Epigenetic responses to drought stress in rice (*Oryza sativa* L.)

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Abstract Cytosine methylation polymorphism plays a key role in gene regulation, mainly in expression of genes in crop plants. The differential expression of cytosine methylation over drought stress response was analyzed in rice using drought susceptible but agronomically superior lines IR 20 and CO 43, and drought tolerant genotypes PL and PMK 3 and their F_1 hybrids. The parents and hybrids were subjected to two moisture regimes viz., one under drought condition and another under control condition. The cytosine methylation polymorphism in genomic DNA was quantified under both the conditions at the reproductive stage of the plant using the Methylation Sensitive Amplified Polymorphism (MSAP) technique devised by Xiong et al. (261:439–446, 1999). The results depicted that under drought condition, hypermethylation was predominant in the drought susceptible genotypes while drought tolerant genotypes presented hypomethylation behavior. While imposing drought, spikelet sterility per cent was positively correlated to percentage of methylation whereas, panicle length, number of seed per panicle, panicle weight, 100 seed weight, and yield/plant were negatively correlated indicating the role of epigenetic regulation in yield attributing traits in response to drought. Thus, methylation can be considered as an important epigenetic regulatory mechanism in rice plants to adapt drought situation. From this study, we speculate that the hyper-methylation may be an indicator of drought susceptibility and the hypo-methylation

for drought tolerance and this methylation polymorphism can be effectively used in drought screening program.

Keywords MSAP - Methylation Sensitive Amplification Polymorphism · AFLP - Amplified Fragment Length Polymorphism · Cytosine methylation · Epigenetics · Drought tolerance

Introduction

Plants are constantly challenged by biotic and abiotic stresses, from which they save themselves by modulating their physiological and developmental machinery through changes in genome wide gene expression (Wang et al. 2011). Epigenetics plays a very important role in modulating the expression of nuclear genes by *switching on/off* mechanisms in tissue or developmental stage specific fashion or in response to the external or internal environment of the cell (Lin et al. 2005). Though epigenetics is known to be hidden influence upon the genes, it has its molecular and chemical basis of origin and inheritance (Ooi and Bestor 2008; Chinnusamy and Zhu 2009; Wang et al. 2011). Epigenetic alterations are typically reversible heritable chemical modifications in chromatin structure and nucleotide sequences of DNA in the genome. There is increasing evidence (Klose and Bird 2006; Matzke et al. 2009; Boyko et al. 2010; Kile et al. 2010) that the regulation of chromatin structure through histone acetylation and DNA methylation might mediate the long lasting changes in the physical, three-dimensional structure of DNA itself which causes the suppression of gene expression. Symmetric cytosine CpG and CpNpG are main target sites of DNA methyl transferases (DNA MTases) enzymes in response to the environment, resulting in associated changes in gene expression without a change to the DNA sequence (Coolen et al. 2007). Though generally methylation results in suppression of gene activity but in some cases it have been reported that methylated genes can be actively transcribed in several invertebrates,

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including different insects (Mandrioli 2004), the sea urchin *Strongylocentrus purpuratus*, the sea squirt *Ciona intestinalis* and the marine annelid *Chaetopterus variopedatus* (Simmen et al. 1999). Cytosine DNA methylation is a major epigenetic modification affecting gene regulation in response to environment and also has evolutionary importance for adapting different environments over long evolutionary period of time. Recent research findings have revealed that imposing different biotic and abiotic stresses to the plant leads to increased gene methylation and thus leading to degeneration of genome activity. In contrast, favourable growth conditions and absence of stress is associated with lower methylation and optimum expression (Labra et al. 2002; Madlung and Comai 2004).

