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DNA METHYLATION PATTERNS ARE DIFFERENTLY AFFECTED BY PLANTING DENSITY IN MAIZE INBREDS AND THEIR HYBRIDS

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ABSTRACT - Hybrid vigor or heterosis refers to the superiority in one or more characters of crossbred organisms relative to their inbred parents. This superiority can be related to increase in size or growth rate and increase in yield. While the biological basis of heterosis remains unknown, accumulated data clearly suggest the significance of quantitative regulation of gene expression in heterotic phenomena. DNA methylation is an epigenetic, genome-wide general regulatory mechanism that affects the expression of many genes important for the manifestation of heterosis. According to previous studies DNA methylation in maize varies among different genotypes (parental inbred lines and hybrids) and developmental stages. Our recent studies indicate that growth conditions affect the level and pattern of DNA methylation. Parental inbred lines and hybrids were grown under two different plant densities: 1.5m (spread) and 0.25m (dense) distance between individual plants with a density of 0.513 plants/m^2 and 18.5 plants/m^2, respectively. The effect of density-induced stress on the pattern of methylation in certain sites of DNA was examined using the Coupled Restriction Enzyme Digestion and Random Amplification (CRED-RA) technique. The results suggested that hybrids are more resistant to density-related methylation alterations in comparison to their parental inbreds.

KEY WORDS: Heterosis; Methylation; Stress; CRED-RA.

INTRODUCTION

Heterosis is a genetic phenomenon, in which hybrids manifest superiority over the inbred parental genotypes for several quantitative characters including yield. While plant breeders and agronomists achieved an impressive increase in maize yield utilizing heterosis, the biological basis of the phenomenon remains unknown. Previous attempts to understand physiological and biochemical aspects of heterosis demonstrated that maize hybrids possess superior physiological and biochemical properties than those of the parental lines (for review see TSAFTARIS, 1995). Farmers prefer F1 hybrids for their high and stable yield while growing in different fields and in different years. Genetic stability (homeostasis) refers to reduced genotype X environment interaction, particularly under stress conditions. The selection of adapted single crosses with high yield and high stability in high planting density has been responsible for the consistent increase in maize yields in USA since their introduction in the early 1960s (DUVICK, 1997; FASOULA and FASOULA, 1997; JANICK, 1998; TSAFTARIS and POLIDOROS, 2000). High planting density is considered as a stress condition and it is worthy to examine the parameters involved in hybrid resistance to density-induced stress.

Research performed in different laboratories clearly suggests the significance of regulation of gene expression in manifestation of complicated phenomena such as heterosis (TSAFTARIS and POLIDOROS, 1993; DE VIENNE *et al.*, 1996; TSAFTARIS and KAFKA, 1998; SONG and MESSING, 2003; WU *et al.*, 2003). One mechanism involved in regulation of gene expression is DNA methylation. Nuclear DNA is associated with histone proteins in a highly organized chromatin structure that can be divided into active euchromatin and heterochromatin, which is largely transcriptionally inert. Both forms of chromatin have distinct structural and biochemical properties that are thought to provide epigenetic marks for the formation of higher order structure and for maintaining gene activity and/or gene silencing. DNA methylation, along with diverse covalent modifications of histones and specific associations with non-histone proteins are the major determinants of

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chromatin states. The recent discovery of small RNAs able to direct DNA methylation and histone modification has provided a model for regulation of gene activity where RNA molecules play a central role (for review see TARIQ and PASZKOWSKI, 2004). Methylation in plants occurs in cytosine residues within the dinucleotide sequence CpG and the trinucleotide CpNpG (N is equal to any nucleotide except G). The extent and distribution of genomic DNA methylation is correlated with the rate of expression of many genes examined not only in plants but in all higher organisms (MATZKE *et al.*, 1989; BIRD, 2002).

