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# Variation and Patterns of DNA Methylation in Maize C-type CMS Lines and their Maintainers

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**DNA methylation plays an important role in gene expression regulation during biological development in plants. This study adopted methylation sensitive amplification polymorphism (MSAP) to compare the levels and patterns of cytosine methylation at CCGG sites in maize genome. The tissues assayed included seedlings and tassels of C-type cytoplasmic male sterility (C Huang Zao Si, C 48-2) and its maintainer lines. For each tissue, both C Huang Zao Si and C 48-2 were more methylated than their corresponding maintainers not only on MSAP ratios, but also on the full methylation levels. In different nuclear backgrounds, the two tissues were more methylated in Huang Zao Si than in 48-2, although the two lines shared the same cytoplasm. Full methylation of internal cytosine was the dominant type in the maize genome. In addition, four different classes of methylation patterns were identified in tassels between C-CMS lines and their maintainer lines; these were specific-methylation, demethylation, hypo-methylation, and hyper-methylation. The results obtained demonstrated the power of the MSAP technique for large-scale DNA methylation detection in the maize genome, and suggested the possible association between DNA methylation polymorphism and C-type cytoplasmic male sterility.**

***Key words***: DNA methylation, maize, normal cytoplasm, cytoplasmic male sterility, methylation sensitive amplification polymorphism.

Cytoplasmic male sterility (CMS) is a maternally inherited trait in higher plants that results in the inability of the mature plant to produce functional pollen, but it does not affect female fertility. In maize, CMS has been extensively studied due to its potential application in hybrid seed production. CMS of maize was classified as types T, C, S based on the specific restoration of nuclear gene. Normal (N) cytoplasm allows development of viable pollen in absence of fertility restorer genes.

CMS is caused by disturbances in the nuclear-mitochondrial interaction, clearly demonstrated by the maternal inheritance of the male-sterile phenotype and the ability to suppress male-sterility by nuclear Restorer-of-fertility (*Rf*) genes (1). Pollen abortion in CMS lines is accompanied by expression of novel open reading frames (ORFs) encoding in mitochondrial DNA (2). Alterations in the mitochondrial DNA expression could result in changed expressions of certain nuclear genes, which lead to the dysfunction of the cells (3). However, there are probably few mechanisms by which changed expression of mitochondrial DNA could impair male reproductive function without affecting vegetative development or female gametogenesis.

In fact, the CMS involved in male flower or pollen development, not only depends on tissue-specific gene expression, but also correlates with genetic regulation of floral organ development. Till date, many studies demonstrated that DNA methylation participates in gene expression regulation to adjust plant growth and development (4-6). The bulk of DNA methylation involves transfer of methyl groups from S-adenosine-L-methionine to cytosines and adenines, by DNA methyltransferases, after DNA duplication. In higher plants, cytosine methylation existed mainly in nuclear DNA, and also in mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) (7). At different stages of plant growth, the changes in DNA methylation level play an important role in response to variation of heredity and the environment (8, 9). DNA methylation may suppress gene expression. Actively transcribed sequences are often found to be less methylated than the promoters and certain coding regions

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*Abbreviations*: MSAP – methylation sensitive amplification polymorphism, CMS – cytoplasmic male sterility, Rf – Restorer-of-fertility, ORFs – open reading frames, AFLP – amplified fragment length polymorphism.

of silent genes (10). Demethylation may make some silent genes active and expressive (11). For example, plant vernalization results in DNA demethylation that induces or accelerates flowering (12, 13). Moreover, alterations of DNA methylation patterns have been widely found in plants displaying phenotypic defects, notably in cases of aberrant floral morphogenesis (14) and in somaclonal variants (15). In Arabidopsis (16) and tobacco (17), demethylation in transgenic plants was also observed to be associated with drastic morphological changes. Therefore, we were interested in searching for DNA methylation variation associated with CMS lines of maize which have defective male flower organs.

In this study, we analyzed DNA cytosine methylation of two C-type CMS maize lines and their maintainer lines in the tissues collected from two developmental stages using methylation sensitive amplification polymorphism (MSAP) (18). The major aim of this work was to explore the characterization and regularity of variation in DNA methylation in maize CMS. The work presented here also provides new insights into understanding of the role of DNA methylation in gene expression regulation of CMS lines.