Methylation level contributes greatly to plant's ability to respond to stress (Boyko and Kovalchuk 2008). The hyper or hypo methylation changes in the hybrids, when compared to parents, are an indicator of the suppression or expression of stress related genes under drought (Chen and Chen 2008; Shen et al. 2012). The availability of different methods to study amount of cytosine methylation and its nucleotide sequence specificity is critical for understanding the role of DNA methylation in a variety of biological systems. Methylation Sensitive Amplification Polymorphism is one of the techniques that can be used for quantification of CpG and CpNpG methylation sites in genomic DNA (Xiong et al. 1999).

In this study, we tried to find out total amount of methylation and differential methylation under drought and controlled conditions using MSAP. Evaluation study for various yield attributing traits also has been undertaken to correlate them with the cytosine methylation level under drought and control conditions.

Material and methods

Plant material

Among the four rice genotypes selected for the study, two female lines viz., IR 20 and CO 43 were drought susceptible locally adapted high yielding rice varieties and two drought tolerant male lines viz., PMK 3, a locally adapted upland variety and local upland land race, Paiyur Local (PL). Four types of crosses, IR 20 × PMK 3, IR 20 × PL, CO 43 × PMK 3 and CO 43 × PL, were made and the hybrids (F₁s) were

confirmed by using parental polymorphic SSR primers (see supplementary Table 8 for list and other details of identified polymorphic primers) (Figs. 1 and 2).

Two sets of F₁s and parents were transplanted in pots kept under rainout shelter at Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore and were exposed to two sets of treatments. First treatment being the drought and the second was control condition with four replications each (Fig. 3). Drought was imposed at the panicle initiation stage of plants for drought treatment by withholding regular watering in pots. Relative water content (RWC) at 60 % level used as the standard for drought treatment. The leaf samples for DNA isolation were taken from drought treated plants when RWC reached to 60 % and then plants were revived by normal watering. RWC measuring protocol was followed by Smart and Bingham (1974), measuring RWC in a day interval for each individual plant under drought treatment.

Methylation-sensitive amplification polymorphism (MSAP) analysis

Total genomic DNA was extracted from penultimate leaf of drought imposed and control parent and hybrid rice plants according to the protocol developed by Gawel and Jarret (1991). The MSAP protocol was adopted from Xiong et al. (1999) with minor modifications. Two restriction digestions were carried out for both the drought stressed and control genomic DNA samples of each hybrid and parent. In the first restriction digestion, 1 µg of high quality genomic DNA was digested with two restriction enzymes *EcoRI* (15U) and *HpaII* (15U) simultaneously in a single tube and was made up to 50 µl with 1X restriction-ligation buffer and then incubated for 6 h at 37 °C. The second digestion reaction was performed in exactly the same way, where only *HpaII* was replaced with *MspI*.

The forward and reverse strands of the *EcoRI* and *HpaII* - *MspI* adapters were diluted separately in the sterile double distilled water and then were mixed and heated to 95 °C for 10 min followed by slow cooling at room temperature to synthesize double stranded adapter, and these adapters were used in ligation reactions.

The above said restriction digestion mixtures of genomic DNA were used for ligation with the adapters by adding 10 µl

Fig. 1 Parental polymorphism: Primer RM131 showing polymorphism between parental lines

Fig. 2 Hybrid fixing using RM131 primer: individuals 12, 13 and 15 are hybrids representing alleles from both the parents IR20 and PMK3

ligation mixture containing buffer, 5 mM DTT, 0.5 pmol *EcoRI* adapter, 5.0 pmol *HpaII-MspI* adapter (Table 1), 1 mM ATP, 10U T4 DNA ligase and incubated at 37 °C for 4 h. The ligation reaction was stopped by incubating at 65 °C for 8 min. This ligated mixture was then diluted to 120 µl and used in the pre-amplification PCR.

