Euphytica (2010) 175:303–314
DOI 10.1007/s10681-010-0167-2

Alterations in inheritance pattern and level of cytosine DNA methylation, and their relationship with heterosis in rice

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Received: 25 June 2009 / Accepted: 5 April 2010 / Published online: 27 April 2010
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Abstract Novel hybrid-specific/heterotic gene expression patterns observed from expression studies suggest the need to characterize the underlying regulatory mechanism(s) to reveal the biological basis of heterosis in crop plants. To gain an insight into the molecular basis of heterosis in rice, we investigated the inheritance pattern and level of cytosine methylation, a major epigenetic regulatory mechanism, in the leaf tissue of an elite Indian rice hybrid KRH2 and its parents at three stages (15 day-old, 35 day-old seedling and flag leaf) and their correlation with heterosis using methylation-sensitive amplification polymorphism (MSAP) technique. In contrast to parents, the level of methylation was high during initial growth stages and reduced as the hybrid grew. Even though, a majority of cytosine methylation profiles were transmitted to hybrid at all stages, a considerable level (25.4%) of cytosine methylation pattern was observed to be novel in hybrid and some of these altered loci were identified to code for known/hypothetical proteins. It was also observed that demethylation events occurred more predominantly during early stages than hypermethylation while the frequency of demethylation events decreased in flag leaf with a remarkable increase in hypermethylation in the hybrid. These bi-directional events in methylation in KRH2 were validated in six hybrids with different levels of grain yield heterosis and the level of cytosine methylation in the hybrid was observed to be significantly correlated with heterosis.

Keywords Heterosis · Rice · Molecular basis · DNA methylation · MSAP

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Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops in the world. It has been projected that we need to produce 70% more rice from reduced land area with less labor and less water by the year 2025 to feed an ever increasing population (Khush 1997).

Hence, it is inevitable to enhance the productivity levels under limiting resources. Exploitation of heterosis is the best available strategy to break the existing yield barriers in rice. Despite the significant contributions realized by exploiting heterosis to improve crop productivity, the biological mechanism of heterosis is not clearly understood. Understanding the mechanism will enhance our ability in future selection programs towards a more targeted exploitation of heterosis and also help in more efficient ways of predicting heterosis (Xiong et al. 1998), thereby enhancing the efficiency of hybrid breeding. In addition, it may help us to understand the role of heterosis in evolution and the domestication of crop plants (Lippman and Zamir 2006).

With the advent of molecular markers and construction of linkage maps, attempts were made by different scientific groups to characterize the genetic basis of heterosis (Xiao et al. 1995; Yu et al. 1997; Li et al. 2001; Luo et al. 2001; Zhang et al. 2001; Xing et al. 2002; Hua et al. 2002, 2003; Frascaroli et al. 2007) and to understand the relationship of heterosis with molecular heterozygosity in rice (Zhang et al. 1994, 1995, 1996; Saghai Maroof et al. 1997; Liu et al. 1998; Zhao et al. 1999). However, these studies have provided no information about the genes and/or the molecular mechanisms involved in the manifestation of heterosis. While it was hoped that the new genomic technologies such as global gene expression profiling using microarrays would reveal the genetic and molecular basis of heterosis, several studies in maize, rice, and Arabidopsis involving microarrays (Guo et al. 2003, 2006; Vuylsteke et al. 2005; Huang et al. 2006; Swanson-Wagner et al. 2006; Stupar and Springer 2006; Meyer et al. 2007) have only reported the differential gene expression in hybrid and proposed molecular models based on classical genetic hypotheses. No consensus was emerged as to how those differential expression patterns arose, how they relate to heterosis (Zhang et al. 2008a) and what are the underlying causative regulatory mechanisms of these differential expression patterns in hybrids. Characterization of the mechanisms that regulate these expression polymorphisms will certainly advance the fundamental understanding of biological processes underlying growth and development leading to phenotypic diversity; thereby enhance our understanding of heterosis (Huang et al. 2006). The observed differential gene expression could be due to *cis*- or *trans*-acting, or epigenetic regulatory polymorphisms among the parents and hybrid. Among the regulatory mechanisms, DNA methylation, a major epigenetic regulatory phenomenon, has received considerable attention in recent years due to its fundamental role in multiple cellular activities, including orchestrating gene expression during plant development (Rangwala and Richards 2004; Chan et al. 2005), maintenance of genome integrity (Bestor 1998; Colot and Rossignol 1999; Matzke et al. 1999; Cao and Jacobsen 2002; Rapp and Wendel 2005), formation and perpetuation of heterochromatin, and control of genomic imprinting (Finnegan et al. 2000; Bourc'his and Bestor 2004; Rangwala and Richards 2004; Tariq and Paszkowski 2004). Moreover, due to its important role in transcriptional inactivation leading to gene silencing, and gene regulation, there is an increasing interest to study various aspects of cytosine DNA methylation (Dong et al. 2006).

