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Tissue culture-induced DNA methylation variation in maize

(somaclonal variation/epigenetic variation/*Zea mays*)

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ABSTRACT **Twenty-one progeny lines derived from tissue cultures of two embryo sources of maize inbred strain A188 were examined for DNA methylation changes. Total DNA was cut with the isoschizomers *Hpa* II and *Msp* I and probed with 18 single-copy *Pst* I genomic clones and two cDNA clones. Eight of these probes could detect both increases and decreases in methylation. With these probes 39% of the families were found to contain an altered methylation pattern. All changes represented a decrease in methylation. The other 12 probes could detect only increases in methylation; no methylation variation was seen with these probes. Fifteen percent of the methylation changes were homozygous in the original regenerated plant. Changes were stably inherited upon two generations of self-pollination. No sequence variation was observed in *Msp* I-digested DNA from the same 21 progeny lines. Certain probes detected methylation changes much more often than others. Our study provides evidence that demethylation occurs at a high frequency and could be an important cause of tissue culture-induced variation. Occurrence of the frequent homozygous alterations in original regenerated plants implies a nonrandom mutational mechanism.**

The mutagenic nature of tissue culture has been extensively documented and reviewed (1–5). In maize this mutagenesis is manifested as qualitative mutations (6–11, †), quantitative trait variation (11–13), cytological abnormalities usually resulting from chromosome breakage (14–19), and the activation of transposable elements (20–22, ‡, §). A hypothesis to explain the underlying basis of tissue culture-induced mutagenesis must contain mechanisms to explain the high frequency of all of the above types of variation. The hypothesis we are testing, described by Phillips *et al.* (23), states that variation in DNA methylation could be a principal factor in tissue culture-induced mutagenesis. Methylation changes might effect variation in several ways. DNA methylation changes could result in chromatin structure alterations. Such alterations may lead to late replication of heterochromatin and, therefore, to chromosome breakage, and changes in gene expression. Methylation variation at specific sites also could result in changes in gene expression in either a positive (e.g., transposable element activation) or a negative fashion.

A negative correlation between DNA methylation and gene expression generally exists in eukaryotes (24, 25). The relationship between DNA hypomethylation and transposable-element activation has been described (26–28). DNA methylation is also correlated with chromatin structure. The heterochromatic inactive X chromosome in mammalian females is highly methylated (29). Klaas and Amasino (30) have shown that DNase I-sensitive regions, which are usually euchromatic, are undermethylated relative to total DNA in pea, barley, and maize.

Brown (31) and Brown *et al.* (32), using Southern analysis, found that DNA methylation and base sequence changes are frequent in maize callus and among regenerated maize plants. Brown *et al.* (33) and Müller *et al.* (34) have also found a high frequency of methylation and sequence variation among progeny of regenerated rice plants.

The purpose of this study was to analyze the nature, inheritance, and frequency of DNA methylation changes among progeny of regenerated maize plants.

MATERIALS AND METHODS

In a previous study (35), R_1 seed was produced in the following manner (see also Fig. 1). A plant of maize inbred strain A188 was selfed and two embryos (I and J) were induced to form embryogenic callus. The callus was maintained on modified Murashige–Skoog medium (36) for 7 months, at which time plants were regenerated (R_0) and grown to maturity in a glasshouse. R_0-derived R_1 seed produced by selfing these regenerated plants was used in this study. Nine R_0-derived R_1 families from embryo source I and 13 R_0-derived R_1 families from embryo source J were evaluated. The plants were grown for 4 weeks in a glasshouse. The above-ground portions of five plants from each family as well as 15 noncultured control plants were individually harvested and lyophilized. DNA was extracted by the cetyltrimethylammonium bromide extraction procedure (37). The DNA was incubated overnight with 3 units of restriction enzyme per microgram to ensure complete digestion. *Hpa* II was the methylation-sensitive restriction enzyme used to cut the DNA; the isoschizomer *Msp* I was used as a control when appropriate. Both enzymes recognize the sequence CCGG, although only *Msp* I will cut the sequence if the internal C is methylated. The DNA was blotted to Immobilon-N membrane according to the manufacturer's (Millipore) procedures. The blots were probed with 18 single-copy *Pst* I genomic clones [UMC 15, 31, 34, 54, 60, 67, 80, 84, 89, 102, 103, and 137—all restriction fragment length polymorphism (RFLP) probes from the University of Missouri-Columbia—and BNL 3.04, 5.09, 5.62, 5.71, 6.25, and 12.30—RFLP probes from Brookhaven National Laboratory] and two cDNA clones (sucrose synthase 1 and alcohol dehydrogenase 1). Eight of the 10 chromosomes are represented by these probes. Hybridizations were carried out at 65°C using 5× standard saline citrate and no formamide. Stringency washes contained 0.1× standard saline citrate and were done at 65°C for 1 hr.