Pre-amplification PCR

Pre-amplification PCR was done using 3 µl of the above said diluted ligation mixture with *EcoRI* (E+0) and *HpaII-MspI* (HM+0) primers (50 ng each) having no selective nucleotides at 3' end (Table 1). The PCR master mix for a single reaction of 25 µl contained 1X PCR buffer, 0.1 mM of each dNTP and 1U *Taq* polymerase, and final volume was made up with distilled sterile water. The PCR conditions involved were initial denaturation at 95 °C for 5 min and 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, with a final extension at 72 °C for 5 min. The pre-amplified products were then diluted to 100 µl and stored at –20 °C for the selective amplification PCR. The 10 µl of pre-amplified PCR product out of 25 µl obtained in this step was loaded in agarose gel electrophoresis to ensure the amplification of DNA fragments before performing selective amplification.

Selective amplification PCR

Selective amplification PCR was done using 5 µl of above said pre-amplification product with 30 ng of *EcoRI* (E+3) and 40 ng of *HpaII-MspI* (HM + TCAA) primers each having selective nucleotides (Table 1). The PCR master mix for each reaction contained 1X PCR buffer, 0.1 mM of each

dNTP and 1U *Taq* polymerase. Total volume was made up to 20 µl with distilled sterile water. The touchdown cycles PCR conditions were involved in this step as described in the original AFLP protocol (Vos et al. 1995). The denatured PCR products were separated on a 6 % denaturing polyacrylamide gel electrophoresis (PAGE) at 65 V for 3 h (Maughan et al. 1996) (Fig. 4).

Relative quantification of cytosine methylation

Quantification method of cytosine methylation in genomic DNA of rice plants under drought stress and control was adopted from Xiong et al. (1999). The Isoschizomer restriction enzymes *HpaII* and *MspI* are sensitive to cytosine methylation state on their recognition site i.e. 5'C/CGG3'. The position(s) of methylated cytosine on the recognition site in one strand or both the strands of DNA give rise the way for differential recognition of the recognition site for the isoschizomers. The recognition site(s) having distinctions due to cytosine methylation are differentially recognized by isoschizomer restriction enzymes and produces DNA fragment polymorphism which is amplified in PCR and visualized in the PAGE after staining (presence/absence of fragments) (Table 2 and Fig. 4). The polymorphism arose this way in the PAGE used to quantify the relative amount of the cytosine methylation of the genomic DNA.

Relative quantification of methylation is represented as methylation percent which is calculated by counting total number of methyl groups present in all the recognition sites of *HpaII* and *MspI* and dividing them by maximum number of methylation sites on cytosine residues (C-5 position on pyrimidine ring of cytosine nucleotide) of the recognition sites of

Fig. 3 Earthen pots with transplanted rice plants which are at the panicle initiation stage (3a) and the drought treated hybrid IR 20 × PL flanked by parents IR 20 and PL which are in control condition (3b)

HpaII and *MspI* in all the loci (the Tables 2 and 3). Since the 5' CCGG3'/3'GGCC5' recognition site sequence has four cytosine residues, maximum of four methyl groups can be added

to it, hence total number of the recognition sites are multiplied by four. The formula devised is given below:

$$\text{Methylation \%} = \frac{\text{No. of Methylation groups present in 5'CCGG3' recognition sites in all the loci of HpaII and MspI}}{4(\text{Total number of CCGG recognition sites in all the loci of HpaII and MspI})} \times 100$$

Relationship between cytosine methylation and agronomic yield attributing traits

A number of directly and indirectly yield attributing traits like days to 50 % flowering, plant height, number of tillers per plant, panicle length, number of seeds per panicle, spikelet sterility percent, panicle weight, hundred seed weight and yield per plant were recorded (see supplementary Table 7). The mean values of four plants of each genotype under each parameter were used to make correlation between methylation percentage and yield attributing traits under drought as well in control condition using SPSS software v.15.