Recently, it has been recognized that DNA methylation has significant implications in plant breeding, too (TSAFTARIS and POLIDOROS, 2000). In the past, genetic variation was considered only as a result of alterations in the primary nucleotide sequences due to mutations and/or gene recombination. However, it is now established that epigenetic systems like DNA methylation could generate epigenetic variation that had never been considered in plant breeding as a source of phenotypic variation (SHAKED *et al.*, 2001; HAN *et a*l., 2003). DNA methylation in maize was found to be genotype, tissue and developmental stage specific (TSAFTARIS *et al.*, 1999). In addition to endogenous (genetic) mechanisms affecting the level of DNA methylation in different tissues and developmental stages, external (environmental) signals also affect the extent of DNA methylation by their interaction with the plant (SHERMAN and TALBERT, 2002; STEWARD *et al.*, 2002). Moreover, stressful growth conditions result in more methylated DNA (less expressed) and, in general, vigorous hybrids are more resistant to such density induced methylation and suppression of genome activity in their genomic DNA (TSAFTARIS and POLIDOROS, 2000). Thus, at least to some extent methylation may underlie some heterotic phenomena.

The aim of this study was to estimate how different planting densities could affect DNA methylation in maize inbred lines and their offspring hybrids, as this could have significant implications in understanding heterosis and stability of hybrid performance. Taking advantage of the honeycomb design of planting (FASOULAS and FASOULA, 1995), and using different sets of inbreds and hybrids we estimated the effects of growth conditions (spread / dense planting) on site-specific DNA methylation with the coupled restriction enzyme digestion and random amplification (CRED-RA) of genomic DNA (CAI *et al.*, 1996). This technique allows amplification of a DNA fragment containing a cut site of a methylation sensitive restriction enzyme, only when the cytosine residue within the site is methylated and protected from digestion. Thus, presence of a PCR band reveals a methylated site(s). Unmethylated site(s) lead to absence of the amplicon (that would be present in uncut DNA) due to a digestion event(s). We report here our results indicating significant methylation differences between inbreds and hybrids, revealing that hybrids are more stable in comparison to their parents.

MATERIALS AND METHODS

Plant material

Plant material consisted of 6 sets of genotypes (Table 1). Each set included two inbred lines bred from the F2 population of the commercial hybrid PR 3183, (Pioneer Hi-Bred, Des Moines, IA) along with their respective hybrid (TOKATLIDIS *et al.*, 1998). In order to study the impact of planting density on DNA methylation, we estimated DNA methylation in 2 month old plants. This developmental stage was selected for two reasons: first because plants have grown enough to undergo density stress in dense planting; and second, in previous studies, we have shown that the highest differentiation in total methylation between different genotypes occurs at this time point (TSAFTARIS *et al.*, 1997). Plants were grown in the field under two different densities: 1.5m (spread) and 0.25m (dense) distance between individual plants with a density of 0.513 plants/m^2 and 18.5 plants/m^2 respectively, using the honeycomb design (FASOULAS and FASOULA, 1995). All the necessary cultivation treatments for plant growth under optimal conditions were performed. Sampling in the field was performed in two consecutive experiments in two years.

CRED-RA assay

Genomic DNA was isolated from the three upper leaves of each genotype according to DELLAPORTA *et. al.* (1983). DNA quantity was estimated spectrophotometrically and its quality was examined in 1.2% agarose electrophoresis. DNA (1 μg) was used for HpaII digestion. After digestion the sample was phenol/chloroform extracted and DNA was ethanol precipitated and resuspended in autoclaved distilled water. An estimated 25-50 ng of digested DNA was added as PCR template. Each set of plant material was tested with 20 random 10mer primers (AB-0320-kit 9 and10, Advanced Biotechnologies, Surrey, UK). Positive control of uncut DNA from one of the materials and negative control with no DNA was tested for each of the primers. PCR was performed according to the following conditions: denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, and extension at 72°C for 2 min. The final extension step was 7 min to ensure completion of PCR in every synthesized product. In order to simplify comparisons and avoid complications due to genotype polymorphisms, PCR products were run on 1.2% agarose gels in sets of two parental inbreds and their respective hybrid, in spread and dense conditions, for each primer tested. Appearance of a PCR band in a genotype in one condition, which is not present in the other

condition, implies that HpaII did not cleave the respective recognition site in the former condition. Consequently, the cytosine residues of the HpaII recognition site were considered to be methylated. Random repetition of a subset of the reactions revealed 100% reproducibility of the results.

