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ORIGINAL PAPER

# Endosperm-specific hypomethylation, and meiotic inheritance and variation of DNA methylation level and pattern in sorghum (*Sorghum bicolor* L.) inter-strain hybrids

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**Abstract** Understanding dynamics and inheritance of DNA methylation represents important facets for elucidating epigenetic paradigms in plant development and evolution. Using four sets of sorghum (*Sorghum bicolor* L.) inter-strain hybrids and their inbred parents, the developmental stability and inheritance of cytosine methylation in two tissues, leaf and endosperm, by MSAP analysis were investigated. It was found that in all lines (inbred and hybrid) studied, endosperm exhibited a markedly reduced level of full methylation of the external cytosine or both cytosines at the CCGG sites relative to leaf, which caused a variable reduction in the estimated total methylation level in endosperm by 6.89–19.69% (11.47% on average). For both tissues, a great majority of cytosine methylation profiles transmitted to F1 hybrids, however, from 1.69 to 3.22% of the profiles showed altered patterns in hybrids. Both inherited and altered methylation profiles can be divided into distinct groups, and their frequencies are variable among the cross-combinations, and between the two tissues. The variations in methylation level and pattern detected in the hybrids were not caused by parental heterozygosity, and they could be either non-random or stochastic among hybrid individuals. Homology analysis of isolated bands that showed endosperm-specific hypomethylation or variation in hybrids indicated that diverse sequences were involved, including known-function cellular genes and mobile elements. RT-PCR analysis of six genes representing endosperm-specific hypomethylation in MSAP profiles indicated that all showed higher expression in endosperm than in leaf, suggesting involvement of methylation state in regulating tissue-specific or tissue-biased expression in sorghum. Analysis on leaf-RNA from 5-azacytidine-treated plants further corroborated this possibility.

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## Introduction

It has been proposed that cytosine DNA methylation represents an important epigenetic modification of eukaryotic chromatin, which plays an essential role in orchestrating gene expression across plant development (Rangwala and Richards 2004; Chan et al. 2005), and in maintaining genome integrity (Bestor 1998; Matzke et al. 1999; Colot and Rossignol 1999; Cao and Jacobsen 2002; Rapp and Wendel 2005). In recent years, this proposition has been

supported by compelling empirical evidence documenting that disturbance of intrinsic DNA methylation patterns can lead to pleiotropic developmental abnormality (Kakutani 2002; Finnegan et al. 1996) and genome instability (Comai et al. 2003; Marfil et al. 2006).

In spite of its ancient origin, several characteristics distinguish cytosine DNA methylation in plants from that in animals (Cao and Jacobsen 2002; Kakutani 2002; Martienssen and Colot 2001; Riddle and Richards 2002). Among these is the meiotic heritability of methylation state (level and pattern) in plants versus their often "erasure and reset" dynamics in each organismal generation of animals (Reik et al. 2001; Geiman and Robertson 2002; but also see Chong and Whitelaw 2004). For example, the genome-wide hypomethylation in *Arabidopsis* induced by *ddm1* or *met1* mutation exhibited faithful trans-generational inheritance (Kakutani et al. 1996, 1999; Finnegan et al. 1996). The naturally occurred differential cytosine methylation states at several studied genomic regions, including two major ribosomal loci, in different ecotypes of *Arabidopsis* are also largely transmitted from parents to inter-strain hybrids as Mendelian traits (Riddle and Richards 2002). Nonetheless, apparent non-inheritance or re-modeling of parental methylation patterns may occur in certain situations, like in several plant inter-specific hybrids, allopolyploids and introgression lines (Madlung et al. 2002; Levy and Feldman 2004; Liu and Wendel 2003; Liu et al. 2004; Salmon et al. 2005; Lukens et al. 2006; Marfil et al. 2006), in progenies of some inter-strain *Arabidopsis* hybrids (Riddle and Richards 2005), and in certain developmentally terminated tissues like endosperm (Lauria et al. 2004; Kinoshita et al. 2004). Notwithstanding these interesting findings, the causing factors for, and generality of, methylation dynamics in plants, particularly with reference to ontogenic development, hybrid formation and trans-generational heritability, remained largely mysterious.

It has been showed that the inheritance of epigenetic state in plants relies largely on maintenance of cytosine methylation (particularly CG methylation) through sporophytic mitosis, meiosis and postmeiotic mitosis, giving rise to gametophytes (Takeda and Paszkowski 2006). A hallmark of angiosperms is the double-fertilization process that leads to the production of embryo and endosperm. In the process, one haploid sperm nucleus fuses with the egg cell nucleus, and the zygote develops to a diploid embryo. The other sperm nucleus fuses with the diploid nucleus of the central cell to begin the development of a triploid endosperm. Therefore, endosperm nuclei contain two maternal chromosome sets and one paternal chromosome set, and this 2 m:1p ratio is crucial for the proper development of the endosperm, clearly suggesting the involvement of epigenetic regulations (Luo et al. 2000). Indeed, several imprinting genes (showing parent-of-origin effect) were identified in endosperm development, which are associated with alterations in cytosine methylation (Vielle-Calzada et al. 1999; Adams et al. 2000; Autran et al 2005; Takeda and Paszkowski 2006; Julliena et al. 2006). Therefore, to further study the epigenetic paradigms in plants, endosperm provides an ideal and unique tissue for comparison with normal sporophytic tissues.

A recent study in maize (*Zea mays* L.) showed that endosperm exhibited a 13% reduction in total cytosine methylation compared with leaf and embryo, which was mainly because of maternal-specific, unidirectional hypomethylation (Lauria et al. 2004). This event was suggested to play an important role in the parent-of-origin effect on maize endosperm development (Lauria et al. 2004). Similar studies have not been reported in other plants, leaving generality of the phenomenon unknown. Sorghum (*Sorghum bicolor* L.) is a staple food crop in Africa and to much of the developing world largely owing to its superior tolerance to arid growth conditions (Feltus et al. 2006). It is also a close relative of maize (Draye et al. 2001), but with a much reduced genome size (735 vs. 2,600 Mb) and complexity largely due to lack of some evolutionarily recent bursts of retrotranspositional events in maize (SanMiguel and Bennetzen 1998). It thus will be interesting to find out whether sorghum endosperm will experience a similar process of global hypomethylation, and whether this contributes to endosperm-specific or enhanced gene expression. An added significance to study epigenetic inheritance and variation in sorghum is, like in maize, F1 heterosis or hybrid vigor has been widely used in its grain production. Although the molecular basis of heterosis is largely obscure, epigenetic regulatory mechanism-mediated allele-specific or differential expression has been implicated to play an important role (Birchler et al. 2003; Sun et al. 2004; Varshney et al. 2005; Swanson-Wagner et al. 2006; Springer and Stupar 2007). Therefore, it is interesting to investigate the inheritance or variation of cytosine methylation level and pattern in sorghum F1 hybrids relative to their inbred parents.