In the summer of 1990, R_1 families were selfed to produce R_2 plants. Selfed progeny of five R_1 families were analyzed as

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†Woodman, J. C. & Kramer, D. A., Sixth International Congress on Plant Tissue Cell Culture, Aug. 3–8, 1986, Minneapolis, p. 215 (abstr.).

‡Evola, S. V., Tuttle, A., Burr, F. & Burr, B., First International Congress on Plant Molecular Biology, Oct. 27–Nov. 2, 1985, Savannah, GA, p. 10 (abstr.).

§Evola, S. V., Burr, F. A. & Burr, B., 11th Annual Aharon Katzir-Katchalsky Conference, Jan. 8–13, 1984 (abstr.).

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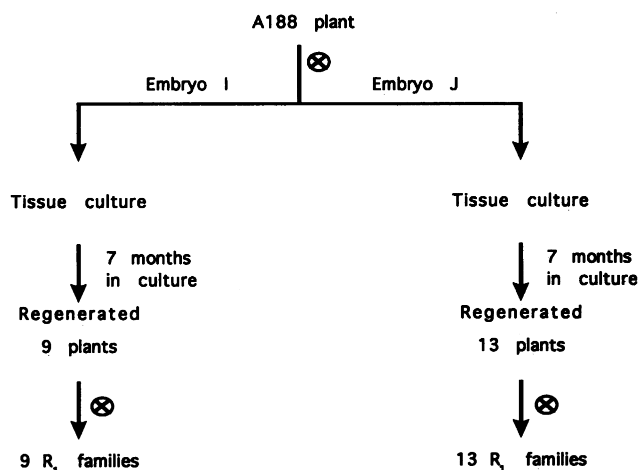

FIG. 1. Origin of the regenerant-derived families from two A188 embryo sources.

described above to determine the stability and inheritance of the methylation changes found in the R_1 families.

RESULTS AND DISCUSSION

Fig. 2 shows an autoradiograph of a blot containing DNA from five individual plants, each representing a different R_1 family from embryo source I. The blot was probed with BNL 5.09. This figure shows the range of variation among families derived from a single explanted embryo. The fact that all altered bands are of a lower molecular weight than the control illustrates the important observation that all observed changes represented decreases in methylation. In every case in which methylation variation was found it could be attributed to a loss of methylation in at least one site. Fifteen noncultured control plants tracing back to a sib plant to the donor of the I and J embryos were invariant for methylation and sequence pattern with all probes and enzymes used in this study (data not shown).

In the analysis of culture-derived lines, the probes were divided into two classes based on Southern blot patterns obtained with noncultured controls. Class I probes were those where the *Hpa* II band(s) was larger than the *Msp* I band(s), indicating that at least one CCGG site in or around these probe sequences was methylated. Class I probes, therefore, could detect both increases and decreases in methylation in the regenerant-derived progenies. When the class II probes were used on DNA from noncultured control plants, the *Hpa* II band(s) was equal in size to the *Msp* I band(s) indicating that CCGG sequences within and immediately adjacent to the probe sequence were not methylated. Only increases in methylation would be detected with these probes. Class I probes detected a high frequency of variation, and class II probes detected no variation (Table 1). These data strongly indicate that only decreases in methylation had occurred. The probes UMC 54 and BNL 5.09 detected the most variation (Table 1).