## Materials and Methods

***Plant materials and DNA extraction* —** The materials used in this study were two C-type CMS lines of maize (C Huang Zao Si and C 48-2) and their maintainer lines (N Huang Zao Si and N 48-2), which were maintained at the Maize Research Institute of Sichuan Agriculture University, China. Tissues assayed included young seedlings and pre-emergent tassels. Genomic DNA was extracted from fresh tissues using the CTAB method (19).

***MSAP analysis of DNA methylation* —** MSAP assay used in the study was similar to AFLP, except that the "frequent cutter" enzyme *Mse*I, was replaced by isoschizomers *Hpa*II and *Msp*I, which displayed differential sensitivity to DNA methylation (20, 21). The MSAP system was consisted of digestion and ligation reactions, preamplification and selective amplification reactions, and detection reactions. The adapter and primers for *Eco*RI were the same as that used in standard AFLP analysis, while the *Hpa*II -*Msp*I adapter and primers were designed according to Xiong (21), as reported in Table 1.

To detect MSAP, each of the DNA samples was separately restricted with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I (Promega, Madison WI, USA). Both double digestion and ligation were completed in one step in order to minimize discrepancy caused by experimental factors. The digestion-ligation reaction was performed in a volume of 25 µl containing 250 ng DNA template, 3 units *Eco*RI, 3 units *Hpa*II (or *Msp*I), 1.5 units $T_4$ DNA ligase (Promega, Madison WI, USA), 5 pmol *Eco*RI adapter, 50 pmol *Hpa*II/*Msp*I adapter, and 2.5 µl 10× $T_4$-ligase buffer. The mixture was incubated at 37°C overnight, inactivated at 70°C for 15 min, and stored at -20°C.

Pre-amplified PCR reactions were conducted in a final volume of 20 µl containing 2 µl of ligation products, 40 ng of E00 and HM00 Preamplified primer (Table 1), 1 unit of *Taq* polymerase, 1.6 µl of dNTPs (2.5 mM each), 1.2 µl of $MgCl_2$ (25 mM), and 2 µl of 10× PCR buffer. DNA fragments were amplified for 25 cycles of 94°C for 90 s, 56°C for 30 s, and 72°C for 1 min. After checking the presence of a smear of fragments (100-1000 bp in length) by agarose electrophoresis, the preamplification product was diluted 1 to 20 (v: v) with $ddH_2O$ for the next selective amplification.

**Table 1.** Adapter and primer sequences

| | *Eco*RI (E) | *Hpa*II/*Msp*I (HM) |
|---|---|---|
| Adapter 1 | 5′-CTCGTAGACTGCGTACC-3′ | 5′-GACGATGAGTCCTGAG-3′ |
| Adapter 2 | 5′-AATTGGTACGCAGTC-3′ | 5′-CGCTCAGGACTCAT-3′ |
| PreAmp primer | 5′-GACTGCGTACCAATTC-3′ (E00) | 5′-GATGAGTCCTGAGCGGC-3′ (HM00) |
| Selective primers | (E00+3) | (HM00+3) |
| | 1 E00+AAC (E32) | HM00+CAA (HM1) |
| | 2 E00+AAG (E33) | HM00+CAC (HM2) |
| | 3 E00+ACC (E36) | HM00+CAG (HM3) |
| | 4 E00+ACG (E37) | HM00+TAA (HM4) |
| | 5 E00+AGC (E40) | HM00+TCC (HM5) |
| | 6 E00+AGG (E41) | |

Selective amplification process followed. A volume of 1.5 µl of these diluted samples was mixed with the PCR buffer, 40 ng of E00+3, and HM00+3 selective primer, which had three additional selective nucleotide bases compared to the preamplified primer (Table 1), 1 unit of *Taq* polymerase, 1.6 µl of dNTPs (2.5 mM each), 1.2 µl of $MgCl_2$ (25 mM), and 2 µl of 10× PCR buffer in a final volume of 20 µl. The PCR conditions were as follows: 13 cycles at 94°C for 30 s, 65°C for 30 s (reduced by 0.7°C each cycle), 72°C for 1 min; 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The final PCR products were denatured, and electrophoresed on 5% denaturing polyacrylamide sequencing gels at 75 W for 1.5 h. After silver stain detection, only clear and reproducible bands were scored.