Understanding the dynamics and inheritance patterns of DNA methylation is essential for elucidating epigenetic paradigms in plant development, evolution (Zhang et al. 2007) and heterosis. The possible role of methylation in the expression of heterosis was first suggested by Tsaftaris et al. (1997) in maize. Later, Tsaftaris and Polidoros (2000) have suggested that DNA methylation could be considered as genome-wide regulatory mechanism that affects the global expression of many genes involved in the manifestation of heterosis. Although the molecular bases 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). Of the various approaches to study global methylation, several studies have strongly demonstrated the consistency and reproducibility of MSAP (methylation-sensitive amplification polymorphism) technique in detecting global cytosine methylation patterns and levels in rice and other crops (Cervera et al. 2002; Portis et al. 2004; Sha et al. 2005; Dong et al. 2006; Fang and Chao 2007; Zhang et al. 2007; Zhao et al. 2007; Li et al. 2008; Salmon et al. 2008; Zhang et al. 2008b).

Despite the tremendous progress in genetics and genomics of rice, very little or no information is available on the epigenetic aspect, particularly cytosine methylation or its relationship to heterosis. Xiong

et al. (1999) have studied the extent and patterns of cytosine methylation in a rice hybrid and its parents using MSAP technique and demonstrated the utility of this technique in studying global methylation status although the relationship between methylation and heterosis was not examined. As it is clear from the recent microarray based expression-studies that characterization of regulatory mechanisms and their interaction is important in elucidating molecular bases of heterosis, the present study aimed to characterize one such regulatory mechanism such as DNA methylation and its relationship to heterosis. The main objective of the present study is to characterize DNA methylation in order to reveal level, pattern and direction of DNA methylation likely to be associated with heterosis during growth and development of an elite rice hybrid KRH2 (a widely grown hybrid with consistently proven heterotic potential).

Materials and methods

Plant material and field evaluation

The parental lines and hybrids used in the present study are listed in Table 1. In addition to the elite rice hybrid KRH2 and their parents, three released public-bred hybrids and three highly heterotic experimental hybrids along with their parents were studied for their performance and mid-parent heterosis. All the hybrids were grown in the test cross nursery along with their parents to study heterosis in randomized block design (RBD) with three replications during *rabi* 2005 at the Maharajpet farm of Barwale foundation, Hyderabad. The field layout was designed by adopting a plot size of 3 m × 0.2 m (single row of 20 plants per plot) with 20 cm × 15 cm spacing and single seedling per hill. Standard agronomic practices and need-based plant protection measures were adopted uniformly to raise a good crop. Observations were recorded on five randomly selected plants per replication for single plant yield and on all the plants from the plot for plot yield.

Heterosis calculation and correlation analysis

Estimates of heterosis in F_1 hybrids over the mid-parental value (MP) and standard checks were calculated by methods of Turner (1953) and Hayes et al. (1955) and the significance of heterosis was tested by a 't' test using the formula suggested by Wynne et al. (1970). The differences in the magnitude of heterosis

Table 1 List of CMS lines, restorer lines and hybrids used in the study

S. No	Genotype	Varietal type	Pedigree/parentage	Source
1	IR58025A	A-line	IR48483A × Pusa167-120-3-2	IRRI, Philippines
2	IR62829A	A-line	IR46828A × IR29744-94-3-2-2-3	IRRI, Philippines
3	Pusa 6A	A-line	–	DRR, Hyderabad
4	KMR3R	R-line	–	DRR, Hyderabad
5	BR827-35R	R-line	–	DRR, Hyderabad
6	IR40750R	R-line	IR19660-274-3-3-1-3 × IR22082-41-2	IRRI, Philippines
7	PRR78R	R-line	–	DRR, Hyderabad
8	29R	R-line	IR65515-56-1-3-19R	IRRI, Philippines
9	1A × 29R	Highly heterotic experimental hybrid	IR58025A × IR65515-56-1-3-19R	Barwale Foundation, Hyderabad
10	1A × PRR78	Highly heterotic experimental hybrid	IR58025A × PRR78R	Barwale Foundation, Hyderabad
11	2A × PRR78	Highly heterotic experimental hybrid	IR62829A × PRR78R	Barwale Foundation, Hyderabad
12	KRH2	Public bred commercial hybrid	IR58025A × KMR3R	DRR, Hyderabad
13	Pusa RH10	Public bred commercial hybrid	Pusa 6A × PRR78R	DRR, Hyderabad
14	DRRH1	Public bred commercial hybrid	IR58025A × IR40750R	DRR, Hyderabad
15	Sahyadri	Public bred commercial hybrid	IR58025A × BR827-35R	DRR, Hyderabad