## Materials and methods

### Plant materials

Four sets of sorghum hybrids, designated as AE, BF, CH and CI (for all crosses, the first and second letters denoting maternal and paternal parents respectively), and their corresponding inbred parental lines, YN336 (A), YN510 (E), YN338 (B), YN507 (F), YN323 (C), YN185 (H) and YN213 (I), were used in this study. All seven inbred lines had been maintained in our hands by strict self-pollination for many generations, whilst the hybrids were made by careful manual pollination.

### DNA isolation

Genomic DNA was first isolated from expanded leaves at the 9–10th leaf-stage and endosperms harvested at 15 days after pollination of pooled sorghum plants of the various inbred lines and hybrids by a modified CTAB method (Kidwell and Osborn 1992). For the purpose of analyzing uniformity or variation of methylation alterations among different hybrid individuals, and to detect possible heterozygosity in the parental inbred lines, genomic DNA was also isolated from expanded leaves of the same stage individual plants of hybrids and parental inbred lines.

### MSAP analysis

MSAP is a modified version of the standard AFLP (amplified fragment length polymorphism) fingerprinting technique (Vos et al. 1995), by incorporating *Hpa*II and *Msp*I, a pair of isoschizomers that recognize the same restriction site (5′-CCGG) but have different sensitivity to methylation of the cytosines. Specifically, *Hpa*II will not cut if either of the cytosines is fully (double-strand) methylated, but will cut if the external cytosine is hemi-methylated (single-strand); in contrast, *Msp*I will not cut only if the external cytosine is fully- or hemi-methylated (McClelland et al. 1994). Thus, for a given DNA sample, two major methylation states at the CCGG sites, i.e., full methylation of the internal cytosine, or hemi-methylation of the external cytosine, will be readily recognized in the MSAP profiles (Reyna-Lopez et al. 1997; Cervera et al. 2002). Nonetheless, some methylation states at the CCGG sites (e.g., full methylation of the external cytosine or both cytosines) can be differentiated by this method only in a situation where two or more tissues or developmental stages of the given genotype being examined. This is because under such a situation any difference in the MSAP profile should reflect differential methylation state at the CCGG sites, and hence, will allow the estimation of full methylation of the external cytosine and both cytosines in one versus the other tissue or between developmental stages for the fixed genotype.

The MSAP protocol used in this study was essentially as reported (Reyna-Lopez et al. 1997; Xiong et al. 1999). The restriction enzymes *Eco*RI, *Hpa*II and *Msp*I were purchased from the New England Biolabs Inc. (Beverly, Mass.). In total, one pair of pre-selective primers and 24 pairs of selective primers were used for amplifications (Supplementary Table 1). Silver stained sequencing gel was used to resolve and visualize the amplification products. Only clear and reproducible bands that appeared in two independent PCR amplifications (starting from the digestion-ligation step, i.e., the first step of MSAP) were scored. The scored MSAP bands represent three major cytosine methylation states: (1) hemi-methylation of the external C, which are bands present in *Hpa*II, but absent from the corresponding *Msp*I-digest, i.e., pattern H/M = +/−; (2) full methylation of the internal C, which are bands absent from *Hpa*II, but present in the corresponding *Msp*I-digest, i.e., pattern H/M = -/+, and; (3) full methylation of the external C or both Cs, which are bands absent from both *Hpa*II- and *Msp*I-digest, but present in the alternative tissue of the same genotype i.e., pattern H/M = -/- in tissue1 versus H/M = +/+ in tissue2, and vice versa. Statistical analysis was performed whenever possible by the *t* test.

### Recovery and sequencing of MSAP bands

Bands showing various patterns of methylation alteration in a hybrid relative to its parents, or hypomethylation in endosperm relative to leaf, were eluted from the silver-stained MSAP gels and re-amplified with the appropriate selective primer combinations. Sizes of the PCR products were verified by agarose gel electrophoresis, and then cloned into the AT cloning vector (Takara Biotech. Inc., Dalian, China). The cloned DNA segments were sequenced with vector primers by automated sequencing. The Advanced BlastN and BlastX programs at the NCBI website (http://www.ncbi.nlm.nih.gov/) were used for homology analysis of the cloned DNA sequences that gave quality-reads.

### Reverse transcriptase (RT)-PCR analysis

The protocol was essentially according to Liu et al. (2004). Specifically, total RNA was isolated from leaves and endosperms of the same pooled sorghum plants, of hybrid AE and its parents A and E, as those used for MSAP analysis, described earlier, and from leaves of 5-azacytidine (Sigma, St. Louis, MO, USA) or 5-AC-treated plants (Liu et al. 2004), by the Trizol Reagent (Invitrogen), following the manufacturer's protocol. The RNA was treated with DNaseI (Invitrogen), reverse-transcribed by the SuperScriptTM RNase H-Reverse Transcriptase (Invitrogen), and subjected to RT-PCR analysis using gene-specific primers. Six sequenced MSAP bands that bear significant homology to annotated genes in sorghum or other plants were selected for RT analysis. A sorghum actin gene (Genbank accession X79378) was used as a control for RNA input. The primers for these six genes were designed by the Primer 3 program (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and given in Supplementary Table 2. DNA contamination was tested by inclusion of RNAs without RT. From 25 to 28 cycles were used for the various studied genes (20 cycles for the actin gene), which ensured that the amplifications were within the linear range for each gene. The amplicons were visualized by ethidium bromide staining after electrophoresis through 2% agarose gels. Three batches of independently prepared total RNAs were used as technical

replications. The data were quantified by densitometry using the UTHSCSA ImageTool software (http://www.ddsdx.uthscsa.edu/), and the values (in arbitrary unit) were normalized against those of the corresponding actin gene, and the means and standard deviations were calculated.

## Results

### Difference in cytosine methylation levels revealed by MSAP at the CCGG sites between leaf and endosperm, and between hybrids and inbred lines in sorghum

