

THE USE OF RESTRICTION LANDMARK GENOME SCANNING (RLGS) FOR ASSESSMENT OF *NOI*-SITE METHYLATION IN MAIZE

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ABSTRACT - Epigenetic phenomena and particularly DNA methylation play a significant role in regulating gene expression in eukaryotes. Restriction Landmark Genomic Scanning (RLGS), which in a single assay displays the methylation status of more than a thousand genomic regions containing *NoI* recognition sites, was used to evaluate the status of global methylation in maize parental inbred lines and hybrids, and the effects of density induced stress on the methylation status of a hybrid selected for its stability of performance. Examining the RLGS patterns of two S_5 inbreds it was determined that they differed in total DNA methylation by 20%. Their F_1 hybrid displayed methylation patterns similar to its parents for the majority of the detected spots, although some *NoI* landmarks were genotype-specific. Comparison of the RLGS profile of a stable F_1 hybrid grown under low and high planting density (density induced stress) showed that its methylation pattern was influenced by conditions of growth. However, density induced stress caused only a slight increase in the copy number of methylated fragments, indicating that the stable hybrid could be resistant to stress-induced methylation changes. Our results suggest that different levels of global genomic DNA methylation might be related to the field performance potential of specific maize inbred lines and hybrids, and resistance of the hybrids to methylation changes induced by density stress could be part of hybrid superiority and stability of performance facing different stress conditions.

KEY WORDS: Heterosis; Maize; Methylation; RLGS; Stress.

INTRODUCTION

Epigenetic phenomena and particularly DNA methylation are receiving much attention and complement classical genetics in many fields including

molecular and developmental biology, epidemiology, cancer biology, breeding and evolution. DNA methylation, catalyzed by DNA methyltransferases (FINNEGAN and KOVAC, 2000), is observed in such distinct organisms as bacteria and humans. The most abundant methylated base in eukaryotes, 5-methylcytosine, acts together with other chromatin modifications to maintain the silent state of some genomic regions, enabling the transcription only of genes that are required for cell activity (RAZIN, 1998). Consequently, changes in DNA methylation patterns are hallmarks of development, evolutionary adaptation, defense and disease in organisms. For example, in plants altered genomic cytosine methylation has been correlated with processes as different as gametic imprinting, paramutation, phenotypic variation, stress responses, and changes in the activity of transposable elements and transgenes in transgenic plants (FINNEGAN *et al.*, 2000).

Environmental factors induce DNA methylation changes that can be transmitted to progeny (KAEPPLER and PHILLIPS, 1993). In addition to being inherited, genomic methylation is reversible, which makes it a prime candidate adaptational mechanism. Adaptation and stability of performance under changing environmental conditions, which are usually faced by plants during their life cycle, is of utmost importance for stress tolerance and a steady target of plant breeding efforts (DUVICK, 1992). Recent data suggest that grain yielding ability and stability of performance in widely successful maize hybrids is due primarily to improved stresses tolerance and particularly the stress imposed by high density planting (for review see TSAFTARIS and POLIDOROS, 2000). Therefore, defining the role of DNA methylation in stable genomic responses to stress is of fundamental importance for plant breeding programs.

Hybrids, the progeny of crossing diverse inbred lines, exhibit greater biomass, speed of develop-

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ment, and yield than both parents. This phenomenon termed heterosis (a quantitative measurement expressed as the ratio of hybrid performance to the best parent or mid-parental performance) or hybrid vigor (a term describing the better performance of the hybrid) has been exploited extensively in crop production and has been a powerful force in plant evolution. The genetic basis of heterosis has been investigated for nearly a century, mainly examining the participation of dominance versus overdominance in the manifestation of heterosis, but little consensus has emerged. To formulate a molecular model of heterosis, simple broad alternatives need to be tested so that more refined and targeted hypothesis testing can focus on the detailed mechanism (BIRCHLER *et al.*, 2003). Recent biochemical and molecular investigations on heterosis indicate that quantitative variation of gene expression may be important in vigor manifestation (for review see TSAFTARIS and POLIDOROS, 2000; BIRCHLER *et al.*, 2003). These results suggest a shift in gene regulation in hybrids and support the significance of regulatory mechanisms involved in the quantitative modulation of gene expression particularly in global ways.