were tested following the procedure given by Panse and Sukhatme (1967). In addition to KRH2 and its parents, the data on cytosine methylation at flag leaf stage, grain yield and heterosis of six heterotic hybrids were used for the analysis. The correlation between methylation levels (x) and performance/heterosis (y) was computed using the formula $r_{xy} = \frac{\mathrm{Cov}(x.y)}{\sqrt{V(x)\cdot V(y)}}$, where, r_{xy} = correlation coefficient between x and y, $V(x)$ = variance of x and $V(y)$ = variance of y.

DNA extraction and MSAP analysis

DNA was extracted from fresh tissue using the method described by Murray and Thompson (1980). Tissues assayed included 15-day old and 35-day old seedling, and fully expanded flag leaves at the day of heading. The MSAP assay procedure described in Xiong et al. (1999) was adopted. All adapters and primers were synthesized by Sigma BioSynth (Bangalore, India) and their sequences are given in Supplementary Table 1. The PCR amplification reactions with 24 primer combinations were performed using the touch-down cycles as described in the original AFLP protocol (Vos et al. 1995). The reactions starting from restriction digestion to selective amplifications were carried out in duplication. The selective amplification products were denatured, resolved on 6% polyacrylamide gel, and visualized by silver staining (Panaud et al. 1996).

Scoring and data analysis

The scoring of differential methylation pattern was based on the presence and absence of bands (Supplementary Table 2), assuming each band represented a restriction site of CCGG. *Hpa*II is sensitive to full methylation (methylation of both strands) of either cytosine residues at the recognition site while *Msp*I is sensitive only to methylation at the external cytosine. Hence, a band detected in the *Eco*RI + *Msp*I digest but not in the *Eco* RI + *Hpa*II indicates the occurrence of internal-full methylation (both strands methylated). Conversely, a band detected in *Eco*RI + *Hpa*II digest but not in the *Eco*RI + *Msp*I digest indicates the occurrence of hemi-methylation (single strand methylated). The number of full and hemi-methylation sites were counted in each genotype, and expressed in percentage against the total number of bands detected (Xiong et al. 1999; Dong et al. 2006; Zhang et al. 2007). The cumulative level of hemi-methylation and internal-full methylation was added together to constitute the total methylation level. The changes in methylation status in hybrids were compared with the parents and were recorded as: demethylation—when amplification occurred in the hybrid but not in both parents for a particular enzyme digestion; hypermethylation—when amplification occurred in both the parents but not in the hybrid for a particular enzyme digestion (Joel and Zhang 2001a). The increased or hypermethylation and decreased or demethylation at a particular site in the hybrid was critically analyzed by comparing the parents and the direction of methylation was deduced.

Cloning, sequencing and in silico mapping analysis of differentially methylated fragments

Differentially amplified fragments between parents and hybrids were identified and ten such fragments were eluted and re-amplified with appropriate selective primer combinations. The purified DNA fragments were cloned using a commercial cloning Kit (Invitrogen) and sequenced at Mahyco Life Science Research Center, Jalna, India. The sequences were used as queries to search against the nucleotide databases for homology using the BlastN tool at the NCBI web site (www.ncbi.nlm.nih.gov/BLAST).

Results

Heterosis in the hybrids tested

The grain yield per plant among the seven hybrids varied from 25.4 (Pusa RH10) to 29.8 g (KRH2). An experimental hybrid 1A x PRR78 showed highest mid-parent heterosis for grain yield per plant. The hybrid KRH2 ranked top in plot yield, per hectare grain yield and heterosis for plot yield (Table 4).

Cytosine methylation status among parental lines and hybrid

Heterosis or hybrid vigor is manifested early in the seedling stage of the hybrid and the flag leaf stage marks the near-completion of most of the growth and developmental activities (Joel and Zhang 2001a). Therefore, we have analyzed the extent of cytosine methylation using the leaf tissues collected at three

stages viz., 15-day-old and 35-day-old seedling stages, and flag leaf stage of KRH2 hybrid and its parents IR58025A and KMR3 (Table 2; Fig. 1a–c). In 15-day-old seedling, the per cent hemi-methylation, internal-full methylation and total methylation were found to be high in the hybrid as compared to both parents. In contrast, the hybrid was found to be less methylated (both hemi and total methylation) than both parents at 35-day-old seedling stage. The percent hemi-methylation and internal-full methylation was high in the male parent KMR3. With respect to flag leaf, KRH2 exhibited less internal methylation as well as less hemi-methylation than both the parents while the percent total methylation was almost equal to both parents. Irrespective of parent or hybrid, the flag leaf exhibited a markedly reduced level of internal-full methylation and hemi-methylation of cytosines at the CCGG sites relative to seedling stages, which caused a variable reduction in the estimated total methylation level by 12.9–16.4% (14.6% on average).