By using 24 pairs of *Eco*RI + *Hpa*II/*Msp*I selective primer combinations (Supplementary Table 1), from 1,363 to 1,459 clear and reproducible bands for the four sets of sorghum hybrids and their inbred parental lines, in leaf and endosperm, were amplified by MSAP (Table 1). Based on the MSAP patterns, various bands representing non-methylation, hemi-methylation of external C, full-methylation of internal C and full methylation of external C or both Cs (see Materials and methods for rational of band-scoring) were tabulated (Table 1). Data showed that among the sorghum lines analyzed, the total methylation levels (calculated by adding up the various patterns) of leaf and endosperm are, respectively, in the range of 27.54–31.94 and 22.76–29.07%, which comprises 6.90–8.02% (leaf) and 8.68–9.76% (endosperm) hemi-methylation of external C, 10.90–13.60% (leaf) and 12.75–17.14% (endosperm) full-methylation of the internal C, and 7.75–11.41% (leaf) and 1.03–3.24% (endosperm) full methylation of the external C or both Cs (Fig. 1; Table 1). Among the methylation patterns, the one showing the most striking difference between the two tissues is full methylation of external C or both Cs at the CCGG sites, with endosperm possessing a significantly lower value than its corresponding leaf tissue for a given inbred or hybrid genotype, indicating marked hypomethylation in endosperm (Table 1). This endosperm-specific hypomethylation was mainly attributable to appearance of many novel bands in the MSAP profiles of endosperm relative to the leaf, in one or both enzyme digestions (exemplified in Fig. 1 as solid circles). On the other hand, the slight increase of hemi-methylation of the external C and full-methylation of the internal C in endosperm relative to leaf was apparently due to their appearance in endosperm from otherwise full methylation of external C or both Cs in leaf. Notably, because the close relatedness of the parental lines used, the number of polymorphic MSAP profiles was small, which, unlike in the case of maize (Lauria et al. 2004), did not allow an estimation on the parental contribution to the hypomethylation in endosperm (e.g., Fig. 1). Notwithstanding this limitation, a general hypomethylation in sorghum endosperm is unequivocal, which has rendered the total methylation level at the CCGG sites of endosperm being significantly lower than that of leaf ($P < 0.01$) in all plant lines studied, by 11.47% (Table 1). Another interesting generalization regarding methylation level that can be obtained from the MSAP analysis is that the hybrids always showed lower levels of the various profiles, including total methylation, hemi-methylation of external cytosine, full methylation of internal cytosine and full methylation of external cytosine or both cytosines, than those of their corresponding mid-parent values in both leaf and endosperm (Table 1). However, statistical analysis showed that only the total methylation levels in leaf were significantly different between the hybrids and the inbred parental lines ($P < 0.01$), whereas all other differences between the hybrids and the parental inbred lines, although obvious (Table 1), did not reach a statistically significant level ($P > 0.05$).

### Inheritance and variation in locus-specific DNA methylation patterns revealed by MSAP in sorghum hybrids

The MSAP profiles enable monitoring of inheritance or variation of parental methylation patterns in hybrid progenies. It was found that a great majority (from 96.78 to 98.31%, depending on crosses) of the methylation profiles in sorghum inbred lines transmitted to the inter-strain hybrids; however, from 1.69 to 3.22% of the profiles in the hybrids exhibited variation from the expected parental additivity (Fig. 2 and Supplementary Table 3). Both inheritance and variation of methylation profiles can be categorized into various types, as exemplified in Fig. 1 and summarized in Fig. 2 and Supplementary Table 3. Specifically, seven types of profiles can be recognized for inheritance, which include: $IA_1$—referring to bands in hybrids that appeared only in *Hpa*II-digest, which were inherited from the maternal parent (data not shown); $IA_2$—referring to bands in hybrids that appeared only in *Msp*I-digest, which were inherited from the maternal parent (Fig. 1, marked as $IA_2$); $IA_3$—referring to bands in hybrids that appeared in both *Hpa*II- and *Msp*I-digests, which were inherited from the maternal parent (Fig. 1, marked as $IA_3$); $IB_1$—referring to bands in hybrids that appeared only in *Hpa*II-digest, which were inherited from the paternal parent (data not shown); $IB_2$—referring to bands in hybrids that appeared only in *Msp*I-digest, which were inherited from the paternal parent (Fig. 1, marked as $IB_2$); $IB_3$—referring to bands in hybrids that appeared in both *Hpa*II- and *Msp*I-digest, which were inherited from the paternal parent (Fig. 1, marked as $IB_3$), and; IC—referring to bands in hybrids that appeared in both *Hpa*II- and *Msp*I-digest, which were inherited from either or both of the maternal and paternal parent—this type apparently comprises the

**Table 1** Levels of cytosine methylation at the CCGG sites in leaf and endosperm of sorghum inbred lines and inter-strain hybrids

| Inbreds and hybrids | Total sites | Non-methylated CCGG sites (number and frequency) | | Methylated CCGG sites (number and frequency) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Total | | Hemi-methylation of the external Cs | | Full methylation of the internal Cs | | Full methylation of the external Cs or both Cs | |
| | | Leaf | Endosperm | Leaf | Endosperm | Leaf | Endosperm | Leaf | Endosperm | Leaf | Endosperm |
| A | 1,371 | 965 (70.39%) | 1045 (76.22%) | 406 (29.61%) | 326 (23.78%) | 103 (7.51%) | 119 (8.68%) | 183 (13.35%) | 190 (13.86%) | 120 (8.75%) | 17 (1.24%) |
| E | 1,363 | 937 (68.75%) | 1015 (74.47%) | 426 (31.25%) | 348 (25.53%) | 108 (7.92%) | 133 (9.76%) | 178 (13.06%) | 201 (14.75%) | 140 (10.27%) | 14 (1.03%) |
| Mid-parental value | 1,367 | 951 (69.57%) | 1030 (75.34%) | 416 (30.43%) | 337 (24.66%) | 106 (7.72%) | 126 (9.22%) | 181 (13.20%) | 196 (14.30%) | 130 (9.51%) | 16 (1.13%) |
| AE | 1,459 | 1055 (72.31%) | 1127 (77.24%) | 404 (27.69%) | 332 (22.76%) | 108 (7.40%) | 130 (8.91%) | 183 (12.54%) | 186 (12.75%) | 113 (7.75%) | 16 (1.10%) |
| B | 1,368 | 936 (68.42%) | 998 (72.95%) | 432 (31.58%) | 370 (27.05%) | 108 (7.89%) | 125 (9.14%) | 186 (13.60%) | 213 (15.57%) | 138 (10.09%) | 32 (2.34%) |
| F | 1,359 | 925 (68.06%) | 964 (70.93%) | 434 (31.94%) | 395 (29.07%) | 109 (8.02%) | 118 (8.68%) | 178 (13.10%) | 233 (17.14%) | 147 (10.82%) | 44 (3.24%) |
| Mid-parental value | 1,364 | 931 (68.24%) | 981 (71.94%) | 433 (31.76%) | 383 (28.06%) | 109 (7.96%) | 122 (8.91%) | 182 (13.35%) | 223 (16.36%) | 143 (10.45%) | 38 (2.79%) |
| BF | 1,442 | 1030 (71.43%) | 1071 (74.27%) | 412 (28.57%) | 371 (25.73%) | 105 (7.28%) | 127 (8.81%) | 170 (11.79%) | 207 (14.36%) | 137 (9.50%) | 37 (2.57%) |
| C | 1,378 | 972 (70.54%) | 1009 (73.22%) | 406 (29.46%) | 369 (26.78%) | 99 (7.18%) | 129 (9.36%) | 163 (11.83%) | 221 (16.04%) | 144 (10.45%) | 19 (1.38%) |
| H | 1,359 | 949 (69.83%) | 984 (72.41%) | 410 (30.17%) | 375 (27.59%) | 96 (7.06%) | 124 (9.12%) | 168 (12.36%) | 233 (17.14%) | 146 (10.74%) | 18 (1.32%) |
| Mid-parental value | 1,369 | 961 (70.18%) | 997 (72.81%) | 408 (29.82%) | 372 (27.19%) | 98 (7.12%) | 127 (9.24%) | 166 (12.10%) | 227 (16.69%) | 145 (10.60%) | 19 (1.35%) |
| CH | 1,449 | 1050 (72.46%) | 1080 (74.53%) | 399 (27.54%) | 369 (25.47%) | 102 (7.04%) | 132 (9.11%) | 172 (11.87%) | 218 (15.04%) | 125 (8.63%) | 19 (1.31%) |
| C | 1,378 | 972 (70.54%) | 1009 (73.22%) | 406 (29.46%) | 369 (26.78%) | 99 (7.18%) | 129 (9.36%) | 163 (11.83%) | 221 (16.04%) | 144 (10.45%) | 19 (1.38%) |
| I | 1,376 | 960 (69.77%) | 989 (71.88%) | 416 (30.23%) | 387 (28.13%) | 95 (6.90%) | 129 (9.38%) | 164 (11.92%) | 221 (16.06%) | 157 (11.41%) | 37 (2.69%) |
| Mid-parental value | 1,377 | 966 (70.15%) | 999 (72.55%) | 411 (29.85%) | 378 (27.45%) | 97 (7.04%) | 129 (9.37%) | 164 (11.87%) | 221 (16.05%) | 151 (10.93%) | 28 (2.03%) |
| CI | 1,450 | 1044 (72.00%) | 1072 (73.93%) | 406 (28.00%) | 378 (26.07%) | 102 (7.03%) | 130 (8.97%) | 158 (10.90%) | 220 (15.17%) | 146 (10.07%) | 28 (1.93%) |