Epigenetic mechanisms, such as chromatin remodeling, RNA interference and particularly DNA methylation could be considered as genome-wide general regulatory mechanisms that affect the expression of many genes important for the manifestation of heterosis (TSAFTARIS *et al.*, 1998; TSAFTARIS and KAFKA, 1998; TSAFTARIS *et al.*, 1997; PHILLIPS, 1999). In maize, DNA methylation patterns differ between tissues, developmental stages and are influenced by growth conditions (TSAFTARIS *et al.*, 1998; TSAFTARIS and KAFKA, 1998; TSAFTARIS *et al.*, 1997; BANKS *et al.*, 1988; ROSSI *et al.*, 1997; STEWARD *et al.*, 2002; STEWARD *et al.*, 2000; COCCIOLONE *et al.*, 2001; STURARO and VIOTTI, 2001; TANI *et al.*, in press). Results from several studies employing global methylation measurements using high performance liquid chromatography (HPLC) and local methylation of random sequences using coupled restriction enzyme digestion - rapid amplification (CRED-RA) in our lab indicated that: (a) hybrids are, in general, less methylated than their parental inbreds; (b) heterotic hybrids are less methylated than related non-heterotic hybrids; (c) old, low-yielding inbreds are more methylated; (d) most modern inbreds, especially those selected for high and stable yield under low density planting in the absence of competition (FASOULA and FASOULA, 1997; FASOULA and FASOULA, 1995), have lower percentage of methylation in comparison with old prog-

enitor lines (TSAFTARIS and POLIDOROS, 2000; TSAFTARIS *et al.*, 1998; TSAFTARIS and KAFKA, 1998; TSAFTARIS *et al.*, 1997; TSAFTARIS *et al.*, 2001). It was also shown using the CRED-RA technique, that inbreds displayed a higher percentage of methylation changes compared to hybrids growing in low density versus high-density conditions (TANI *et al.*, in press).

These findings support the hypotheses made by several researchers (reviewed in STUBER, 1998) that systematic selfing for development of inbred lines, with emphasis only to combining ability of inbreds (leading to line inbreeding depression), could also lead to gradual accumulation of more methylated sites, which possibly will then be released and/or re-patterned when the selfed lines are crossed to generate hybrids. Selection of inbred lines could moderate line-inbreeding depression (TOKATLIDIS *et al.*, 1998; TSAFTARIS *et al.*, 2001). While approaching homozygosity, selecting with emphasis in line performance per se, degenerative alleles (possibly methylated and/or even mutated genes) could be constantly selected out in homozygous inbreds. The efficiency of this kind of selection in inbreds approaching homozygosity could be correlated with the lower methylation levels of plants selected for line performance per se (TSAFTARIS *et al.*, 1998; TSAFTARIS and KAFKA, 1998; TSAFTARIS *et al.*, 1997).

To further test these hypotheses and analyze global DNA methylation patterns in maize we used the optimized Restriction Landmark Genomic Scanning (RLGS) method. The RLGS uses methylation-sensitive restriction enzyme sites as landmarks and allows simultaneous analyses of the methylation status and copy number of thousands of CpG sites in a single assay (HATADA *et al.*, 1991). We estimated the polymorphism of methylation patterns in inbred lines and hybrids. We also examined the effects of high-density growth on their global genome methylation status in a maize hybrid selected for its stable performance.

MATERIALS AND METHODS

Plant material, growth conditions and yield measurements

Plant material used in this study was a) the old maize hybrid B73 x Mo17, b) the S₅ recombinant inbred lines A9-13 and B1-22, derived from the commercial maize hybrid Lorena by honeycomb selection for potential yield per plant in the absence of competition (FASOULAS and FASOULA, 1995) and c) their second cycle F₁ hybrid B1-22 x A9-13 selected for its stable performance.