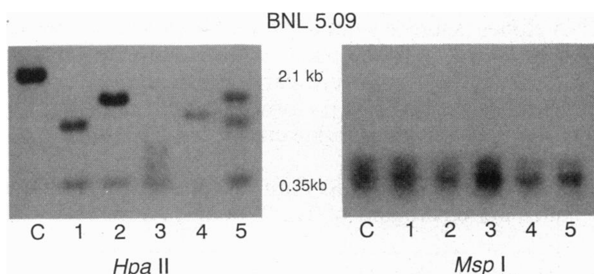

FIG. 2. Extent of variation detected with probe BNL 5.09. DNA samples from individual R_1 plants each representing a different regenerant-derived family originating from embryo source I were digested with *Hpa* II or *Msp* I as indicated. Lanes C, noncultured control; lanes 1–4, plants homozygous for a methylation change; lanes 5, a plant heterozygous for a change. Heterozygosity and homozygosity were determined by analysis of additional plants from the same R_0-derived R_1 families.

BNL 5.09 is a particularly interesting sequence. Our results indicate that there are at least six CCGG sites within this 2.3-kb *Pst* I fragment, as well as two more sites close to, but outside, the probe sequence (data not shown). Five of the internal sites are methylated in most cells of the control A188 (Fig. 2, fragments shorter than 300 base pairs were not detectable with the procedures used). One or more of these methylated sites were demethylated in each of the regenerant-derived families. The BNL 5.09 region could be classified as a CpG or "*Hpa* II tiny fragment" island. Such regions are often involved in the control of gene expression. BNL 5.09 and UMC 54 may both represent CCGG-rich regions and therefore detect more methylation variation because many sites are tested. However, the probes UMC 31 and UMC 84 also appear to recognize regions with several CCGG sites, since they hybridize to multiple bands in *Msp* I digests, yet they detect relatively little variation. Perhaps certain regions are much more subject to demethylation. Methylation may be more important in some regions than in others and may therefore need to be more faithfully maintained in some regions than in others.

Table 1. Variation in DNA methylation observed with class I and class II probes when hybridized to *Hpa* II digests of 21 R_1 families

Probe	Chromosome	No. of variant families	No. of nonsegregating families*	No. of segregating families
Class I†				
Adh 1	1	13	1	12
UMC 84	1	4	0	4
UMC 137	2	1	0	1
UMC 60	3	1	0	1
UMC 31	4	3	0	3
UMC 54	5	18	3	15
UMC 89	8	4	0	4
BNL 5.09	9	21	6	15
Mean		8.13	1.25	6.88
Class II‡				
BNL 5.62	1	0	0	0
UMC 34	2	0	0	0
UMC 102	3	0	0	0
UMC 15	4	0	0	0
BNL 6.25	5	0	0	0
BNL 5.71	5	0	0	0
UMC 67	5	0	0	0
UMC 80	7	0	0	0
BNL 12.30	8	0	0	0
UMC 103	8	0	0	0
sus1	9	0	0	0
BNL 3.04	10	0	0	0
Mean		0	0	0

Data from embryo source I and embryo source J have been pooled. Adh 1, alcohol dehydrogenase 1 cDNA. sus1, Sucrose synthase 1 cDNA.

*Nonsegregation implies that the R_0 plant was homozygous for the DNA methylation change as determined by analysis of five or more seedlings within the R_0-derived R_1 family.

†Higher molecular weight band(s) with *Hpa* II digestion than with *Msp* I digestion of noncultured control DNA.

‡Equal molecular weight band(s) with *Hpa* II and *Msp* I digestion of the noncultured controls.

A second important result (Table 1) is that some families had methylation changes but were not segregating (Fig. 3*A*). This implies that these families were derived from regenerated plants homozygous for the methylation change. The probability that five out of five plants from a selfed heterozygous individual would each be homozygous for the altered band is <0.001. Five more plants were tested in 6 of the 10 cases where a family was not segregating for a change with a certain probe. In all cases, lack of segregation was confirmed.