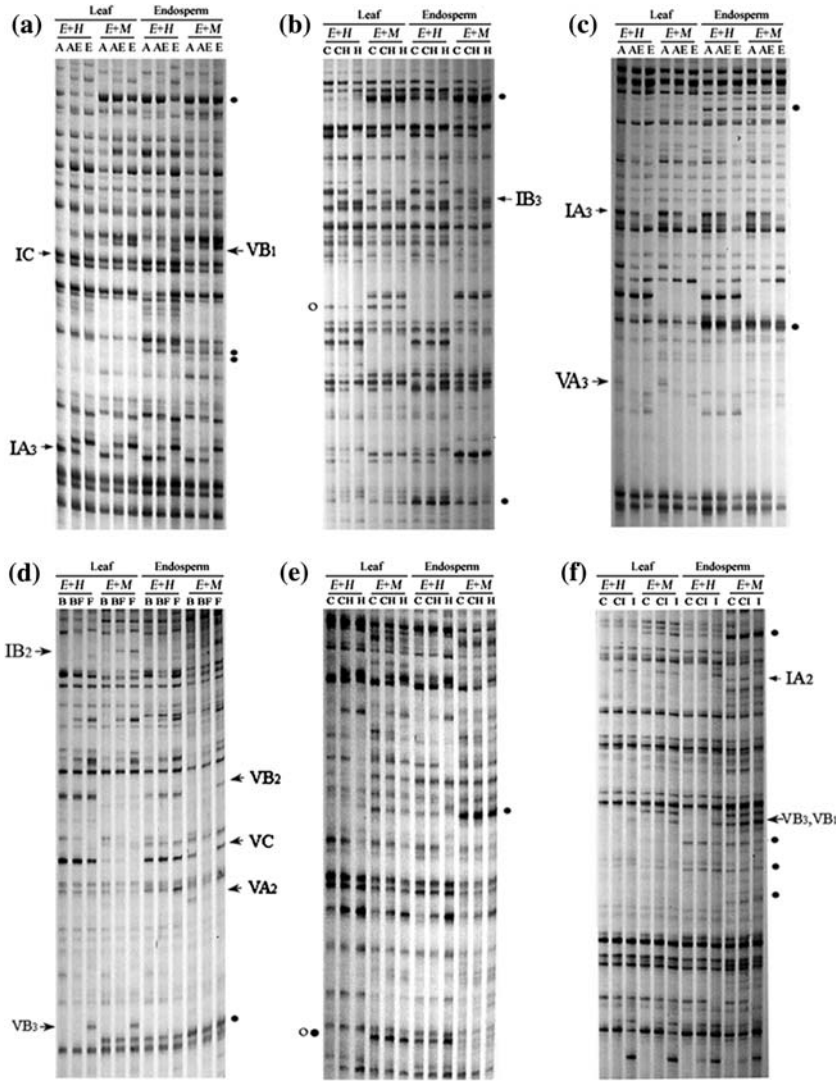


**Fig. 1** Examples of MSAP profiles showing the various types of locus-specific DNA methylation inheritance and variation in sorghum inter-strain hybrids relative to their corresponding inbred parental lines, and difference in methylation between leaf and endosperm in DNA methylation patterns. Primer combinations are *Eco*RI + AGG/*Hpa*II(*Msp*I) + TTG (**a**), *Eco*RI + AGG/*Hpa*II(*Msp*I) + TTA (**b**), *Eco*RI + AGG/*Hpa*II(*Msp*I) + TCT (**c**), *Eco*RI + AAC/*Hpa*II(*Msp*I) + TCT (**d**), *Eco*RI + AAC/*Hpa*II(*Msp*I) + TTA (**e**) and *Eco*RI + AAG/*Hpa*II(*Msp*I) + TTC (**f**). Inheritance or variation of the various methylation patterns are denoted by the following codes: $IA_2$—bands in hybrids that appeared only in *Msp*I-digest, which were inherited from the maternal parent; $IA_3$—bands in hybrids that appeared in both *Hpa*II- and *Msp*I-digests, which were inherited from the maternal parent; $IB_2$—bands in hybrids that appeared only in *Msp*I-digest, which were inherited from the paternal parent; $IB_3$—bands in hybrids that appeared in both *Hpa*II- and *Msp*I-digest, which were inherited from the paternal parent; IC—referring to bands in hybrids that appeared in both *Hpa*II- and *Msp*I-digest, which were inherited from either or both of the maternal and paternal parent. $VA_2$—alterations occurred in *Msp*I-digest of hybrids, wherein band(s) was not inherited from the maternal parent; $VA_3$—alterations occurred in both *Hpa*II- and *Msp*I-digest of hybrids, wherein band(s) was not inherited from the maternal parent; $VB_1$—alterations occurred in *Hpa*II-digest of hybrids, wherein band(s) was not inherited from the paternal parent; $VB_2$—alterations occurred in *Msp*I-digest of hybrids, wherein band(s) was not inherited from the paternal parent; $VB_3$—alterations occurred in both *Hpa*II- and *Msp*I-digest of hybrids, wherein band(s) was not inherited from the paternal parent; VC—alterations occurred in *Hpa*II- or *Msp*I-digest of hybrids, wherein band(s) was not inherited from both the maternal and the paternal parents. Note that three codes ($IA_1$, $IB_1$ and $VA_1$) not detected in these images are described in the main text. The endosperm- and leaf-specific MSAP bands are marked, respectively, by *solid* and *open circles*

greatest majority, as all monomorphic bands belong to this type (Fig. 1, marked as IC; Fig. 2; Supplementary Table 3).