For statistical analysis of the differences between the two materials, following formula was used (22):

$$P = \frac{y_1 + y_2}{n_1 + n_2},\ q = 1 - p,\ s_{p1-p2} = \sqrt{pq\left(\frac{1}{n_1} + \frac{1}{n_2}\right)},\ U = \frac{P_1 - P_2}{s_{p1-p2}}$$

where $n_1$ is the total amplified sites for a given sample; $n_2$ the total amplified sites for another sample; $y_1$ the total methylation sites, hemi-methylation sites (only one strand methylation) or fully methylation sites (methylation of both strands) for a given sample; $y_2$ the total methylation sites, hemi-methylation sites or fully methylation sites for another sample; $p_1$ the percentage of total methylation sites, hemimethylation sites or fully methylation sites for a given sample; $p_2$ is the percentage of total methylation sites, hemimethylation sites or fully methylation sites for another sample.

## Results

Isoschizomers *Hpa*II and *Msp*I recognize and digest 5′-CCGG-3′ sites, but display differential sensitivity to DNA methylation. *Hpa*II is inactive if either cytosine is fully methylated (methylation of both strands), even as it cleaves the hemi-methylated sequence (only one strand methylation). *Msp*I is sensitive only to methylation at the external cytosine. It cuts in the case of inner cytosine methylation (CmCGG), but not in the case of outer cytosine methylation (mCCGG). Therefore, different band patterns from PCR amplification can reflect the methylation status and level at the site.

According to the presence or absence of a band, methylation patterns of *Hpa*II- and *Msp*I- digested genomic DNA could probably be divided into four types with the methylation status as follows: Type I, bands present for both enzymes, which means hemi-methylation (single strand) at internal cytosine or no methylation; Type II, bands present for *Hpa*II, but absent for *Msp*I, which means hemi-methylation at external cytosine or at both cytosines; Type III, bands present for *Msp*I, but absent for *Hpa*II, which means full methylation (double strands) at internal cytosine; Type IV, bands absent for both enzymes, which means full methylation at external or both external and internal cytosines.

***Methylation level of C-type CMS lines and their maintainer lines —*** We used 30 pairs of selective primers obtained from six *Eco*RI primers (E32, E33, E36, E37, E40, and E41) in combination with five *Hpa*II/*Msp*I primers (HM1-5), to analyze DNA methylation at CCGG sites in tassel tissues and seedling tissues from N Huang Zao Si, C Huang Zao Si, N 48-2, and C 48-2, respectively. The methylation sensitive amplified polymorphisms, namely the methylation level of total amplified sites in tassel were 22.65%, 25.26%, 21.59% and 23.70% for N Huang Zao Si, C Huang Zao Si, N 48-2, and C 48-2, respectively, and 23.04%, 23.26%, 22.15%, and 22.49% in seedling, among which the corresponding fully methylated ratios in tassel were 17.16%, 20.14%, 16.67% and 19.30%, respectively, and 17.54%, 18.17%, 17.27% and 17.85% in seedling (Table 2).

Data showed that there was a certain degree of difference in tassels between normal cytoplasm and C-type male sterile cytoplasm under the two nuclear backgrounds. Both C Huang Zao Si and C 48-2 were more methylated than its corresponding maintainers not only on the MSAP levels, but also on the full methylation levels. The changing tendency was similar in seedlings, but the difference between C-CMS line and its maintainer line was very slight. Meanwhile, a significant difference in genomic 5mdC content has been found in different nuclear genetic backgrounds. MSAP ratios and fully methylated ratios of the two tissues were lower in 48-2 than in Huang Zao Si although both lines shared the same cytoplasm. Specifically, for the total level of methylation, different materials used in the study showed various levels (increase or decrease) no matter in tassel or seedlings, but none of the change was statistically significant. For full methylation, the tassels of CMS lines showed significant higher values (20.14% and 19.30%) than those of the