Statistical analysis

Analysis of data was performed using the z criterion for comparing the percentages of new methylated sites in inbred lines and hybrids under different planting conditions.

RESULTS AND DISCUSSION

The aim of this study was to record, evaluate, and compare methylation alterations in inbred lines and their hybrids when planted under spread and dense planting conditions. A representative example of recording HpaII site methylation alterations in a set of materials (1st set, Table 1) with one primer is shown in Fig. 1. A 630 bp amplicon absent in spread planting in the inbred B11-19, appeared under dense planting, demonstrating that one (or more) HpaII site(s) was unmethylated (thus cleaved) in spread planting while it was methylated (not cleaved) in dense planting. The inbred A11-7 and the hybrid did not show any changes in the band pattern under both planting densities. Table 2 summarizes the occurrence of methylated sites in both spread and dense planting regarding all the genotype and primer combinations tested. This table summarizes the results of 720 different PCRs (18 genotypes X 20 primers X 2 planting densities). Corrections of the observed numbers in inbreds were made to take into account that they resulted after testing twice as many genotypes than hybrids.

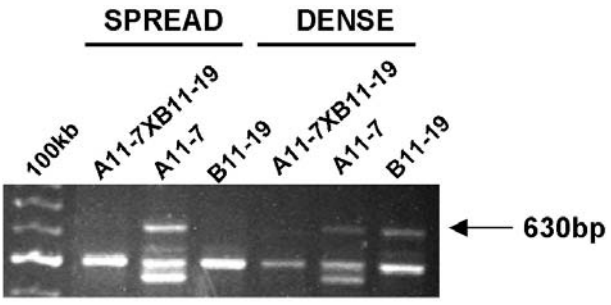

FIGURE 1 - An example of the CRED-RA analysis using one set of genotypes (two inbred lines A11-7 and B11-19 and their hybrid A11-7XB11-19), grown under spread and dense conditions. The B11-19 inbred line showed a new band at 630bp (denoting presence of one or more methylated HpaII sites). The inbred A11-7 and the hybrid maintained the same pattern in both spread and dense planting.

TABLE 1 - *Sets of plant material involving inbreds and their hybrid used for CRED-RA analysis.*

Set	Parental Inbreds		Hybrid
1st	A11-7	B11-19	A11-7 X B11-19
2nd	A5-8	B4-12	A5-8 X B4-12
3rd	A9-6	B10-19	A9-6X B10-19
4th	A3-10	B11-1	A3-10 X B11-1
5th	A7-14	B12-13	A7-14X B12-13
6th	A9-6	B5-19	A9-6X B5-19

TABLE 2 - *Occurrence of methylated sites only in spread, only in dense planting, and as total, in inbred lines and their hybrids. Observed is the number of bands recorded in each condition. For inbreds this number was divided by 2 and is shown in the corrected column, to take into account that it resulted after testing twice as many genotypes in comparison to hybrids. The percentage (%) column displays percentage of bands in each category in relation to the total number of bands recorded in this study. Percentages were estimated from the corrected value in inbreds and the observed in hybrids to the corrected total which is 45 (29 inbreds + 16 hybrids).*

	Inbreds			**Hybrids**	
	Observed	Corrected	%	Observed	%
Dense	48	24	53.4	11	24.4
Spread	10	5	11.1	5	11.1
Total	58	29	64.5	16	35.5