For methylation patterns that showed variation in hybrids relative to their parents, at least seven types are detected, which include: $VA_1$—referring to alterations occurred in *Hpa*II-digest of hybrids, wherein band(s) was not inherited from the maternal parent (data not shown); $VA_2$—referring to alterations occurred in *Msp*I-digest of

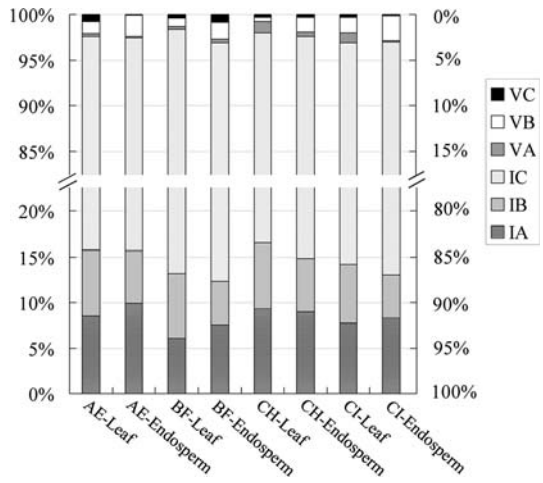


**Fig. 2** Frequencies of inheritance and variation of the three main types of cytosine methylation patterns at the CCGG sites in two tissues, leaf and endosperm, of four sorghum inter-strain hybrids (AE, BF, CH and CI)

hybrids, wherein band(s) was not inherited from the maternal parent (Fig. 1, marked as $VA_2$); $VA_3$—referring to alterations occurred in both *Hpa*II- and *Msp*I-digest of hybrids, wherein band(s) was not inherited from the maternal parent (Fig. 1, marked as $VA_3$); $VB_1$—referring to alterations occurred in *Hpa*II-digest of hybrids, wherein band(s) was not inherited from the paternal parent (Fig. 1, marked as $VB_1$); $VB_2$—referring to alterations occurred in *Msp*I-digest of hybrids, wherein band(s) was not inherited from the paternal parent (Fig. 1, marked as $VB_2$); $VB_3$—referring to alterations occurred in both *Hpa*II- and *Msp*I-digest of hybrids, wherein band(s) was not inherited from the paternal parent (Fig. 1, marked as $VB_3$); and VC—referring to alterations occurred in *Hpa*II- or *Msp*I-digest of hybrids, wherein band(s) was not inherited from both the maternal and paternal parents (Fig. 1, marked as VC).

For variation frequency, although a wide-ranging difference (from 0 to 1.46%) was detected across the genotype/tissue/variation-type combinations (Supplementary Table 3), three generalizations can be made: (1) with regard to the hybrid genotypes, variation occurred in all four studied F1 hybrids in both tissues (Fig. 2; Supplementary Table 3, the right-most column). (2) With regard to the three variation types i.e., VA (including VA1, VA 2 and VA 3), VB (including VB1, VA 2 and VA3) and VC (see the foregoing paragraph for detailed description), VB occurred at the highest frequency, followed by VA, and then by VC, when the two tissues and four hybrids were considered together (Fig. 2; Supplementary Table 3, the lower-most row). (3) With regard to the two tissues, if all the variation types were considered together, the total methylation variation frequency in endosperm appeared to be higher than that in leaf for each hybrid (Fig. 2 and Supplementary Table 3); however, the difference is not statistically significant ($P > 0.05$). If the different types of variation (VA, VB and VC) were considered separately, sharp difference exists in the two tissues, whereas a higher variation frequency in leaf than in endosperm is evident in at least three of the four hybrids (AE, CH and CI); for VA the opposite is true for VB in all four hybrids (Fig. 2; Supplementary Table 3), both of these differences are statistically significant ($P < 0.05$); for VC, higher frequency occurred in leaf than in endosperm in two hybrids (AE and CI), no difference in one hybrid (CH), and the opposite is true in the remaining hybrid (BF) (Fig. 2; Supplementary Table 3).

## The DNA methylation alteration in sorghum inter-strain hybrids appeared either non-random or stochastic and was not caused by parental heterozygosity

In the foregoing analysis, DNA from pooled plants (10–15 individuals) was used; thus the inheritance or variation of DNA methylation patterns should reflect the *majority* of the hybrid plants rather than *all* plants. In addition, the major type of variation found in this study, i.e., loss of parental bands, would have escaped from detection if not all hybrid plants showed the same variation. Thus, to test if inheritance and variation in DNA methylation patterns occurred in all plants (henceforth being referred to as non-random) or in only some plants (henceforth being referred to as stochastic) for a given hybrid, we analyzed 14 randomly selected individual plants for each of the four hybrids, with eight MSAP primer pairs (Supplementary Table 1) that showed the most alterations in hybrids. It was found that in all primer pairs, both non-random and stochastic alterations are evident in the hybrids (e.g., Fig. 3 and data not shown). Another indirect line of evidence for occurrence of non-random methylation alteration is that, based on the sequencing data (Supplementary Table 4), several MSAP profiles were actually being isolated independently from hybrids of different cross-combinations.

An apparent concern for variation in the MSAP profiles in the hybrids is the possibility of pre-existing parental heterozygosity. The inbred nature of the seven parental lines used (they had been strictly selfed for many successive generations in our hands), argues against this possibility. To further clarify this concern, we randomly chose 21 individual plants for each of the seven inbred lines and subjected them to the same MSAP analysis using the same eight primer combinations (Supplementary Table 1). It was found that in all primer combinations, complete uniformity in banding patterns was observed for each of the seven lines (e.g., Fig. 4 and data not shown), indicating that it is unlikely that parental heterozygosity has been a major contributory factor in the methylation alterations detected in hybrids. This analysis has also verified the high reproducibility of the MSAP method we used.

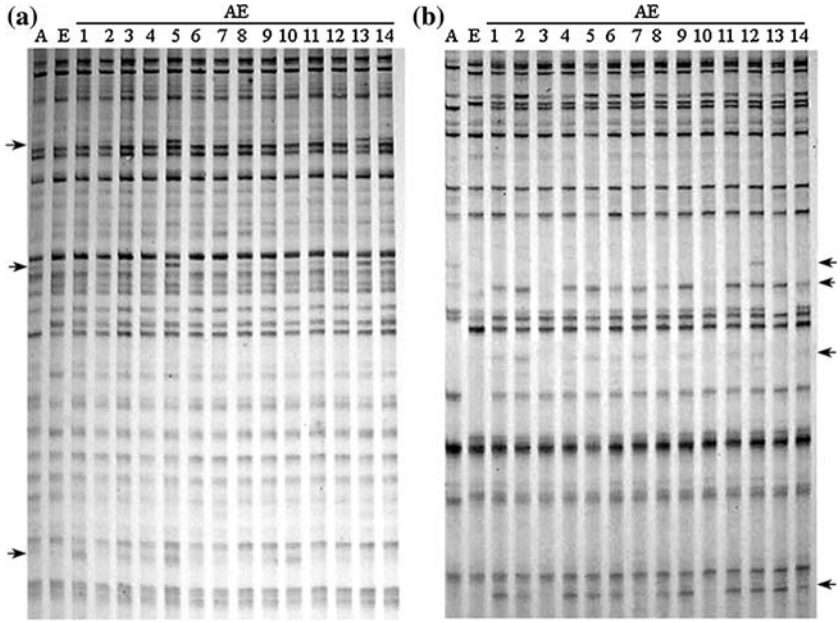


**Fig. 3** An example of stochastic variation in MSAP patterns (marked by *arrows*) in individual hybrid plants of cross combination AE. The primer combinations are *Eco*RI + AAC/*Hpa*II(*Msp*I) + TTA (**a**) and *Eco*RI + AGG/*Hpa*II(*Msp*I) + TTG (**b**)