Results

Relative cytosine methylation level in rice hybrids and their parents

Table 1 Details of primers and adapters used in the study

S.No.	Primers ^a /adapters	Sequence 5' to 3'
1.	<i>HpaII</i> - <i>MspI</i> primer (HM+0)	CATGAGTCCTGCTCGG
2.	<i>HpaII</i> - <i>MspI</i> primer (HM+TCAA)	CATGAGTCCTGCTCGGTCAA
3.	<i>EcoRI</i> primer (E+0)	GACTGCGTACCAATTC
4.	<i>EcoRI</i> primer (E+3)	GACTGCGTACCAATTC AAG GACTGCGTACCAATTCACA GACTGCGTACCAATTCACC GACTGCGTACCAATTCAAA GACTGCGTACCAATTC AAC
5.	<i>EcoRI</i> forward adapter	CTCGTAGACTGCGTACC
6.	<i>EcoRI</i> reverse adapter	AATTGGTACGCAGTC
7.	<i>HpaII</i> - <i>MspI</i> forward adapter	GATCATGAGTCCTGCT
8.	<i>HpaII</i> - <i>MspI</i> reverse adapter	CGAGCAGGACTCATGA

^a The selective primer 5'-CATGAGTCCTGCTCGGTCAA (HM+TCAA) was used in combination with each of the 5 E+3 primers listed for selective amplification. The core sequence of the *EcoRI* primer is exactly the same as that used in the AFLP protocol by Vos et al. (1995)

The rice genome of drought stressed and control plants were relatively quantified for cytosine methylation using MSAP. A number of studies in plants have shown that epigenetic mechanisms like DNA methylation, histone modifications and RNA interference assist plants to cope up with different kind of biotic and abiotic stresses. These epigenetic mechanisms modulate the gene expression in response to the change in environment *via* activating specific defense mechanisms and various adaptation processes (Kovalchuk et al. 2004; Raja

Fig. 4 PAGE of MSAP profile of IR 20, PL and their hybrid under drought stress (S) and control condition (C). 'H' and 'M' pre-amplified products further amplified using selective *HpaII*-*MspI* primer (HM+TCAA) with *EcoRI* primer (E+AAG). Arrow marks indicate the polymorphism arises due to methylation at 5'CCGG3' recognition site of the Isoschizomers enzymes (see Table 2 for more details)

Table 2 *HpaII* and *MspI* isoschizomers differential restriction due to methylation

Recognition site C/CGG GGC/C	Methylation state		Presence of fragment in the gel	
			<i>HpaII</i>	<i>MspI</i>
CCGG GGCC	Nil Methylation	NM	Yes	Yes
C ^m CGG GG ^m CC	Full Internal Methylation	FIM	No	Yes
^m CCGG GGCC	Hemi External Methylation	HEM	Yes	No
^m C ^m CGG GG ^m C ^m C	Full Methylation	FM	No	No

‘m’ indicates the CH₃ group on 5th C of cytosine

et al. 2008; Chinnusamy and Zhu 2009; Boyko et al. 2010; Uthup et al. 2011; Mirouze and Paszkowski 2011; Downen et al. 2012; McCue et al. 2012; Tricker et al. 2012; Saze et al. 2012). It suppresses activity of specific genes *via* methylating them and thereby conserves energy for survival of the plants in adverse conditions like drought (Huizinga et al. 2008; Wang et al. 2011; Boyko et al. 2010; Cao et al. 2011; Grativol et al. 2012). Agreeing with the above fact, we found that drought stress causes locus specific methylation and de-methylation of cytosine residues at the recognition sites of *HpaII* and *MspI* (5'CCGG3'). These methylation alterations were evaluated to find out a sensible

relationship between methylation changes and drought tolerance and susceptibility in rice. The quantified cytosine methylation percent and differential cytosine methylation percent for all the four parents and their hybrids strongly revealed that there exists cytosine methylation polymorphism between the drought stressed and control plant genomic DNA (Table 4).