**Table 2.** Methylation level in two C-type CMS maize lines and their maintainer lines

| Types | N Huang Zao Si | | C Huang Zao Si | | N 48-2 | | C 48-2 | |
|---|---|---|---|---|---|---|---|---|
| | Tassel | Seedling | Tassel | Seedling | Tassel | Seedling | Tassel | Seedling |
| I (1 1) | 1439 | 1390 | 1349 | 1352 | 1380 | 1347 | 1333 | 1322 |
| II (1 0) | 101 | 98 | 90 | 89 | 86 | 84 | 75 | 78 |
| III (0 1) | 301 | 296 | 319 | 309 | 280 | 289 | 297 | 281 |
| IV (0 0) | 15 | 17 | 35 | 9 | 11 | 8 | 32 | 19 |
| Total amplified bands | 1841 | 1784 | 1758 | 1750 | 1746 | 1720 | 1705 | 1681 |
| Total methylated bands[1] | 417 | 411 | 444 | 407 | 377 | 381 | 404 | 378 |
| MSAP (%) | 22.65 | 23.04 | 25.26 | 23.26 | 21.59 | 22.15 | 23.70 | 22.49 |
| Fully methylated bands[2] | 316 | 313 | 354 | 318 | 291 | 297 | 329 | 300 |
| Fully methylated ratio (%) | 17.16 | 17.54 | 20.14 | 18.17 | 16.67 | 17.27 | 19.30 | 17.85 |

[1]Total methylated bands = II+III+IV; [2]Fully methylated bands = III+IV

corresponding maintainer lines (17.16% and 16.67%; U values: 2.29 and 2.02, and $U_{0.05}$ = 1.96).

Moreover, methylation status at 5′-CCGG-3′ sites in C-CMS lines and their maintainers was also compared between seedlings and tassels. The MSAP ratios of tassels were lower than seedlings in normal cytoplasm lines, N Huang Zao Si and N 48-2. The reverse was the case for CMS lines, C Huang Zao Si and C 48-2, in which methylation extent of tassels appears to be a greater than that in seedlings.

Table 2 showed that type III (inner methylation of double-stranded DNA) has the largest number of bands. The number of bands in tassels from N Huang Zao Si, C Huang Zao Si, N 48-2, and C 48-2 were 301, 319, 280 and 297, respectively. The corresponding ratios to total methylated sites were 72.18%, 71.85%, 74.27% and 73.51%, respectively. In the seedling tissue, 296, 309, 289 and 281 bands attributed to this type, and the corresponding ratios were 72.02%, 75.92%, 75.85% and 74.34%, respectively. These results showed that full methylation of internal cytosine occurred most often in all types at 5′-CCGG-3′ sites in the maize genome.

***Methylation patterns of C-CMS lines and their maintainer lines —*** C-CMS lines and their corresponding maintainer lines were compared for the patterns of differential amplification using tassel and seedling tissues. When both DNA strands were methylated at the inner cytosine, *Hpa*II did not recognize nor cleave, but *Msp*I did. Therefore, “0, 1, 0, 1” amplified pattern was produced in H/M lanes of two certain materials. If only one DNA strand was outer methylated, *Msp*I did not recognize nor cleave, but *Hpa*II did. Thus, “1, 0, 1, 0,” amplified pattern was produced. In this study, the two types (“0, 1, 0, 1” or “1, 0, 1, 0”) described earlier, belonged to the monomorphic sites, in which the methylation statuses were identical in C-CMS lines and their maintainer lines (Fig·1).

In the tassels, 360 monomorphic sites were amplified from N Huang Zao Si and C Huang Zao Si, and 336 sites from N 48-2 and C 48-2 by 30 pairs of selective primers, indicating that 78.09% of methylation statuses were identical in N Huang Zao Si and C Huang Zao, and 78.50% of methylation statuses were consistent in N 48-2 and C 48-2. In the seedlings, the site numbers of monomorphism for Huang Zao Si and 48-2 were 347 and 311, respectively, the corresponding ratios were 85.05% and 84.28% (Table 3). This data showed that more than 84% of the methylation in the seedling remained consistent in C-CMS and its maintainer lines.