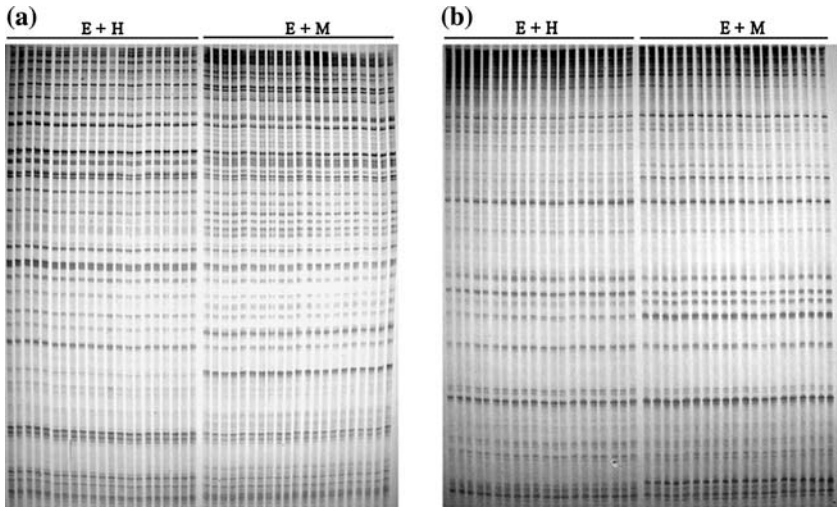


**Fig. 4** Examples of MSAP analysis on possible heterozygosity in the inbred parental lines A [primer combination *Eco*RI + AAC/*Hpa*II(*Msp*I) + TTA] (**a**) and E [primer combination *Eco*RI + AAC/*Hpa*II(*Msp*I) + TAC] (**b**)

Sequences underlying DNA methylation alterations in sorghum inter-strain hybrids and sequences showing endosperm-specific hypomethylation

BlastX analysis of 77 sequenced MSAP bands representing various patterns of methylation alteration in the four sets of sorghum inter-strain hybrids (Figs. 1, 3; Supplementary Table 4) showed that diverse sequences are involved in methylation alterations accompanying hybrid formation (Supplementary Table 4). Of these, 12 and 17 bands, respectively, from leaf and endosperm origin, showed significant homology to known-function cellular genes. The 12 leaf bands included three putative kinase proteins (A21, A26 and A48), a DRE-binding protein (A20), a putative, mariner-like transposon protein (A33), a C2H2 zinc-finger transcription factor (A41), a putative reverse transcriptase (C13), a leaf senescence-related protein (H5-3), a putative alcohol dehydrogenase (A34), an IcmB protein (A1-6), a putative RIRE2 orf3 (A17), a putative cytochrome P450-like protein (A40). Most of the bands isolated from endosperm-bearing similarity to known-function genes are metabolism-related proteins, like those involved in photosynthesis and respiration (E30, E36, H5-2, E12, E28, E26, C2-1, C2-2, E2 and E41) and a putative sulfate transporter (E5). In addition, a pathogenesis-related protein (E24), a methyl-binding domain protein (E19), a C2 domain-containing protein (E17), an ABA-induced gene (A1-3) and two putative retroelements (C2-3 and E35) are also identified. Three bands (two from leaf and one from endosperm) showed homology to hypothetical proteins. The remaining 35 bands showed no homology to the database sequences (Supplementary Table 4).

Thirty-six sequenced MSAP bands that represent endosperm-specific genomic loci for a given genotype (Fig. 1) also included diverse sequences (Supplementary Table 5). Of these, nine bands showed significant homology to known-function cellular genes including three starch synthesis-related enzymes (C2-3, C5-2 and D5-3), two putative senescence-associated proteins (E6-3 and G10-2), a putative nuclear ribonucleoprotein (E1-2), a malate dehydrogenase (A6-3), a putative 22 kDa kafirin cluster (G1-1), and a ADP-glucose pyrophosphorylase subunit SH2 (B5-1). Five bands (H5-1, A8-1, F5-1, H6-2 and B4-1) are related to putative transposon or retrotransposon proteins. Two bands bear homology to hypothetical proteins. The remaining 20 bands showed no homology to database sequences.

Sequence analysis confirmed that 78 out of the 113 isolated MSAP bands have only one of their termini with a CCGG site and the other with a GAATTC site, indicating that these bands were resulting from *Eco*RI and *Hpa*II/*Msp*I double digestions (Supplementary Tables 4, 5). Notably, the remaining 35 bands contained one or more internal CCGG site(s). This suggests that the methylation levels calculated based on MSAP are underestimated to an extent.

### Endosperm-specific hypomethylation in some genic sequences is accompanied with increased transcript accumulation

Six sequenced MSAP bands (E6-3, E1-2, D5-3, C2-3, H1-4 and C5-2) that showed endosperm-specific hypomethylation in one or more of the sorghum inbred lines or hybrids (Supplementary Table 5), and that bear significant homology to annotated genes of sorghum or other plants (Supplementary Tables 2, 5) were selected for expression analysis. Transcript accumulation was measured by semi-quantitative RT-PCR on three batches of independently isolated RNAs from leaf and endosperm tissues of two inbred lines (A and E) and its hybrid (AE), as well as from leaves of 5-azacytidine (5-AC)-treated plants. As shown in Fig. 5, all six genes exhibited an increased level of steady-state transcript accumulation in endosperm than in leaf, and which is true for both inbred parental lines and the hybrid. Specifically, four genes (E6-3, E1-2, D5-3 and C2-3) showed an enhanced expression in endosperm relative to leaf in a quantitative manner, whereas the rest two genes (H1-4 and C5-2) in a complete on/off (endosperm vs. leaf) manner, i.e., endosperm-specific expression. To test if the increased or specific expression of these genes in endosperm is associated with their hypomethylated state relative to that of leaf, RNA was isolated from leaves of 5-AC-treated sorghum plants. As shown in Fig. 5, of the four genes that showed enhanced expression in endosperm in a quantitative manner, two genes (E6-3 and E1-2) exhibited an apparent increase in transcript amount in leaf as a result of 5-AC treatment; of the two endosperm specifically expressed genes, one (H1-4) showed activation in leaf by 5-AC treatment in one inbred parental line (A), whereas it remained silent in the other inbred line (E) and the hybrid (AE), suggesting striking genotypic difference in response to demethylation by the drug treatment. No transcriptional activation was detected for gene C2-3. Taken together, this experiment suggests that for some but not all genes, their endosperm-specific or enhanced expression is likely associated with their tissue-specific loss of methylated cytosines. Another notable observation from this analysis is that for all six genes, the expression level in the hybrid (in both tissues, leaf and endosperm, as well as in leaf from 5-AC treated plants) is very close to the calculated mid-parent values as would be expected from parental additive expression, although unequal parental transcript contribution cannot be ruled out (Fig. 5b).