Plants were grown in a honeycomb pattern (FASOULAS and FASOULA, 1995) in the field at either low density (LD) with each plant separated from the other by 1.5 m (i.e., 0.513 plants/m²),

or at high density (HD) with an interplant spacing of 25 cm (i.e., 18.5 plants/m²). The F₁ hybrid B1-22 x A9-13 was used for molecular analyses of density stress experiments. This hybrid has been classified, using existing criteria (FASOULA and FASOULA, 1997), as a stable one regarding its performance under various growth conditions.

DNA extraction and RLGS method

Total DNA was isolated from three apical leaves of 60-day-old plants using a CTAB protocol optimized for rice (http://www.geocities.jp/millet_kawase/RLGS/protocol.html). DNA samples were treated with RNase (Promega) and tested for quality both by spectrophotometrical analysis and electrophoresis.

The RLGS was done as previously described with minor modifications (HATADA *et al.*, 1991). For the first RLGS step, the DNA end labeling, 5 µg of genomic DNA was digested to completion with 50 units of *NotI* and *EcoRV* in NEB buffer 3 (New England Biolabs). The cohesive ends were then filled in using 1.3 units of Sequenase ver. 2.0 (USB) in the presence of 166 nM [α -³²P] dGTP (3000 Ci/mmol) and 83 nM [α -³²P] dCTP (6000 Ci/mmol). The second RLGS step is the 2D electrophoresis of genomic DNA. For the first dimension (1D), we used 0.9% agarose gels (SeaKem GTG, FMC) made in 61 cm long Teflon tubes with a 2.4 mm diameter (Habiateknofour, Sweden). Labeled samples were electrophoresed in the 1D buffer (100 mM Tris-HCl, 40 mM NaOAc, 35 mM NaCl, 3 mM EDTA, pH 8.0) at 100 V for 2 h followed by 230 V for 22 h. The gel rods were then transferred to Falcon tubes, incubated in NEB buffer 3 for 20 minutes, and set into a plastic tray for *in situ* digestion with 6000 units of *PstI* in NEB buffer 3. After digestion was complete, gel was incubated for 10 minutes in the second dimension (2D) buffer (50 mM Tris-HCl, 60 mM boric acid, 1 mM EDTA, pH 8.0). For the separation in the 2D, gels rods were transferred onto 5% polyacrylamide gels that were electrophoresed until the bromphenol blue tracking dye reached the bottom of the gel. The gels were dried on Watman 3MM paper under vacuum at 75°C for 2.5 hours and exposed with intensifying screens at -80°C to Kodak XAR films for 2 to 5 days. All samples were analyzed at least three times and on average more than 1000 spots per gel were observed. The matching of spots was based on non-polymorphic landmark spots. Only spots that were reproducible were taken into account for comparisons. Gels of samples under comparison were running simultaneously under identical conditions. Spot detection and analysis was performed on scanned autoradiograms printed on transparencies that were sequentially superimposed one to another and spot patterns were visually compared in specific areas defined by a grid.

Distribution of *NotI* restrictions sites in maize

Representative GenBank maize sequences were used for analysis of the distribution of *NotI* restrictions sites and CpG islands in the maize genome. A total number of 104 sequences with length more than 10,000 bp were retrieved. From these only defined sequences (that contained at least one repeat, gene or trasposon) were analysed. The total length of analysed sequences was 3,851,434 bp.

For the computational analysis of CpG islands, the CpG island searcher software was used (TAKAI and JONES, 2003). All searches were performed using the lower limit values 500bp for length, 55% for GC content and observed CpG/expected CpG ratio greater than 0.65, i.e. stringent criteria that were shown to lead to a better association between CpG islands and genes (TAKAI and JONES, 2003).

RESULTS

Characterization of global DNA methylation pattern in maize by RLGS analysis

To optimize the RLGS analyses, we used DNA isolated from leaves of either an inbred A9-13 or a hybrid B73 x Mo17 material. The DNA was double-digested with a methylation-sensitive restriction enzyme and a second enzyme that generated DNA fragments of the size suitable for electrophoresis in the first dimension. Similarly to optimized RLGS methods for mammals, rice, tobacco and arabidopsis (HATADA *et al.*, 1991; MATSUYAMA *et al.*, 2000; MATSUYAMA *et al.*, 2003), the methylation-sensitive restriction enzyme *NotI* proved to be the best first landmark restriction enzyme for maize samples. When *EcoRV* was used as a second restriction enzyme, the generated DNA fragments were electrophoretically separated into well-resolved spots. Therefore, we used the *NotI/EcoRV* restriction enzyme cocktail in all further experiments. Before the separation of the genomic DNA in the second dimension, DNA was digested with *PstI*, a C-methylation sensitive enzyme.