The drought susceptible genotypes viz., IR 20 and CO 43, both exhibited hyper-methylation; 72.22 % under drought stress condition, while under controlled conditions recorded 52.78 and 36.11 % respectively (Table 4). The drought tolerant genotypes PL and PMK 3 displayed hypomethylation in response to drought stress. The PL recorded cytosine methylation percent of 80.56 and 69.44 under control and drought condition respectively. Similarly, the moderately drought tolerant variety PMK 3 depicted a reduction of cytosine methylation percent from 66.67 in drought condition to 60.35 under control. It has also been observed that both methylation and de-methylation events are taking place independently in the genome in response to drought stress. The maximum deviation in cytosine methylation level between the two environments was found in CO 43 followed by IR 20, PMK3 and PL with the values of 36.11, 19.44, –6.32 and –11.12 respectively. Hybrids also showed differential methylation pattern for drought stressed and unstressed samples. The cytosine methylation percent for hybrids in drought stressed genomic DNA samples ranges from 61.11 % in CO 43 × PL to 83.33 % in IR 20 × PL, and under control condition it was 54.44 % in CO 43 × PMK 3 to 88.89 % in CO 43 × PL. Maximum influence of drought stress

Table 3 Method of scoring cytosine methylation and calculation of methylation percent

Locus	Genotype 1						Genotype 2					
	Drought stressed			Control			Drought stressed			Control		
	<i>EcoRI</i> + <i>HpaII</i>	<i>EcoRI</i> + <i>MspI</i>	No. of CH ₃	<i>EcoRI</i> + <i>HpaII</i>	<i>EcoRI</i> + <i>MspI</i>	No. of CH ₃	<i>EcoRI</i> + <i>HpaII</i>	<i>EcoRI</i> + <i>MspI</i>	No. of CH ₃	<i>EcoRI</i> + <i>MspI</i>	<i>EcoRI</i> + <i>MspI</i>	No. of CH ₃
1	–	+	2	+	+	0	+	+	0	+	+	0
2	–	+	2	–	+	2	–	–	4	+	+	0
3	–	+	2	–	+	2	–	–	4	+	+	0
4	+	+	0	+	+	0	–	–	4	+	–	1
5	+	–	1	+	–	1	–	–	4	+	+	0
6	–	–	4	–	–	4	+	–	1	+	+	0
7	–	+	2	–	+	2	+	–	1	+	–	1
8	+	–	1	+	+	0	–	–	4	–	–	4
9	+	+	0	+	+	0	+	–	1	+	–	1
10	–	–	4	+	–	1	+	+	0	+	+	0
Methylation %	45			30			57.5			17.5		
Difference in methylation %	15.00						45.00					

+ ...presence of DNA band in PAGE, –...absence of DNA band in PAGE, CH₃ ... methyl group

Table 4 Cytosine methylation percent and differential methylation percent in parents and their hybrids

Parents and hybrids	Stress/control	Methylation %	Difference in methylation level between stressed and control (%)
IR 20	S	72.22	19.44
	C	52.78	
CO43	S	72.22	36.11
	C	36.11	
PMK3	S	60.35	-6.32
	C	66.67	
PL	S	69.44	-11.12
	C	80.56	
IR 20 × PMK 3	S	68.64	4.78
	C	63.86	
IR 20 × PL	S	83.33	4.74
	C	78.59	
CO 43 × PMK3	S	77.78	23.34
	C	54.44	
CO 43 × PL	S	61.11	-27.78
	C	88.89	

on the cytosine methylation level was observed in the hybrid CO 43 × PL followed by CO 43 × PMK3 with a difference of -27.78 and 23.34 % respectively (Table 4).

Correlation between different agronomic traits and relative methylation level

Bivariate correlation between the different quantitative agronomic yield traits and methylation percentages were worked out using their mean values of parents and their hybrids in drought stress and control environment. In controlled condition, methylation percent is negatively correlated with yield per plant (-0.678) and panicle weight (-0.525) and positively correlated with the spikelet sterility percent (0.694). Moreover, under the drought condition, methylation percent is negatively correlated with yield per plant (-0.743) and panicle weight (-0.590) and positively correlated with the spikelet sterility percent (0.626) (Tables 5 and 6).