Patterns of cytosine methylation

Different classes of patterns of methylation were observed among parents and hybrid. Class A included patterns that were monomorphic to methylation, i.e. the same sites/bands were detected in both parents and the hybrid. Class B includes differentially methylated sites between two parents. When amplification occurred only in parents and not in hybrid, such sites were grouped under class C which implies the increased level of methylation or hypermethylation in the hybrid compared to parents. The class D patterns consist of the sites whose amplification occurred only in hybrid but not in parents (decreased level of methylation or demethylation in the hybrid compared to the parents). Methylation polymorphism (class B, C and D) was detected at 211, 148 and 62 sites in 15-day-old, 35-day-old seedling and flag leaf stage, respectively (Table 3). High level of methylation polymorphism was observed between the parents and hybrid than between the parents alone. Moreover, the level of methylation polymorphism was high during early stage of plant and reduced as they progressed towards later stage of development. Fragments of class C patterns (hypermethylation) and class D patterns (demethylation) were detected to be more in 15-day-old seedling followed by 35-day-old seedling and flag leaf stage. The class D patterns were

Table 2 Levels of cytosine methylation at CCGG sites in 15-day old (15 DAS) and 35-day old (35 DAS) seedling, and flag leaf stages of KRH2 hybrid and its parents

Genotype	Methylated CCGG sites (number and frequency)								
	Hemi-methylation			Internal full methylation			Total methylation		
	15 DAS	35 DAS	Flag leaf	15 DAS	35 DAS	Flag leaf	15 DAS	35 DAS	Flag leaf
IR58025A	64 (13.8%)	77 (15.7%)	42 (11.2%)	70 (15.1%)	72 (14.6%)	31 (8.2%)	134 (28.8%)	149 (30.3%)	73 (19.4%)
KMR3R	95 (20.4%)	85 (17.3%)	32 (8.5%)	61 (13.1%)	108 (22.0%)	38 (10.1%)	156 (33.5%)	193 (39.2%)	70 (18.6%)
KRH2	100 (21.5%)	50 (10.2%)	31 (8.2%)	82 (17.6%)	90 (18.3%)	39 (10.4%)	182 (39.1%)	140 (28.5%)	70 (18.6%)
Parental mean/mid-parent	79.5 (17.1%)	81 (16.5%)	37 (9.8%)	65.5 (14.1%)	90 (18.3%)	34.5 (9.2%)	145 (31.2%)	171 (34.8%)	71.5 (19.0%)

The total number of fragments amplified is 465, 492 and 376 for 15DAS, 35 DAS and flag leaf stages, respectively