Polymorphism reflected DNA methylation patterns differed at the same site of a C-CMS line and its maintainer line. In the two tissues polymorphic sites were detected, but a smaller proportion of the sites were found to be differentially methylated in seedlings. Thus, we further analyzed different methylation patterns in the tassels that act as an important reproductive organ, with marked difference in methylation levels between C-CMS lines and their maintainer lines. Comparison of banding patterns in gels between C-CMS lines and their maintainer lines revealed 10 polymorphic types, which could be classified into four classes, A, B, C, D (Table 4), according to the change from normal cytoplasm to C-type male sterile under the two nuclear backgrounds.

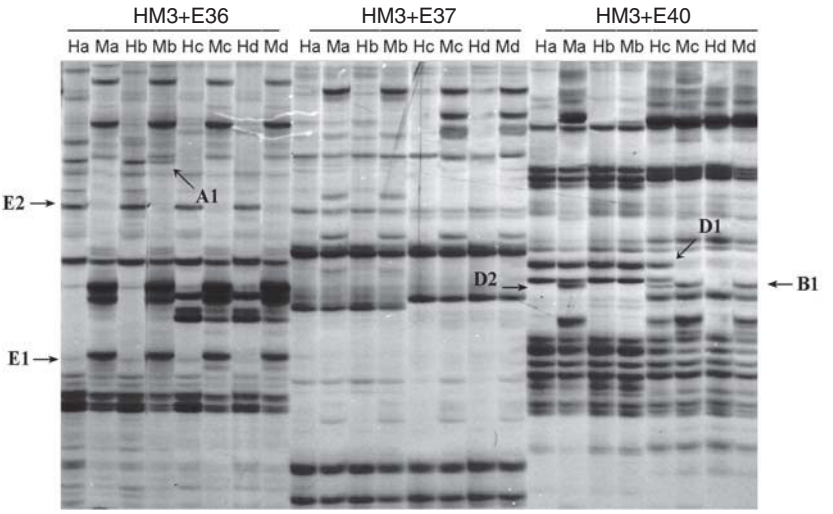


**Fig.1.** MSAP profile of the tassels from C-type CMS maize lines and their maintainer lines. H and M indicate the enzyme combinations of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I; a, b, c, and d: N Huang Zao Si, C Huang Zao Si, N 48-2, and C 48-2, respectively; H3+E36, H3+E37, and H3+E40 are the primer combinations; A1, B1, D1, D2: different methylation patterns of the tassels between C-type CMS maize lines and their maintainer lines which were listed in Table 4; E1 and E2: methylation monomorphism.

**Table 3.** Number of monomorphic and polymorphic fragments in Huang Zao Si and 48-2

| Types | Huang Zao Si | | 48-2 | |
|---|---|---|---|---|
| | Tassel | Seedling | Tassel | Seedling |
| Monomorphism | 360 (78.09%) | 347 (85.05%) | 336 (78.50%) | 311 (84.28%) |
| Polymorphism | 101 | 61 | 92 | 58 |
| Total | 461 | 408 | 428 | 369 |

Class A, demethylation type, that is, N Huang Zao Si or N 48-2 belonged to semi-methylation, full methylation type, or the sites could not be recognized or cleave by either *Msp*I or *Hpa*II, but no methylation in the same sites of the corresponding C-CMS (Fig·1). The site numbers of this class for Huang Zao Si and 48-2 were 17 and 24, respectively. The corresponding ratios to total methylation sensitive sites were 3.69% and 5.61%. On the contrary, Class B indicated methylation only existed in C Huang Zao Si and C 48-2, but not in their maintainers (Fig·1). This class accounted for totally 9.33% and 8.41% methylated sites in Huang Zao Si and 48-2, respectively. Class C, hypo-methylation type, that is, partial demethylation happened to C Huang Zao Si and C 48-2 and thus, the methylation levels of C-CMS lines were lower than those of maintainers at the sites. The ratios of hypo-methylation sites were 2.60% and 1.87%, respectively. Class D indicated hyper-methylation type. This is, Huang Zao Si and 48-2 with normal cytoplasm belonged to semi-methylation or full methylation type, whereas the two cytosines of double strands at the sites were both methylated during changing from normal cytoplasm to C-CMS and thus could not any longer be recognized and cleft by either of the two enzymes (Fig·1). The ratios of hyper-methylation sites were 6.29% and 5.61% in Huang Zao Si and 48-2, respectively. Demethylation and hypomethylation (class A + class C), together with the ratios of about 6.29% and 7.48% in Huang Zao Si and 48-2, represented decrease in the level of methylation in C-CMS lines compared to their maintainers. The other two classes, together with the ratios of 16.62% and 14.02%, reflected the increase of methylation levels in C-CMS lines. Although both methylation and demethylation occurred in CMS lines, methylation seems to be predominant.

