34. Epigenetic variation in rice as revealed by methylation-sensitive amplicon polymorphism (MSAP) technique

K. SAKTHIVEL[1,2)*], K. GIRISHKUMAR[1,3)], V. V. SHENOY[1,4)], G. RAMKUMAR[1,2)], S. T. KAJJIDONI[5)] and P. M. SALIMATH[5)]

1) Barwale Foundation, A.G. Heights, Road No.12, Banjara Hills, Hyderabad 500034 India
2) Directorate of Rice Research, Rajendranagar, Hyderabad 500030 India
3) Nunhems Seeds Pvt. Ltd., Bangalore India
4) Monsanto India Ltd., Hyderabad India
5) Department of Genetics and Plant Breeding, University of Agricultural Sciences, Dharwad 580005 India

DNA in most of the eukaryotic genomes is modified by methylation of cytosine residues (^{5m}C) within the di-nucleotide C_pG and the tri-nucleotide C_pN_pG sequences. DNA methylation, a major epigenetic phenomenon, has been implicated in many biological functions *viz*., transcriptional inactivation leading to gene silencing, gene regulation and genomic imprinting. In addition to being the cause of epigenetic variation, methylation is also found to be associated with induction of mutation and generation of genetic variation (Rasmusson and Phillips, 1997). Methylated cytosines frequently deaminates to thiamine, thus ^{5m}Cytosines are considered as hot spots for mutations, providing an interconnection between epigenetic and genetic variation. In the past, nucleotide modified by methylation was 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. In this way, epigenetic information systems, like DNA methylation, could generate epigenetic variation/epi-alleles that had never been considered as the cause of phenotypic variation and this could be of particular importance in creation of variation in plants, single plant heritability, hybrid vigor etc. (Tsaftaris and Polidoros, 2000).

Although there has been tremendous progress in genome research, mapping and molecular genetic studies of rice, very little research has been done on methylation status of the rice genome. In the present study, an attempt was made to study the methylation variation/epigenetic variation among rice genotypes and to assess the extent of cytosine methylation in the rice genome.

The plant materials used in the present study include three elite varieties, three male sterile lines and six restorer lines of rice (see Table 1). Methylation status was assessed by means of the methylation-sensitive amplified polymorphism (MSAP) technique using the isoschizomers *HpaII* and *MspI*, which can detect cytosine methylation as internally (C ^{5m}CGG), externally (^{5m}CCGG), hemi-(single strand) or fully methylated (both strands) in the restriction site 5' CCGG. Tissues assayed included 35-day old seedling and fully expanded flag leaves at the day of heading. The MSAP assay procedure described by Xiong et al. (1999) was adopted. The scoring of differential methylation pattern was based on the presence and absence of bands, assuming each band represented a restriction site of CCGG. The number of full and hemi-methylation sites were counted in each genotype, and expressed in percentage against the total number of bands detected. The cumulative level of hemi-methylation and internal-full methylation was added together to derive total methylation.

Amplification in 12 rice genotypes resulted in a total of 1402 and 1514 scorable bands in the seedling and flag leaf tissues, respectively. Significant inter-individual variation for both internal-full methylation and hemi-methylation was found in both stages of all the genotypes studied (Table 1). In 35-day old seedling, the level of hemi-methylation ranged from 7.00% (IR62829A) to 19.02% (BR827) and internal methylation ranged from 11.95% (PRR78) to 31.50% (Pusa 6A) and the level of total methylation ranged from 20.51% (2A) to 49.17 (Pusa 6A). The mean hemi-methylation, internal methylation and total methylation in seedling leaf tissues accounted for 13.23%, 20.16% and 33.39%, respectively. In flag leaf, the hemi-methylation level ranged from 4.69% (2A) to 31.05% (15R), internal methylation level ranged from 3.76% (29R) to 26.92% (Pusa 6A) and total methylation level ranged from 12.63% (29R) to 45.28% (Pusa 6A). The mean hemi-methylation, internal methylation and total methylation in flag leaf tissues accounted for 13.43%, 13.80% and 27.23%, respectively. The overall hemi-methylation level of two stages was 13.33%, whereas the internal full methylation and total methylation was 16.98% and 30.31%, respectively. Consolidating the methylation level in all the tissues analyzed, the overall methylation level (based on 5'-CCGG sites) in rice genome was 30.31%. The proportion of cytosine residues that are methylated varies widely, ranging from about 6% in *Arabidopsis* (Kakutani et al., 1999) to as much as 33% in rye (Thomas and Sherratt, 1956). Gruenbaum et al. (1981) reported that in general 20 to 30% of all cytosines may be methylated in plants. Thus, the finding on extent of methylation in rice genome is in accordance with the earlier reports.