Statistical comparisons of genotypes under the two different planting conditions are shown in Table 3. Overall, the data show that hybrids experience significantly less methylation alterations in comparison to their parental inbred lines ($z=2,87>P_{0.01}$). Comparing the two planting conditions in inbreds, it was found that the percentage of methylated sites was significantly higher under dense than under spread planting (z=4,81>P0.01), while in hybrids the difference was not significant ($z=1,67<P_{0.05}$). Comparing inbreds and hybrids in each planting condition, it was estimated that significantly higher percentage of methylated sites occurred in dense planting conditions in the inbreds ($z=2,95>P_{0.01}$), while in spaced planting the difference between inbreds and hybrids was not signifi-

TABLE 3 - *Comparison of the percentage of methylated sites recorded in inbreds and hybrids under spread and dense planting. Statistical significance using the z criterion is indicated by two asterisks (at P0.01) or NS when non-significant. In the comparison column each condition/genotype combination is denoted by two letters meaning D: dense, S: spread, T: total (dense+spread), I: inbred, H: hybrid.*

Comparison	Z	Significance level
DI-DH	2,95	* *
SI-SH	0	NS
TI-TH	2,87	* *
DI-SI	4,81	* *
DH-SH	1,67	NS
DI-TI	–1,07	NS
SI-TI	–6,25	* *
DH-TH	–1,15	NS
SH-TH	–2,86	* *

cant ($z=0<P_{0.05}$). Consequently, when comparing the percentage of methylation changes in dense or spread planting with the total methylation changes in inbreds or hybrids, it was obvious that most of the total methylation alteration was due to increased methylation under dense planting (Table 3). In conclusion, inbred lines experience high percentage of induced methylation under dense planting, while hybrids are more stable under both planting conditions. Additionally, hybrids tend to inherit the methylation condition from the parent that shows the lower level of methylation as shown, for example, in Fig 1.

These data are in agreement with results from other studies revealing an impact of the environmental conditions on DNA methylation. Temperature changes, for instance, altered the activity and methylation state of the transposon *Tam3* in *Antirrhinum* (HASHIDA *et al.*, 2003). Planting density also affected the methylation state of the *Ac* element in maize (TSAFTARIS and KAFKA, 1998). Results obtained for three consecutive years revealed that demethylation (activation) of a methylated *Ac* element was significantly more frequent in plants grown under spread than dense planting. In addition, total DNA methylation measured by High Pressure Liquid Chromatography (HPLC) increased in inbred lines but remained stable in hybrids when compared in plants grown under spread and dense planting conditions (TSAFTARIS *et al.*, 1998).

Resistance of hybrids in induced genome methylation alterations under different stresses could be at the core of high and, maybe more importantly, F1 stable yield, especially if critical cytosine residues (e.g. regulatory genes, promoter regions of protein-coding genes) are preferably involved. Evidence has been provided that developmental changes of DNA methylation and chromatin structure at, or close to, the promoter region of a gene are responsible for epigenetic regulation of expression (HOEKENGA *et. al.*, 2000). Other regions of the genome, (e.g. heterochromatic DNA), remain highly methylated permanently, throughout plant growth. This could explain why slight changes of total methylation are concomitant with significant changes in gene expression. Results from this study support the hypothesis that hybrids perform better than inbred lines as they resist alterations in methylation under stress.

Obviously, experimental validation of this hypothesis requires analysis of higher number of possibly methylated sites than those examined. In subsequent studies we have used Restriction Landmark Genomic Scanning (RLGS) that is a method capable of screening in a single assay the methylation status of more than a thousand genes. The RLGS analysis agreed with this study demonstrating that a stable hybrid had a stable methylation pattern, that did not change significantly under stress (Kovacevic, Polidoros and Tsaftaris, unpublished results). Further experiments are underway to identify genomic regions and critical genes whose methylation is differentially affected by planting density in inbred lines and hybrids, and examine their role in the manifestation of heterosis and stability of hybrid performance.

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