The frequency of DNA methylation variants that were homozygous in the regenerated plants is 15%, based on the data in Table 1. Not shown in the table is the fact that 60% of the segregating families were segregating for two new bands (e.g., Fig. 3*B*). This indicates a high frequency of methylation change, especially in certain regions. However, the frequency is probably not high enough based on random mutagenesis to account for the observed percentage of nonsegregating variant families. Several other potential explanations could account for these results. Somatic recombination, gene conversion, or mismatch repair following a methylation change could result in homozygosity in part of the culture. Another explanation is that culture-induced DNA-binding proteins could bind both chromatids equally and block methylation after DNA replication, resulting in the homozygous condition. A final possibility is that these sequences are not methylated in callus and that remethylation is inexact upon regenerating plants, perhaps because a gametophytic stage is bypassed.

The high frequency of homozygous changes might also be the result of a more regional mechanism which would potentiate methylation change at a number of sites. For instance, chromatin in plants has been shown to be organized into loops which are anchored at scaffold attachment regions (39). Reorganization of the arrangement of these loops could affect the methylation of many sites within the altered chromatin segments. We have not tested these hypotheses.

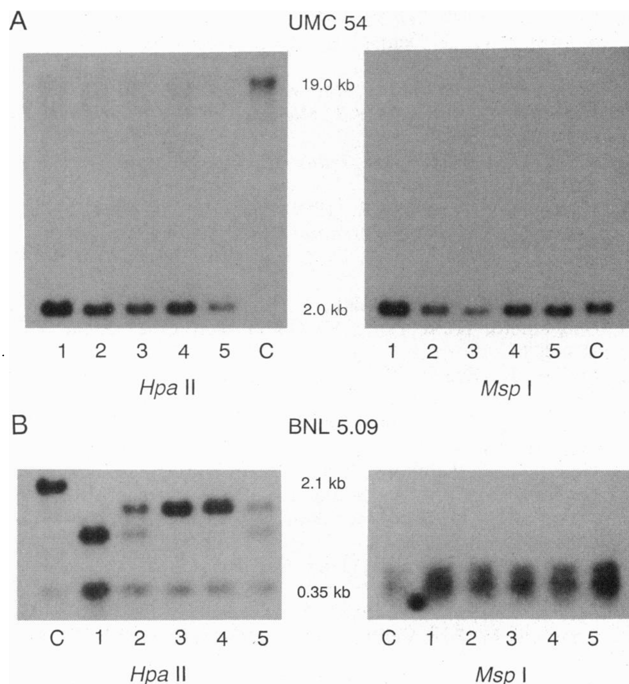

FIG. 3. Examples of nonsegregating (*A*) and segregating (*B*) families. Probes UMC54 (*A*) and BNL 5.09 (*B*) were used to detect differences. DNA was digested with *Hpa* II or *Msp* I as indicated. Lanes C, noncultured control; lanes 1–5, five R_1 plants derived from a single R_0 plant.

In some cases a repeatable difference in the relative intensity of bands generated with a specific probe was observed (e.g., the relative intensity of the 0.35-kb band versus the higher molecular weight band in Fig. 2). These differences in intensity may be due to tissue-specific methylation patterns such as those described by Silva and White (38). Although variation in band intensity was observed among families, the relative intensity of bands found within a family was consistent and bands could, therefore, be scored as segregating or nonsegregating (Fig. 3). This indicates that not only are the methylation patterns stably transmitted, but the intensity of the bands as well. This may suggest that there is a separate heritable mechanism controlling the methylation patterns.

An excellent agreement existed in frequency of events between embryos I and J (Table 2). Families derived from the two embryo sources served as replicates of the experiment. On average, 6% of the families had nonsegregating changes and 33% of the families had segregating changes as detected with the class I probes. The frequency of variant families per class I probe was 39%.