To further simplify the RLGS analysis, we omitted the blocking step that prevents the incorporation of radioactive nucleotides in nicks produced during DNA isolation (OKUIZUMI *et al.*, 1997). Labeled nicked DNA fragments accumulated in the upper part of the 2D gel (Fig. 1A) and therefore, each gel required an exposure to multiple layers of film to resolve the RLGS spots of the upper region. Since the presence of a spot corresponds to absence of methylation in the *NotI* restriction enzyme recognition site, *i.e.* it corresponds to a theoretically active gene (WAGNER, 2003; FRANSZ and DE JONG 2002), we concluded that our modified RLGS protocol is sufficiently informative for further analyses of inbred lines and hybrids even without the blocking step. We were able to separate more than 1400 spots in A9-13 and 900 spots in B73 x Mo17 to identify differences distinguishing the two materials.

NotI sites are correlated with genes and CG islands

The frequency and distribution of *NotI* sites and CpG islands in maize were estimated in representative classes of maize genomic sequences sampled from GenBank accessions. Analyzed sequences contained 297 CpG islands located both at 5' and 3' end of genes, transposons and repeats. 64% of CpG islands was associated with genes and 13.5% was as-

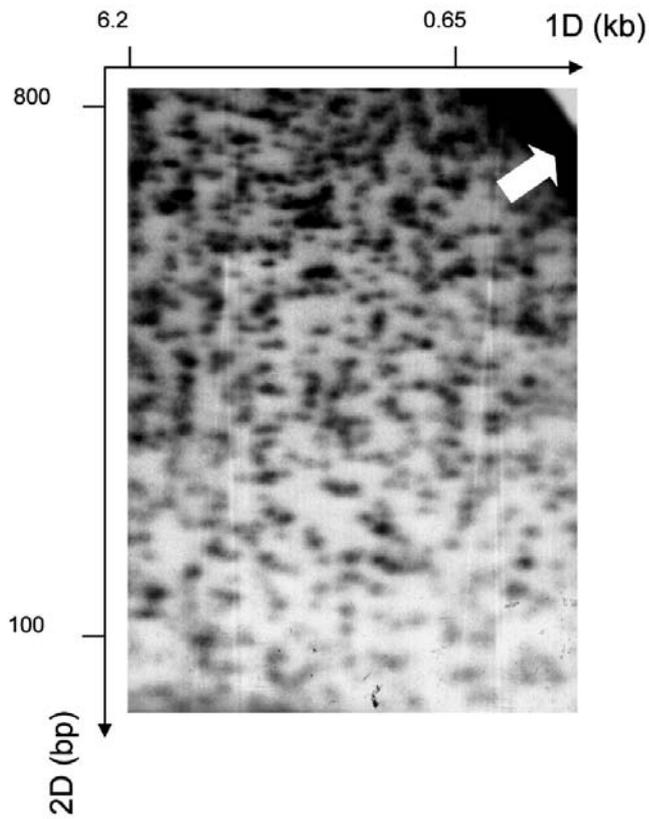
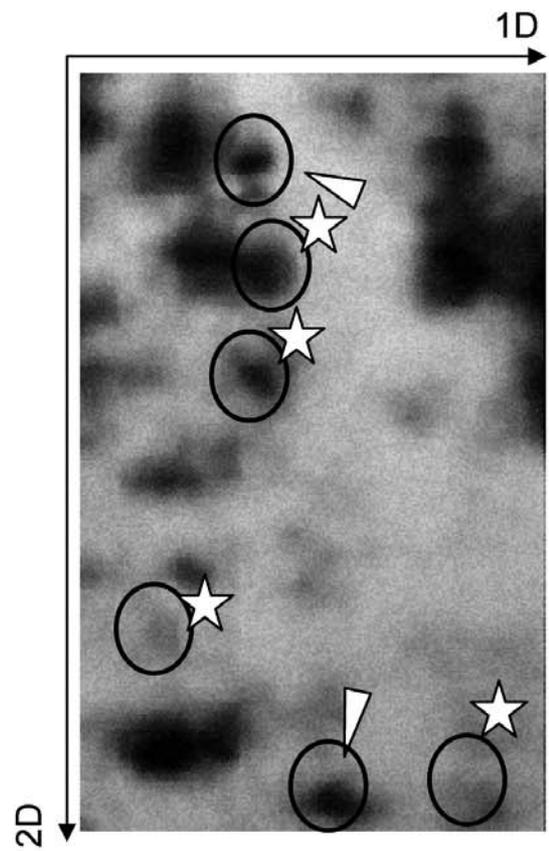
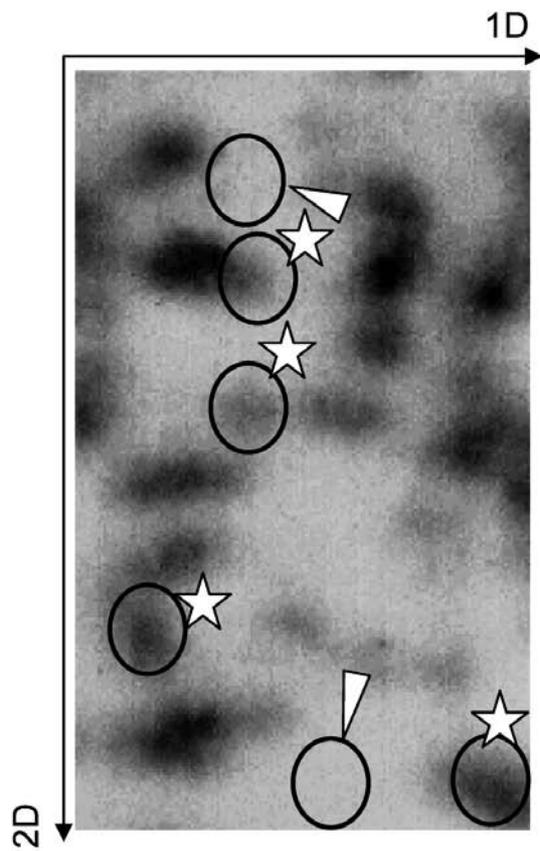