***Specific types of methylation variation in Huang Zao Si and 48-2 —*** Although most sites of methylation variation from normal cytoplasm to C-type CMS under Huang Zao Shi nuclear background was different from 48-2, six out of almost one hundred sites were observed to share the same methylation variation in the tassels from the both nuclear backgrounds. Among them, four sites were specific-methylated simultaneously in C Huang Zao Shi and C 48-2, one site was demethylated, and one site belonged to hyper-methylation type (Table 5). Therefore, methylation variation in the tassels between C-type CMS lines and

**Table 4.** Different methylation patterns of the tassels between C-type CMS maize lines and their maintainer lines

| Class | N | | C | | No. of sites | | Ratios to total methylation sensitive sites | |
|---|---|---|---|---|---|---|---|---|
| | H | M | H | M | Huang Zao Si | 48-2 | Huang Zao Si | 48-2 |
| | 1 | 0 | 1 | 1 | 5 | 7 | | |
| A | 0 | 1 | 1 | 1 | 9 | 14 | | |
| | 0 | 0 | 1 | 1 | 3 | 3 | | |
| Total bands of A type (demethylation) | | | | | 17 | 24 | 3.69% (17/461) | 5.61% (24/428) |
| | 1 | 1 | 0 | 1 | 30 | 23 | | |
| B | 1 | 1 | 1 | 0 | 7 | 5 | | |
| | 1 | 1 | 0 | 0 | 6 | 8 | | |
| Total bands of B type (specific methylation) | | | | | 43 | 36 | 9.33% | 8.41% |
| C | 0 | 0 | 0 | 1 | 8 | 5 | | |
| | 0 | 0 | 1 | 0 | 4 | 3 | | |
| Total bands of C type (hypomethylation) | | | | | 12 | 8 | 2.60% | 1.87% |
| D | 1 | 0 | 0 | 0 | 17 | 13 | | |
| | 0 | 1 | 0 | 0 | 12 | 11 | | |
| Total bands of D type (hypermethylation) | | | | | 29 | 24 | 6.29% | 5.61% |

H and M indicate the enzyme combinations of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I; 0: band absent, 1: band present; N: normal cytoplasm, C: C-type CMS.

**Table 5.** Specific types of methylation variation in Huang Zao Si and 48-2

| N Huang Zao Si | | C Huang Zao Si | | N 48-2 | | C 48-2 | | No. of sites |
|---|---|---|---|---|---|---|---|---|
| H | M | H | M | H | M | H | M | |
| 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 2 |
| 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 2 |
| 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |

H and M indicate the enzyme combinations of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I; 0: band absent, 1: band present.

their maintainer lines in two nuclear backgrounds are site-specific.