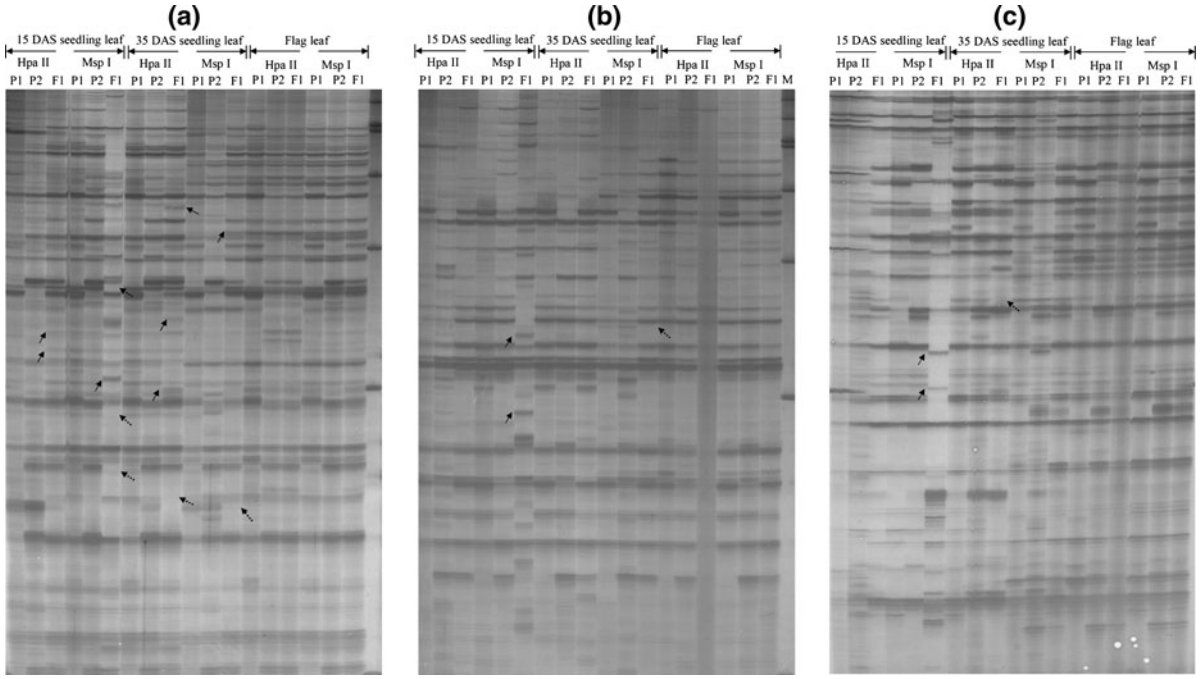

Fig. 1 Differential methylation patterns in leaves collected during three developmental stages of hybrid and its parents. **a** E AGC/HM TCAA, **b** E AAC/HM ACG, **c** E ACG/HM GGC primers. (M-100 bp marker, *black arrows* indicate demethylated site (pattern D) and *dotted arrows* indicate hypermethylated site (pattern C))

Table 3 Patterns and directions of cytosine methylation in rice parental lines and their hybrid detected at 15-day old and 35-day old seedling, and flag leaf stages

Pattern	Number and frequency of sites		
	15-Day old	35-Day old	Flag leaf
Monomorphic (A1 + A2)	254 (54.6%)	344 (69.9%)	314 (83.5%)
Polymorphic (B + C + D)	211 (45.4%)	148 (30.1%)	62 (16.5%)
Differential methylation among parents (B)	20 (4.3%)	20 (4.1%)	13 (3.5%)
Hypermethylation in hybrid (C)	87 (18.7%)	63 (12.8%)	36 (9.6%)
Demethylation in hybrid (D)	104 (22.4%)	65 (13.2%)	13 (3.5%)
Total	465 (100%)	492 (100%)	376 (100%)

found to be more, equal and less, as compared to class C patterns at 15-day-old seedling, 35-day-old seedling and flag leaf stages of hybrid, respectively.

Direction of cytosine methylation and its association with hybrid performance and heterosis

The direction of methylation such as hypermethylation (increased methylation in hybrid compared to parent) and hypomethylation/demethylation (decreased methylation in hybrid compared to parent) were critically analyzed by comparing parents and hybrids in three stages. The results showed that the hypermethylation and demethylation levels were high in 15-day-old seedling stage and got reduced as the hybrid grew (Table 3). In early stages (15-day-old and 35-day-old seedling stage), the occurrence of demethylation event was more frequent than hypermethylation and the direction changes in later stages with an increased hypermethylation levels. In the flag leaf stage, the level of demethylation was greatly reduced (3.5%) in hybrid with a concomitant increase in hypermethylation level (9.6%). To validate this data, a set of six heterotic hybrids were analyzed and found that all the hybrids showed a high level of hypermethylation at

Table 4 Levels of methylation in flag leaf, grain yield and heterosis in rice hybrids

Hybrids	Total methylation (%)	Demethylation (%)	Hyper-methylation (%)	Per plant grain yield (g)	Per plant grain yield heterosis[a] (%)	Plot yield (g)	Plot yield heterosis (%)	Grain yield per hectare (kg)
KRH2	18.6	3.50	9.60	29.8	25.2	1014.2	35.7	6023
DRRH1	20.17	2.90	10.71	26.0	22.6	854.3	34.8	4955
Sahyadri	22.86	4.06	15.10	29.2	30.2	961.5	34.1	5190
PusaRH 10	19.82	1.38	7.47	25.4	22.7	825.2	20.1	4926
1A × 29R	13.21	1.83	2.12	26.9	25.0	861.6	24.2	4825
1A × PRR78	17.97	2.96	3.26	27.9	30.7	826.3	24.3	4627
2A × PRR78	15.03	2.03	2.92	25.8	23.2	850.7	25.4	4764

[a] Mid-parent heterosis calculated based on average grain yield of parental lines of a particular hybrid

Table 5 Correlations of percent total methylation, demethylation and hypermethylation, with performance and heterosis of seven rice hybrids