FIGURE 1 - Examples of RLGs profiles and comparisons between different maize materials.

(A) Total DNA from three upper leaves was isolated, digested with *NotI* and *EcoRV*, end-labeled, separated in the first dimension (1D), digested with *PstI* and separated in the second dimension (2D). The arrow points to the gel zone with labeled nicked DNA. The size of the molecular markers used in 1D and 2D is shown on the top and on the left-hand side of the image.

(B) Indicative example of the comparisons of the RLGs profiles among different materials.

Arrowheads mark encircled spots that were detected only in one sample and asterisks mark encircled spots that differ in intensity between samples.



sociated with repetitive DNA. Approximately 22.5% of CpG islands were mapped to transposable elements. From 72 *NotI* sites that were found 65 were associated to CpG islands (90%). Analysis indicated that most *NotI* sites (82%) were correlated with genes, and 90% of the *NotI* sites were found in CpG islands. A small percentage was correlated with repeats and transposable elements (Fig. 2). Thus, analysis of *NotI* landmarks in maize can be used for assessing the methylation status especially in CpG islands within genes.

Comparison of RLGS patterns in recombinant inbred lines

Since RLGS is a powerful method for systematic detection of DNA methylation in numerous genes (MATSUYAMA *et al.*, 2003) we applied this technique to examine the extent of methylation differences in two S₅ recombinant inbred lines A9-13 and B1-22. The A9-13 and B1-22 RLGS images were compared and approximately 80% of spots were found to be identical. The remaining 20% of spots were either genotype-specific or had a different intensity in each inbred line. Therefore, RLGS can be used as a tool to differentiate between inbred lines of similar pedigree and eventually to possibly correlate their specific traits with a molecular marker.