## Discussion

The modified AFLP technique presented in this study provides a powerful tool to investigate DNA methylation in eukaryotic organisms. The quality of our MSAP profiles were as good as that of standard AFLP analysis, thus making it possible to compare the levels of DNA cytosine methylation as well as to analyze the methylation patterns in C-CMS lines and their maintainer lines. However, it should be pointed out that the technique also has four major constraints associated with resolving power. First, it relies on the template DNA quality. Second, this method can only detect a limited spectrum of bands (50 to 1,500 bp) on the 4% to 6% sequencing gel, shorter or longer DNA fragments cannot be detected (23). Third, the technique can only investigate cytosines in the CCGG sites, restricted to the recognition site of the isoschizomers used, but can not detect other cytosine methylation in CAG and CTG sites. Fourth, both *Hpa*II and *Msp*I are able to recognize unmethylation and inner methylation of a single strand, which produce the same patterns of methylation after amplification. Thus, the types of unmethylation and inner methylation of a single strand can not be distinguished. For these reasons, the results through MSAP analysis possibly underestimate the actual levels of methylation in the genome. Notwithstanding some limits, a high rate of MSAP methylation was observed in the study, which range from 25.26% to 21.59% in maize materials. Similar proportions of methylated CCGG sites were found with the same technique in maize inbred lines (20.24% to 21.78%) and in micropropagated banana (23%) (24, 25).

The major objective of this study was to evaluate the occurrence of DNA methylation changes in C-CMS lines. The results demonstrated that most of sites kept the same methylation pattern in CMS and its maintainer, which might be explained by their same nuclear background and the inheritance of DNA methylation, such as in N Huang and C Huang. Interestingly, genomic methylation in C-CMS lines increased by 2.36% on average compared with the

lines with normal cytoplasm in tassels, but increased by only 0.28% in seedlings. A likely explanation is that the tassels with the more difference were directly correlative with CMS, and displayed different fertility characters between C-CMS lines and their maintainer lines. From the further analysis of tassels, we found ten types of methylation patterns according to the changes found between normal cytoplasm and C-type male sterile cytoplasm, which could be summarized as four classes: specific-methylation, demethylation, hypo-methylation, and hyper-methylation types. It is well known that cytosine methylation plays an important role as a regulator of gene expression in plant development, and methylation in coding regions or promoters can block the expression of these genes, while artificial demethylation will lead to the reactivation of gene expression (26). Therefore, methylation seems to be closely related to transcriptional repression in genes controlled by CMS mechanism. Meanwhile, a small number of hypomethylation and demethylation events were also observed, indicating that some genes are transcriptionally active in CMS. Thus, programmed gene expression in CMS appears to be controlled, at least in part, by the mechanism of both DNA methylation and demethylation events, and the former seems to be predominant.

It should also be noted that the tassels from Huang Zao Si and 48-2 shared the same methylation variation at the six sites. Those specific sites detected both in the two nuclear backgrounds seems to be closely related to specific morphological changes in CMS lines. This was supported by Jaligot *et al* (27), who used specific methylation patterns as markers to discriminate between the "mantled" variation and normal phenotype in oil palm.

Different methylation levels have been observed in the two tissues tested in this study. The tassels were less methylated than seedlings in normal cytoplasms. Our previous study, carried out with maize inbred lines, 18Red and 18White, had also shown that tassels were the least methylated, ear leaf the moderate, and bracteal leaf the most (24). This phenomenon might be due to the complex functions of normal tassel tissue as an important reproductive organ affecting flowering, fertilization, and fruit bearing. Another interesting feature was that the methylation level of the tassels was higher than seedlings in CMS lines, which was opposite to the case in normal cytoplasm lines. DNA methylation is necessary for normal plant growth. If the methylation level is insufficient or redundant, the plants will display abnormalities of biological development and phenotype (14, 16). Accordingly, we may hypothesize that the increase of methylation level in tassels of CMS lines would contribute to repression of gene expression, which was associated with sterile variant phenotype in CMS lines.

In summary, we conclude that the MSAP technique provide an efficient tool for large-scale detection of DNA methylation that should be useful for studying a number of important biological problems. In CMS lines analyzed, such variation of DNA methylation levels or methylation patterns would undoubtedly play an important role in the transcription of some genes, and could regulate transcription efficiency through alteration of methylation status in promoter or open reading region. In principle, an increase in methylation level would lead to repression of gene expression, whereas a decrease in methylation level means activation of gene expression (28). However, an increase or reduction of methylation level is temporary and will alter accordingly, along with the growth process and environmental variation. Therefore, DNA methylation is important in gene expression regulation during biological development and tissue differentiation in plants.

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