The data can also be presented as the number of probes showing variation within each family. The mean number of probes showing variation per family was 2.95. The range was from 2 to 4 variant probes per family.

Five R_1 families representing 14 probe-family variants were examined in the R_2 generation to determine the heritability of the changes; the bands and genotypes expected in the progeny were found. Families scored to have nonsegregating changes in the R_1 generation had the same banding pattern in R_2 and did not segregate. Heterozygous R_1 plants from families scored to be segregating in R_1 were found to be segregating for the variant band pattern in R_2. No unexpected bands resulted from the meiotic generation.

Several controls provide an important perspective on the observed results. First, 15 noncultured control plants tracing back to a sib plant to the donor of the I and J embryos were invariant for methylation and sequence pattern with all probes and enzymes tested. The embryo-donor plant and a self-pollinated sib plant serving as a control were derived from a single selfed plant. It has been our experience that methylation patterns are quite stable upon selfing of noncultured plants, although we have not extensively quantified this stability. In an analysis of seed-derived A188 plants representing 10 generations of sibbing or selfing and having all derived from a single original plant, methylation variation was rare but present. Only increases in methylation were seen. On average, <0.25 variant per probe was detected, giving a rate of change of 0.025 variant per probe per generation. The probes used in this analysis were UMC 54, UMC 80, BNL 3.04, BNL 5.09, BNL 5.62, and the alcohol dehydrogenase 1 cDNA. The DNA was cut with the enzymes

Table 2. Percent variant families in embryo source I and J for class I and class II probes

Variant family type	Embryo source	% variant families: Class I probes	Class II probes
Nonsegregating	I	4	0
	J	7	0
	Combined	6	0
Segregating	I	33	0
	J	33	0
	Combined	33	0
Total	I	37	0
	J	40	0
	Combined	39	0

See footnotes † and ‡ of Table 1 for explanation of probe class.

Hpa II, *Msp* I, and *Hha* I. The nature and frequency of methylation change in these seed-derived materials differ markedly from the results obtained by analysis of the tissue culture-derived lines. Families derived from the same embryo source usually had different variant patterns (Fig. 2), suggesting that the changes had occurred in culture and were not the result of contaminating pollen. Finally, the fact that methylation variation was observed only with probes that could detect decreases in methylation (class I) and not with probes that could detect only increases in methylation (class II) emphasizes that only decreases in methylation were occurring.

In striking contrast to Brown (31), Brown *et al.* (32, 33), and Müller *et al.* (34), we found no base changes in this study—all *Msp* I digests had the control pattern with a particular probe. Perhaps our cultures had undergone less stress prior to plant regeneration than the cultures of those authors.

In conclusion, we found a high frequency of DNA methylation variation among regenerant-derived families, even from the same embryo source. Changes in base sequence were not detected. All changes were the result of decreases in methylation. The changes were heritable and stable upon selfing. Some of the changes were homozygous in the R_0 plant. Certain probes detected variation much more frequently than others; these probes may be especially useful in screening for methylation changes.

Our study provides evidence that methylation changes occur at a sufficiently high frequency to be an important source of tissue culture-induced variation. Methylation variation appears to be much more frequent than sequence variation. The trend toward decreasing methylation indicates that genes such as transposable elements could be turned on by the culture process. The high frequency of variation found with random sequences suggests that many coding regions could be affected. It is possible that decreases in methylation could also affect chromatin structure. This could lead to changes in gene expression due to position effects, changes in recombination rates (40), and changes in the timing of DNA replication, perhaps leading to chromosome breakage (23). It is important to understand whether these changes are directed to certain sequences or are a random response to stress found equally in all sequences. Our results show that certain sequences show more variation than others, suggesting that the variation may not be random even among single-copy sequences. We must also determine whether a change in DNA methylation is the underlying cause of tissue culture-induced variation or just another symptom. A stress response at the DNA level such as methylation variation could represent the underlying mechanism. It will be enlightening to understand the pathway leading to this response. Finally, elucidating the basis for the high frequency of homozygous methylation changes in the primary regenerants will further our understanding of how these changes occur.

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