Levels of methylation (in percentage)	Per plant yield (g)	Per plant yield heterosis (%)	Plot yield (g)	Plot yield heterosis (%)	Grain yieldper hectare (kg)
Total methylation	0.38	0.31	0.49	0.54	0.69*
Demethylation	0.83*	0.72*	0.74*	0.81*	0.47
Hypermethylation	0.41	0.19	0.72*	0.74*	0.91**

* Significance level <0.05; ** Significance level <0.01

this stage (Table 4). A correlation analysis was performed to know if there is any association of the level and changes in methylation in hybrids with heterosis and/or performance of these hybrids. A comparison of data on methylation at flag leaf stage of all the hybrids and their grain yield and heterosis (Table 4) showed that the levels of total methylation, demethylation and hypermethylation were found to be positively correlated with performance and heterosis (Table 5). Significant positive association was observed for percent total methylation and per cent hypermethylation with plot yield, plot yield heterosis and grain yield/ha, and for per cent demethylation with all the characters studied except grain yield/ha.

In silico mapping and homology analysis of differentially methylated fragments between KRH2 hybrid and its parents

In order to identify the genes that are differentially methylated among parents and hybrid, as a preliminary attempt, a total of ten fragments consisting of eight sites that were demethylated in hybrid and two sites that were not methylated in parents (hypermethylated in hybrid) were cloned and sequenced. Homology search localized these sequences to six different chromosomes viz., chromosome 3, 4, 8, 10, 11 and 12 and also to the mitochondrial genome (Supplementary Table 3). The table reveals only the major location of the clones, although most clones detected multiple hits in the japonica rice genomic sequence, presumably because of their repetitive nature. Of the eight demethylated sites, four were identified as protein coding sequences (Table 6). It was not possible to assign any definite function for the rest of the sequences with the current level of genome annotation. The four known sequences were aldolose mRNA, sk20 resistance protein-like, upstream region of NADH dehydrogenase subunit 1 and 5, and a unknown protein. All the above sequences were methylated in parents while they were demethylated in the hybrid. Of the two hypermethylated sites, one was identified as LTR retrotransposon protein, and for the other one it was not

Table 6 Functional classification of clones of differentially methylated fragments between rice parental lines and their hybrid

Category	Number and percentage of altered sites	Gene/sequence	Type of alteration pattern
Known-function gene	3 (30%)	Aldolose mRNA, sk28 resistance protein-like, NADH dehydrogenase sub unit 1and 5	Demethylation in hybrid
Mobile element	1 (10%)	LTR Retrotransposon	Hypermethylation in parents
Unknown protein	1 (10%)	–	Demethylation in hybrid
No similarity	5 (50%)	–	Hyper- or de-methylation in hybrid

possible to assign any function. These sequences were hypermethylated in the hybrid but not in the parents.

Discussion

In the past, nucleotides modified by methylation were not considered to be part of primary nucleotide sequence of an individual. However, since DNA methylation occurs at defined target sequences and not all target sites are methylated, it represents a potentially important form of polymorphism. Epigenetic information systems, like DNA methylation, could generate epigenetic variation that had never been considered as the cause of phenotypic variation (Tsaftaris and Polidoros 2000). The variation in the extent of methylation and characterization of molecular-epigenetic events such as demethylation and hypermethylation in hybrid relative to parents may help explain the phenotypic variation/deviation observed in hybrid compare to parents and thereby may help in understanding the relationship between DNA methylation and heterosis.

In the present investigation, a MSAP analysis was taken up with an elite hybrid, KRH2 and its parents using leaf tissue from three developmental stages *viz*., 15-day old and 35-day-old seedling, and flag leaf. Occurrence of differential levels of DNA methylation not only between parents and hybrid but also among parents (Table 2) clearly indicated the presence of epigenetic variability in the form of DNA methylation. As allelic variation can include sequence differences (alterations in DNA sequence) or regulatory differences (altered expression levels and epigenetic changes) found in different parental genotypes, and as at some level, heterosis is the result of variation between the parental lines, epigenetic variation, like genomic variation, could also combine to produce a heterotic phenotype. In addition, as methylated cytosine can be converted into thymine by deamination to generate a mutation, DNA methylation can be a potent source of new alleles/de novo variation (Rasmusson and Phillips 1997). Tsaftaris and Polidoros (2000) also suggested that the epigenetic information systems, like DNA methylation, could generate epigenetic variation/epialleles that had never been considered earlier as the cause of phenotypic variation and it could be of particular importance in creation of variation in plants, single plant heritability, hybrid vigor, etc.