Comparison of DNA methylation between parental inbreds and their F₁ hybrids

We compared the methylation pattern between a hybrid and its parents using the RLGS method. Total genomic DNA was isolated from parental inbred lines A9-13 and B1-22 and their F₁ hybrid B1-22 x A9-13. A summary of the parallel analyses of the RL-

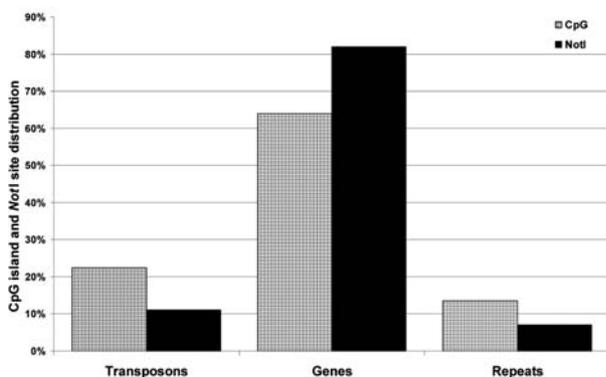


FIGURE 2 - Percentage of CpG islands and *NotI* sites associated with repetitive DNA (repeats), genes and transposable elements (TE).

TABLE 1 - Comparison of A9-13 and B1-22 RLGS profiles with the RLGS profile of their hybrid F₁ B1-22 X A9-13.

Parental lines	Landmarks unique to inbreds	Landmarks unique to the hybrid	Landmarks identical in parents and hybrid
A9-13	6.4%	5.7%	87.9%
B1-22	8.8%	6.7%	84.5%

GS profiles is presented in Table 1. Although, the majority of spots detected in the hybrid could be traced back to one of the parents, significantly more hybrid spots were present also in the male parental line A9-13 ($z=3.484$, $p>0.05$). A number of spots (5-7%) were specific for the hybrid while roughly 5% of the spots of both B1-22 and A9-13 were not present in the hybrid. Therefore, using the RLGS technique, we were able not only to distinguish between methylation patterns of inbred lines and their hybrid, but also to establish that most of the *NotI* landmarks present in the hybrid could also be detected to one or both parents, although a small percentage was selectively methylated in either one of the parents or in the hybrid.

In order to assess if planting density could affect methylation patterns estimated using RLGS we performed the analysis in the hybrid B1-22 x A9-13, grown in LD and HD conditions. Comparison of the RLGS profiles indicated that high-density stress caused changes at the level but not at the pattern of genomic DNA methylation. In the examined sectors of RLGS profile, the number of *NotI* landmarks remained identical under both test conditions. However, approximately 4% of the RLGS spots were more intense in LD-grown plants, suggesting a possible quantitative increase of methylation under stress.

DISCUSSION

Methylation-sensitive restriction enzymes in combination with RLGS are a powerful tool for detection and analysis of differences in methylation patterns at CpG dinucleotides within CpG islands (HATADA *et al.*, 1991; HAYASHIZAKI *et al.*, 1993). Currently in animal model systems, RLGS is used to identify imprinted genes and to analyze hypermethylated CpG islands that are characteristic of vari-

ous types of cancer (KAWAI *et al.*, 1993; MOTIWALA *et al.*, 2003; KREMENSKOY *et al.*, 2003). RLGS protocols have been adapted for some plant species and the Arabidopsis “methylation map” has been established (MATSUYAMA *et al.*, 2000; MATSUYAMA *et al.*, 2003), but this method is not widely used in plants despite its exceptional suitability for epigenetic studies. In this study, we demonstrated that the optimized RLGS could be used as sensitive *NotI*-methylation mapping technique in maize providing information for the methylation status especially in CpG islands within genes.