## Discussion

DNA methylation in the form of cytosine methylation was proposed as an ancient evolutionary device (Colot and Rossignol 1999), which has contributed to genome evolution and plays an important role in maintaining genome integrity and controlling dynamics of gene activity (Tariq and Paszkowski 2004; Rangwala and Richards 2004; Chan et al. 2005). Compared with animals, cytosine methylation is more abundant in plants, particularly at genomic regions containing transposons and their derivatives (Rabinowicz et al. 2005). Similar to the situation in animals, accumulating evidence suggested that DNA methylation plays important roles in normal plant development (Finnegan et al. 2000). For example, both induced and naturally occurring variations in DNA methylation at critical genomic loci may cause wide-ranging developmental abnormalities in several plants investigated (Kakutani et al. 1996; Finnegan et al 1996; Cubas et al. 1999). On the other hand, it is generally believed that, in contrast to animals, plants do not undergo extensive remodeling in cytosine methylation across ontogenic development. However, a recent study clearly showed that plant development can be accompanied by progressive changes in methylation sate (Ruiz-García et al. 2005). In addition, an earlier cytological examination using methylcytosine-specific antibody showed that male gametogenesis in tobacco was associated with a drastic reduction in cytosine methylation content (by ca. 80%) in the pollen generative nuclei (Oakeley et al. 1997). Furthermore, it was documented in maize that, relative to embryo and leaf tissues, endosperm exhibited 13% reduction in methyl-cytosine content, which was mainly caused by extensive maternal-specific hypomethylation (Lauria et al. 2004). This unidirectional hypomethylation in maize was

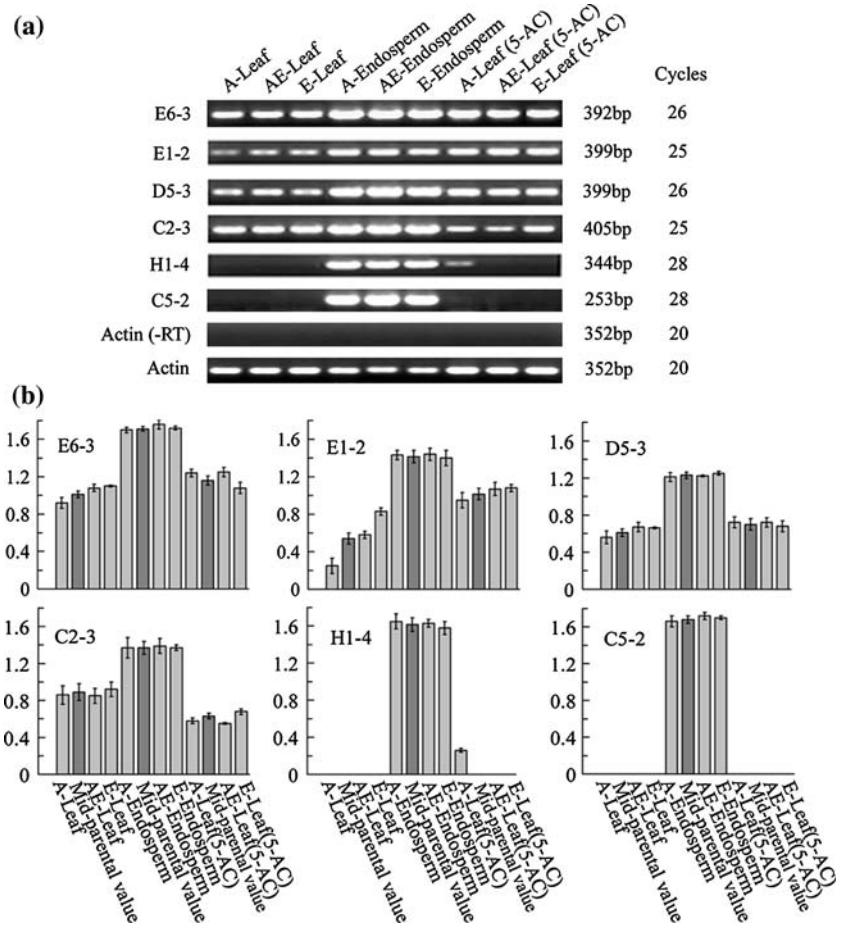


**Fig. 5** RT-PCR analysis of six selected MSAP bands (*E6-3*, *E1-2*, *D5-3*, *C2-3*, *H1-4* and *C5-2*) that underwent endosperm-specific hypomethylation relative to leaf based on MSAP profiles, and that showed significant homology to annotated plant genes, on three batches of independently isolated RNAs from leaf and endosperm of a sorghum hybrid (AE) and its maternal (A) and paternal (E) parents, as well as from leaves of 5-azacytidine-treated plants. A sorghum actin gene (Genbank accession X79378) was used as a control for RNA input, and DNA contamination (on RNAs without RT). One of the three independent experiments is shown in **a**. The gene name, product size and amplification cycles are labeled. Shown in **b** are data of all three experiments after quantification by densitometry using the UTHSCSA ImageTool software (http://www.ddsdx.uthscsa.edu/), and the values (in arbitrary unit) were normalized against those of the corresponding actin gene, and presented as means and standard deviations (*light-gray rectangles*). *Dark-gray rectangles* are calculated mid-parent values. Gene names are labeled

proposed to provide a mechanism conditioning a parent-of-origin, allele-specific effect that is crucial to normal endosperm development (Lauria et al. 2004). Similar findings were made in *Arabidopsis* (Kinoshita et al. 2004). We have showed in this study that in sorghum, a close relative of maize, endosperm also experiences a marked reduction in cytosine methylation level at the CCGG sites (ranging from 6.89 to 19.69%, depending on genotypes) compared with leaf. This endosperm-specific hypomethylation was mainly due to reduction of full methylation of external C or both Cs at the CCGG sites, which were reflected as appearance of many novel bands in the MSAP profiles of endosperm relative to leaf, in one or both *Hpa*II and *Msp*I digestions (Fig. 1, Table 1). It has been established in *Arabidopsis* that CG and CNG methylation states are maintained by two distinct DNA methyltransferases, MET1 and CMT3, respectively (Tariq and Paszkowski 2004; Chan et al. 2005). Therefore, it can be speculated that titration of these enzymes in endosperm versus leaf may cause hypomethylation in the former. Given that the hemi-methylation of the external Cs and full methylation of the internal Cs were not reduced but even slightly increased in endosperm (Table 1), it appears that, if the above is a viable explanation, then the two enzymes probably have not been titrated to the same extent. Alternatively, activation of an active demethylase, like the *Arabidopsis* DEMETER (Gehring et al. 2006), may also result in endosperm-specific demethylation, as in the case of the *Arabidopsis* imprinting gene FWA (Kinoshita et al. 2004). Of course, these two possibilities are not necessarily mutually exclusive.