A comparative analysis of methylation levels among parents and hybrid revealed that the hybrid was more methylated than its parents at 15-day old seedling stage whereas in the later stages the hybrid became less methylated. Mean methylation level in parents increased from 15-day-old to 35-day old seedling and then decreased in the flag leaf stage. In contrast, the level of methylation in the hybrid decreased from early stage onwards (Fig. 2a–c). The observation that the KRH2 hybrid was less methylated than parents at flag leaf stage was found to be well validated in other heterotic hybrids also (data not shown). The varying levels of cytosine methylation during different growth stages clearly indicate that the occurrence of methylation is developmentally regulated. As the role of cytosine DNA methylation in transcriptional inactivation is well established, a decreasing trend leading to a global hypomethylated condition in hybrid might enable derepression and possibly expression of many genes involved in manifestation of heterosis.

The three classes of patterns of cytosine methylation of hybrid identified from the present study could be interpreted as the inherited and altered patterns. Firstly, the methylated sites detected were common in

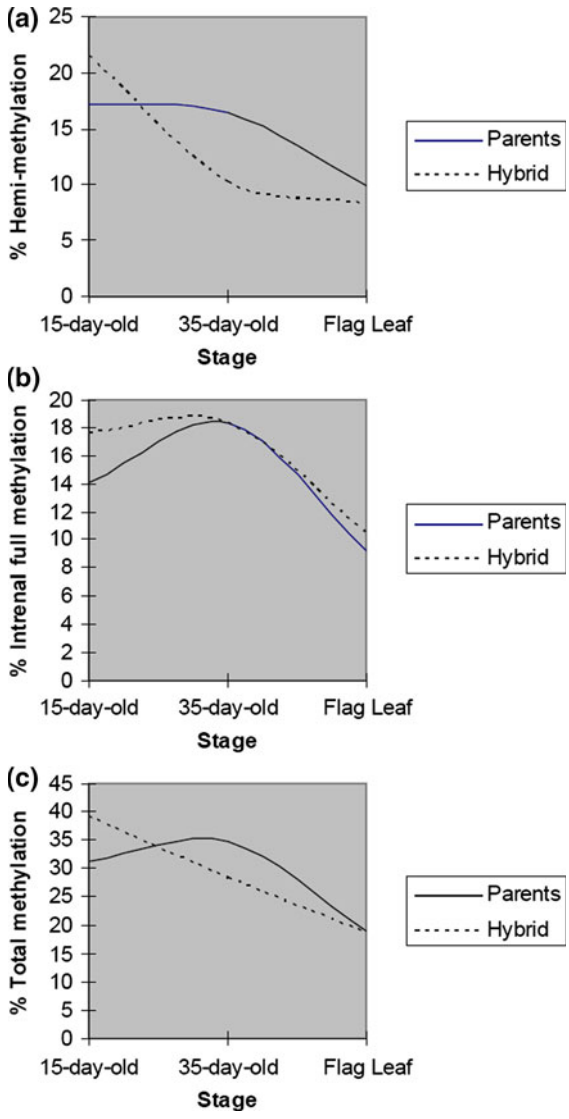

Fig. 2 Differential levels of methylation across three developmental stages of parents (mean values) and its hybrid. **a** percent hemi-methylation, **b** percent internal-methylation, **c** percent total methylation

both parents and hybrid and they are considered monomorphic for methylation. This class of methylation pattern is considered as highly conserved among parents and hybrid and just passed onto the progeny without any change. These conserved genomic regions may be essential for proper plant growth and development and to avoid development-associated irregularities as well as abnormal phenotypes (Joel and Zhang, 2001a). Secondly, the banding patterns were not inherited in a Mendelian fashion and were novel in hybrid. This indicates that certain sites in the genome get reprogrammed for methylation when they are transmitted to the progeny. Bergman and Mostoslavsky (1998) reported that methylation patterns were not inherited from the parental gametes but were established anew in each generation through the series of discrete developmentally regulated steps. It was suggested that the loci which undergo methylation alteration are more likely consequential to hybrid-specific gene expression leading to novel phenotypes (Zhang et al. 2007). Moreover, the transgenerational inheritance and remodeling of parental methylation patterns upon sexual hybridization may have bearings on genome evolution and on the enhanced overall performance of hybrid.