The differential sensitivity of isoschizomers *Hpa*II and *Msp*I revealed the abundance of internal methylated sites in both the stages. The level of internal full-methylation was higher than hemi-methylation irrespective of the two stages. The high level of full methylation than hemi-methylation may be due to the fact that hemi-methylated sites are unstable with respect to methylation status and by the process of maintenance methylation, the hemi-methylated cytosine residues are converted to homo-methylated, i.e full or both strands methylated, as suggested by Otto and Walbot (1990). The total methylation level in two different developmental stages showed the higher level of methylation in the plant's early stage of development and then recedes as it grows towards maturity as evident from the higher level of total methylation in seedling stage than flag leaf stage in all the genotypes studied except for IR65515-56-1-3-19R. This may be to regulate the stage- or developmental-specific gene expression in an efficient way. Another important observation is the existence of inter-individual variation for both internal and hemi-methylation in two tissues of the genotypes which reveals the presence of variability for methylation level in rice. Such a variation beyond DNA sequence variation is referred as 'epigenetic variability' in the form of DNA methylation. Although it remains to be determined whether epigenetic variations generate phenotypic variation, they might be potentially associated with specific phenotype. For example, the first characterized natural mutant of flower symmetry in *Linaria vulgaris* was caused by DNA hyper methylation of the underlying gene *Lcyc* (Cubas et al., 1999).

In conclusion, the present study identified the existence of epigenetic variability in the form of variation in level of DNA methylation across genotypes and estimated the extent of methylated cytosines in rice genome. This study opens up new possibilities of explaining epigenetic perspectives of inter-cultivar phenotypic variation in rice.

Table 1 Extent of cytosine methylation in rice genome of 12 genotypes using two tissues

S. No	Genotype	Hemi-methylation (%)		Internal full-methylation (%)		Total methylation (%)	
		Seedling Leaf Tissue	Flag Leaf Tissue	Seedling Leaf Tissue	Flag Leaf Tissue	Seedling Leaf Tissue	Flag Leaf Tissue
1	Samba Mahsuri	15.02	12.50	20.82	13.02	35.84	25.52
2	Moroberekan	12.09	14.82	19.00	15.00	31.09	29.82
3	Co-39	12.58	12.97	20.66	13.38	33.24	26.35
4	IR58025A	8.55	9.32	17.40	14.41	25.95	23.73
5	IR62829A	7.00	4.69	13.51	13.26	20.51	17.95
6	Pusa 6A	17.67	18.36	31.50	26.92	49.17	45.28
7	IR65515-56-1-3-19R	17.78	8.87	26.92	3.76	44.70	12.63
8	PRR78	14.58	13.17	11.95	8.58	26.53	21.75
9	IR61614-3B-19-3-2R	14.87	31.05	24.00	10.81	38.87	41.86
10	IR40750R	9.32	5.91	14.31	12.11	23.63	18.02
11	KMR3	10.28	11.53	20.46	15.27	30.74	26.80
12	BR827	19.02	17.95	21.42	19.07	40.44	37.01
	Mean	13.23	13.43	20.16	13.80	33.39	27.23
	Mean (Two stages)	13.33		16.98		30.31	

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References

Cubas P., C. Vincent and E. Coen, 1999. An epigenetic mutation responsible for natural variation in floral symmetry, Nature, 401: 157-161.

Gruenbaum T., T. Naveh-Many., H. Cedar and A. Razin, 1981. Sequence specificity of methylation in higher plant DNA. Nature, 292: 860-862.

Kakutani T., M. Kyoko., E. J. Richards and H. Hirochika, 1999. Meiotically and mitotically stable inheritance of DNA hypomethylation induced by *ddm1* mutation of *Arabidopsis thaliana*. Genetics, 151: 831-838.

Otto P. S. and V. Walbot, 1990. DNA methylation in eukaryotes: kinetics of demethylation and *de nova* methylation during the life cycle. Genetics, 124: 429-437.

Rasmusson D. C. and R. L. Phillips, 1997. Plant breeding progress and genetic diversity from *de nova* variation and elevated epistasis. Crop Science, 37: 303-310.

Thomas A. J. and H. S. A. Sherratt, 1956. The isolation of nucleic acid fractions from plant leaves and their purine and pyrimidine compositions. Biochemistry Journal, 62: 1-4.

Tsaftaris A. S and A. Polidoros, 2000. DNA methylation and Plant breeding. Plant Breeding Review, 18: 87-177.

Xiong L. Z., C. G. Xu., M. A. S. Maroof and Q. Zhang, 1999. Patterns of cytosine methylation in an elite rice hybrid and its parental lines detected by methylation sensitive amplification polymorphism technique. Molecular General Genetics, 261: 439-446.