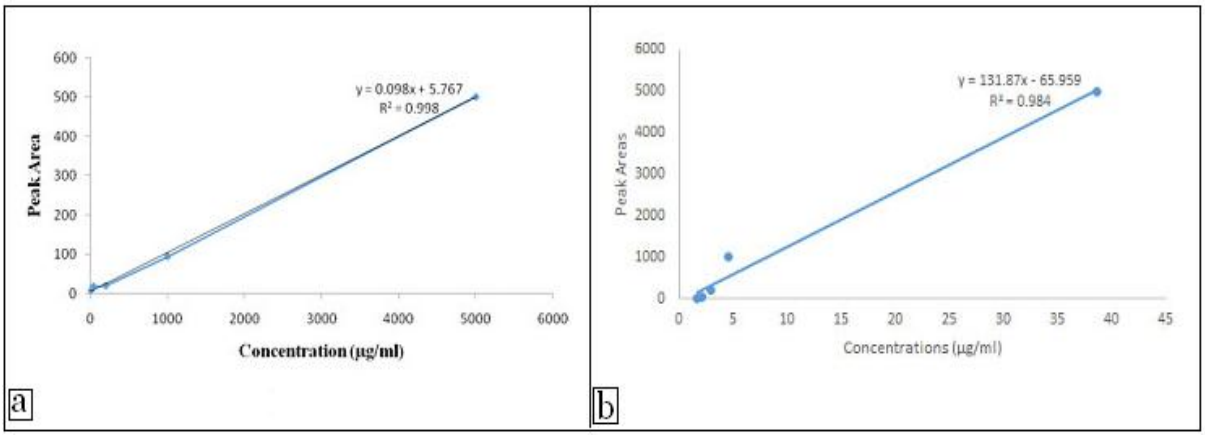

Fig. 1. Standard curves generated for determining the concentration of both standards (cytosine and methylcytosine) (a) Standard curve for cytosine concentration (b) Standard curve for methylcytosine concentration.

This is inconsistent with Huang *et al.* (2012) who proposed the positive correlation between DNA methylation level and concentration of 2,4D, the negative correlation between DNA methylation level and concentration of 6BA as well, during callus induction of *Malus xiaojinensis*. Another scientist Loschiavo *et al.* (1989) reported the increased methylation level with 2,4D concentration, when studying the embryogenesis of carrot.

Based on the HPLC analysis, Gao *et al.* (2014) found the DNA methylation varied obviously during callus induction. Starting with the lowest methylation level (4.33%) at 6 days, two peaks of DNA methylation were detected at 12 days (23.17%) and 30 d (38.7%), respectively. As to DNA methylation during callus induction and differentiation, Gao *et al.* (2014) observed the methylation level generally increases with the elongation of induction time, except for few time points such as 12 d after induction.

The same variation of methylation has been reported in suspension culture of *E. guineensis* (Rival *et al.*, 2013). Fraga *et al.* (2002) reported different methylation level during the life cycle of pine tree as well. For tissue culture of *Cichorium intybus*, DNA methylation level in root tissue is 10–16%, which increases to a maximum level during in vitro culture, and decreases afterwards.

Whereas, methylation level of the shoot tip keeps in 8–13% during developing stage, and increased to 16.6% afterwards (Demeulemeester *et al.,* 1999).

Conclusion

Tissue culture system may bring genetic and epigenetic variations in plants. While for genetic transformation of a plant, it is pre-requisite, the variation must be due to transgene integration and not be due to other factors that could be the tissue culture regime for plant development. Hence, an ideal and proficient *in vitro* regeneration system is, that does not bring any genetic and epigenetic variations. Thus, while establishing tissue culture system it should be considered to evaluate and monitor the established tissue culture system for determining epigenetic variation such DNA methylation.

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