Comparative analysis of various classes of patterns of methylation in parents and hybrid has led to an understanding that the observed methylation changes in hybrid were in both directions such as demethylation of parental-methylated sites; and methylation of parental-non-methylated sites. Of these bi-directional changes, demethylation events were found to be more frequent than hypermethylation during early stages (15-day-old and 35-day-old seedling). This may be due to the fact that higher level of demethylation events might be required for enhanced growth rate during the vegetative stage (cell division and elongation) to realize early seedling vigor in hybrids (Joel and Zhang 2001b). Apart from this, a higher level of initial total methylation observed at seedling stage may also influence the rate of demethylation in hybrid. The predominance of demethylation during early stages of hybrid observed from the present study suggests the importance of these events in the expression of early hybrid vigor. We observed that during later stage the demethylation level decreases significantly with a remarkable increase in hypermethylation (Table 3; Fig. 3). The observation that hypermethylation events were more predominant in later stage was found to be well validated in other heterotic hybrids analyzed at this stage (Table 4). Xiong et al. (1999) reported such decreased methylation in only one Chinese rice hybrid; however this data was neither validated nor associated with heterosis in other hybrids. Hypermethylation is known to cause transcriptional inactivation of CG/CNG sites (Diequez et al. 1998) and has been highly associated with formation of heterochromatin-

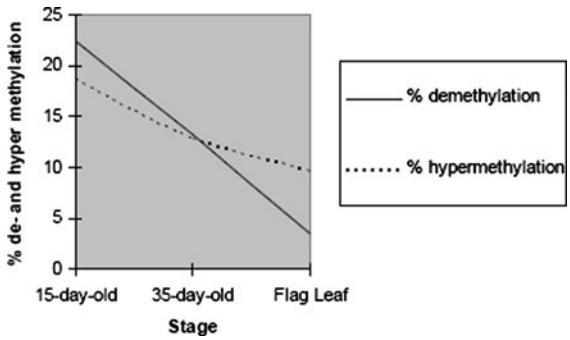

Fig. 3 Trend of demethylation and hypermethylation levels across three stages in the hybrid

mediated gene silencing (Klein and Coasta 1997). The predominance of hypermethylation may be to repress spurious global transcription in order to achieve efficient transcription or complete expression of desirable loci in hybrids. It is also possible that as the flag leaf stage marks the later stage of growth and development, hypermethylation events may be associated with efficient repression of many genes which are not required to be expressed at this stage.

An attempt was made to know whether the change in methylation pattern or the methylation polymorphism observed among parents and hybrid relates to the observed variation in terms of quantitative phenotypes. A strong association of these bi-directional changes in methylation with performance and heterosis was observed in KRH2 and other hybrids (Table 5). Highly significant association of demethylation and hypermethylation with hybrid performance and heterosis suggest that these bi-directional methylation changes may help in maintaining and regulating the gene expression in a hybrid-specific way. In our preliminary study, we observed that four of the eight demethylated sequences were found to code for functional/hypothetical proteins (Table 6; Supplementary Table 3), although their role in heterosis is presently unknown. This not only indicates the functional significance of occurrence of cytosine methylation but also the demethylation of these coding genes may lead to its expression only in the hybrids. Recent results from large scale gene expression experiments suggest that the gene expression in hybrid deviates from mid-parent value for a large number of sequences and novel expression patterns emerge in hybrids (Huang et al. 2006). These novel expression patterns are considered to result from the unique regulatory interactions in hybrid. Based on our observations that demethylation was associated with hybrid performance/heterosis and demethylated sequences in hybrid were corresponding to gene sequences, we suggest that demethylation could be one plausible mechanism to explain novel, hybrid-specific gene expression patterns. To our surprise, a strong association of hypermethylation events with per plot grain yield heterosis was also observed. This implies that apart from demethylation, the hypermethylation of certain genomic regions may also be important for heterosis and thus provide novel insight to the understanding of molecular bases of heterosis in rice. Therefore, we propose that these bi-directional methylation alterations such as demethylation and hypermethylation may provide one plausible mechanism for conditioning hybrid-specific effect that is crucial for heterosis.

In conclusion, the present study characterized demethylation and hypermethylation as the predominant molecular-epigenetic events associated with heterosis. Knowing the regulatory effect of DNA methylation on gene expression, it is plausible that the decreasing levels of cytosine methylation as the hybrid grew coupled with higher rate of demethylation events during early growth stages and a remarkable increase in hypermethylation events at later stages, may indeed contribute to novel hybrid-specific gene expression to achieve heterosis. Further studies focusing on combinations of expression polymorphisms (from global as well specific gene expression data) and their underlying epigenetic regulatory polymorphisms among the parent and hybrid would certainly help to understand the role of DNA methylation in heterosis.

Acknowledgments The fellowship and facilities provided to K.S. by Barwale foundation are gratefully acknowledged. The authors thank Dr Amalraj John Joel for useful discussion and Mahyco Life Science Research Center, Jalna for sequencing facility. Seed material supplied by IRRI, Philippines and DRR, Hyderabad is thankfully acknowledged. We also thank R. M. Sundaram and P. Rajendrakumar for their useful suggestions which helped to improve the manuscript.

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