In plants, 5-methylcytosine is found in CpG and CpNpG sequences (GRUENBAUM *et al.*, 1981). Mendelian inheritance of CpNpG methylation has been reported (VUYLSTEKE *et al.*, 1999). Here, we observed that most of the CpG methylation pattern was also inherited in a similar fashion since about 95% of the spots in the hybrid could be traced to the parents. However, the presence of a parental spot in the hybrid RLGS pattern does not necessarily imply Mendelian inheritance of the methylation pattern. A hypothetical scenario to consider may envisage that a *NotI* site in a gene is methylated in parent A, and unmethylated in parent B, thus represented by a missing spot in A that is present in B. If in the hybrid A X B the alleles exchange methylation status becoming unmethylated if originated from parent A and methylated if originated from parent B a spot should also be present in the hybrid. In the absence of polymorphisms in other restriction sites of employed enzymes (*PstI* and *EcoRV*) the hybrid pattern will appear additive, but there is no inheritance of methylation patterns. By simply looking at the first generation hybrids, it is difficult to rule out this type of scenario.

After digestion with *NotI*, 87.9% of spots in A9-13 and 84.5% of spots in B1-22 were identical with spots in their F₁ hybrid. In addition, we detected a number of polymorphic spots that did not follow Mendelian transmission. Three classes of such spots could be distinguished: *NotI* landmarks present in both parental inbred lines, but absent in the hybrid (4.76%); *NotI* landmarks present in the hybrid but absent in both parents (2.33%); *NotI* landmarks of high intensity present in both parents that displayed a weak intensity in the hybrid (1.33%), suggesting an increase in methylation within the hybrid's tissue that was used for DNA extraction. Similar results of methylation patterns that did not appear to follow simple Mendelian inheritance have been reported in rice (XIONG *et al.*, 1999). The authors suggested that

increased or decreased methylation in the hybrid, compared to parents, might provide an explanation for parent-specific and/or hybrid-specific differential gene expression detected in the tested material.

Some intense parental spots were not detected in the hybrid. Similar results have been obtained in other organisms where the high-intensity non-transmittable spots were identified as ribosomal genes (KIM *et al.*, 2000). The loss of spots that correspond to ribosomal genes of one parent inbred line suggests nucleolar dominance of the other inbred line. Nucleolar dominance is not related to hybrid vigor in maize F₁ hybrids, but it is proof that plants have the capacity for coordinated regulation of genes within chromosomal domains (FINNEGAN *et al.*, 2000).

The existence of both demethylated and newly methylated fragments in the F₁ hybrid supports the original hypothesis that methylation can be released and/or repatterned when inbred lines are crossed to generate hybrids. DNA methylation is a regulatory mechanism that affects the expression of many genes many of which may be important for the manifestation of heterosis (BIRCHLER *et al.*, 2003; TSAFTARIS and KAFKA, 1998). Since our experiments show that part of the methylation inheritance is not Mendelian, novel regulatory circuits related with this repatterned DNA methylation may be formed in the hybrid that may account for quantitative variation in gene expression observed in many other studies (TSAFTARIS and KAFKA, 1998; ROMAGNOLI *et al.*, 1990; LEONARDI *et al.*, 1991; DAMERVAL *et al.*, 1994, reviewed in TSAFTARIS and POLIDOROS, 2000).

When maize seedlings were exposed to cold stress, a genome-wide demethylation occurred in root tissues, suggesting that DNA methylation functions as a common switch of gene expression and that naturally induced changes in DNA methylation may result in heritable epigenetic modification of gene expression (ROSSI *et al.*, 1997). However, other studies revealed methylation increases in the DNA of pea root tips exposed to water deficit, which specifically for the second cytosine of the CCGG target sequence assayed by the MSAP technique, accounted for about 40% of total sites investigated (LABRA *et al.*, 2002). Our data indicated that methylation changes in a stable hybrid growing under HD planting stress were detected as a slight quantitative increase in methylation, which in line with our previous studies indicating that hybrids are more resistant than inbreds to site-specific methylation changes under stress (TSAFTARIS *et al.*, 2001; TANI *et*

al., in press). It is possible that stability of performance is related with increased resistance to changes in methylation patterns under stress, which could explain the differences observed between inbreds and hybrids, as well as between hybrids and the stable hybrid examined in this study.

Overall, data presented here point to a possible involvement of methylation in manifestation of hybrid vigor, and encourage further study of the role of epigenetic inheritance in heterosis, vigor and inbreeding depression.

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