Although the low degree of polymorphic MSAP profiles between the sorghum inbred lines used in this study

did not allow an accurate assessment on the parental contribution to the hypomethylation in endosperm, based on the findings in both maize (Lauria et al. 2004) and *Arabidopsis* (Kinoshita et al. 2004), it is likely that the maternal genome probably plays a greater role than the paternal genome in sorghum endosperm hypomethylation. Because endosperm does not contribute to the next generation, it has been suggested that this maternal-specific, uniparental demethylation provides a novel gene-activation mechanism for regulating expression of genes important for proper endosperm or seed development in higher plants (Kinoshita et al. 2004).

It has been widely recognized that, in contrast to the general rule of "erase-and-reset" cytosine methylation dynamics in each generation in animals, parental methylation states in plants are often stably inherited to sexual progenies (Kakutani 2002; Riddle and Richards 2002; Cubas et al. 1999). Nevertheless, it was observed in a wide array of plant taxa that formation of inter-specific hybrids and allopolyploids is often accompanied by remodeling of the otherwise additive parental methylation patterns (Madlung et al. 2002; Levy and Feldman 2004; Liu and Wendel 2003; Liu et al. 2004; Salmon et al. 2005; Lukens et al. 2006; Marfil et al. 2006). Likewise, it was recently documented in *Arabidopsis* that parental methylation states of at least some genomic loci may also be modified by *trans*-acting modifiers in certain intra-specific hybrids between different ecotypes (Riddle and Richards 2005). We recently showed that this is also the case in several intraspecific maize hybrids (Zhao et al. 2007). Together, these results implicate that the fidelity of epigenetic inheritance in plant DNA methylation patterns can be compromised under certain circumstances, such as when genetically differentiated genomes are brought together into a common nucleus by hybridization.

We have showed in this paper that, in sorghum, although a great majority of the cytosine methylation sites within the CCGG motifs manifested stable inheritance from inbred parents to hybrids, from 1.69 to 3.22% of the sites showed deviation from expected parental additivity. The changing frequencies vary among the parental combinations, and between the two tissues (leaf and endosperm), suggesting that the occurrence and extent of methylation variation are being influenced by both genetic context of the hybrids and probably also development-related epigenetic state. That methylation reconfiguration in inter-strain hybrids is genetically controlled has been elegantly demonstrated in *Arabidopsis* (Riddle and Richards 2005). Together with a general property of transgenerational inheritance, remodeling of parental methylation patterns upon sexual hybridization may have bearings on genome evolution, as well as on the enhanced overall performance of a hybrid relative to its inbred parents, a phenomenon known as heterosis or hybrid vigor (see the following sections for further discussion).

The observation that the methylation alterations in genetically identical sorghum hybrid individuals can be either non-random or stochastic is interesting. Because pooled hybrid plants were used in the present study, and the main type of methylation alteration observed is non-inheritance of parental patterns, it appeared likely that only those MSAP profiles showing non-random methylation alteration (occurred in all pooled hybrid plants) have been characterized in this study (Table 2, Supplementary Table 4). Since it is known that different F1 hybrid individuals for a given cross are largely uniform in phenotype, it can be deduced that the genomic loci undergoing stochastic alterations probably have only negligible, if any, phenotypic consequences. Therefore, those loci that undergo non-random methylation alteration in a hybrid are more likely consequential to hybrid-specific gene expression, and by extension, to novel phenotypes.

An interesting finding from this study is that of the six investigated MSAP bands that undergo endosperm-specific hypomethylation according to their MSAP profiles, and that bear significant homology to annotated plant genes, all showed increased level of expression in endosperm relative to leaf wherein they are hypermethylated. This suggests that DNA methylation state of these genes may underlie the tissue-specific regulation on their expression. Indeed, three of the six genes showed markedly elevated transcript levels in leaves after 5-azacytidine treatment, at least in one genotype, indicating that methylation state likely partition in endosperm-specific or enhanced gene expression in sorghum, but other regulatory mechanisms are apparently also involved.

**Table 2** Functional classification of cloned MSAP profiles showing alteration in sorghum inter-strain hybrids or difference in methylation between leaf and endosperm in DNA methylation pattern based on the MSAP profile

| Category | No. and percentage (%) of MSAP profiles | | |
|---|---|---|---|
| | Alteration in hybrids | | Differential methylation between leaf and endosperm |
| | Leaf | Endosperm | |
| Known-function cellular gene | 12 (32.4 %) | 17 (38.6 %) | 14 (38.9 %) |
| Unknown protein | 2 (5.4 %) | 1 (2.3 %) | 2 (5.6%) |
| No similarity | 23[a] (62.1 %) | 26[a] (59.1 %) | 20 (55.6 %) |
| Total | 37 (100%) | 44 (100%) | 36 (100%) |

[a] Including four MSAP profiles that showed common methylation pattern alterations in leaf and endosperm

Although heterosis has been successfully exploited by plant breeders over many decades, the mechanism underlying the phenomenon remains largely unknown (Birchler et al. 2003, 2005). It was suggested recently that differences in *cis*- or *trans*-acting regulatory factors and epigenetic mechanisms between the inbred parental lines may play a role in regulating novel patterns of gene expression in the resulting hybrid, which contribute to the enhanced performance in the hybrid (Sun et al. 2004; Guo et al. 2004, 2006; Swanson-Wagner et al. 2006; Springer and Stupar 2007). Given what is known about the regulatory effects of DNA methylation on gene expression, it is plausible that the alterations in parental methylation patterns towards a general decrease in methylation level (particularly in many coding genes, Table 2, Supplementary Table 4) may indeed contribute to novel expression in sorghum hybrids, and by extension, to heterosis. In this respect, it is worth mentioning that all four sets of sorghum hybrids used in this study actually are highly heterotic based on multiple "year by location" field test (our unpublished data). Nevertheless, of the six genes investigated here, all showed expression levels comparable to the expected mid-parent values in both leaf and/or endosperm of the hybrid relative to its two parents (Fig. 5b), suggesting that the majority of genes in inter-strain sorghum hybrids, as in maize (Guo et al. 2006), are likely to show additive expression. It remains a formal possibility, however, that the two parental transcripts, at least for some genes, may contribute unequally in the hybrid (Adams and Wendel 2005; Guo et al. 2004), and which may relate to heterosis. Further studies using combinations with contrasting heterotic potential (low vs. high), and employing techniques that enable differentiation of parental transcripts in sorghum hybrids, like those used in maize (Guo et al. 2004, 2006; Springer and Stupar 2007), may help to elucidate possible associations between decreased methylation, parental transcript-contribution and hybrid performance.

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