Discussion

The results of our study supported the fact that the drought stress in plant causes hyper-methylation in susceptible genotypes and hypo-methylation in tolerant genotypes. It is also found that all the recognition sites of the isoschizomer enzymes (*HpaII* and *MspI*) have not given cytosine methylation polymorphism, some are consistently monomorphic in drought as well in control conditions, and hence it is proved that cytosine methylation machinery targets specific site of

the genome. The technique MSAP clearly differentiated known drought susceptible genotypes (IR 20 and CO 43) from drought tolerant genotypes (PMK3 and PL) based on methylation level.

Differential sensitivity of the isoschizomer restriction enzymes, due to positional variations of methylated cytosines on the recognition site, was well utilized in quantification of cytosine methylation at the recognition sites of the whole rice genome. Drought susceptible IR20 and CO43 exhibited hyper-methylation (19.44 % and 36.11 % respectively) under drought treatment, which generally indicates suppression of drought susceptible genes' activity via methylation. PL, a highly drought tolerant locally established land race and PMK3, a drought tolerant variety showed hypo-methylation (-11.12 % and -6.32 % respectively), elucidating the molecular basis of drought adaptive nature of crops (Table 4). Site specific differential methylation pattern has also been observed in response to drought stress i.e. methylation and de-methylation events taking place simultaneously, which indicates possibility of involvement of drought stress responsive epigenetic machinery targeting specific genes or regions of the genome (Bird 2002; Steward et al. 2002; Kile et al. 2010 and Deneberg et al. 2010).

It is well established fact that during drought, the whole plant genome undergoes a state of stress and plant tries to survive by expressing genes related to drought tolerance (Madlung and Comai 2004). During this process of survival and adaptation the genomic cytosine methylation level and site specific differential methylation changes due to different methylating and de-methylating enzymes' activity in response to drought leading to the activation and inactivation of the transcriptional process for specific genes related to drought tolerance (Madlung and Comai 2004; Zhang et al. 2011; Msogoya and Grout 2012; Vining et al. 2012).

The hybrid CO 43 × PL recorded highest hypo-methylation (-27.78 %) values in drought stressed genomic DNA in comparison to the control (Table 4) which indicates possibility of activation of the genes responsible for drought tolerance. The yield per plant, days to 50 % flowering (76 days) also recorded best among all parental lines and hybrids in drought condition, therefore, the hybrid performance of CO 43 × PL is found to be very suitable for drought condition. Hyper-methylation (23.34 %) and reduced agronomic performance in the hybrid CO43 × PMK3 due to drought stress treatment illustrate the vulnerability for drought situation. In the hybrids IR 20 × PL and IR 20 × PMK3, least methylation percent difference has been observed between the two treatments, but agronomic performance has significantly reduced in drought condition than the control, which can be explained by site specific positional methylation variations observed in the hybrid (Fig. 5).

Hybrids have shown hypo-methylation under drought condition than in control, which we articulate as epigenetic

Table 5 Correlation matrix for mean values of different agronomic traits and methylation percent of rice plants under control condition

	DFF	PH	TN	PL	NSP	SP	PW	SW	Y	MP
DF	1									
PH	.289	1								
TN	.191	-.274	1							
PL	-.754*	.212	-.149	1						
NSP	-.423	.187	.181	.840**	1					
SP	-.849**	.104	-.511	.806*	.423	1				
PW	.342	.224	.245	.164	.553	-.241	1			
SW	.128	.599	-.722*	.052	-.064	.231	.235	1		
Y	.336	.191	.803*	-.060	.275	-.477	.565	-.189	1	
MP	-.339	.272	-.695	.222	-.204	.694	-.525	.357	-.678	1

DFF days to 50 % flowering, *PH* plant height (cm), *TN* tiller number/plant, *PL* panicle length (cm), *NSP* number of seeds/panicle, *SP* spikelet sterility %, *PW* panicle weight (g), *SW* 100 seed weight (g), *Y* yield/plant (g), *MP* methylation %

* Correlation is significant at the 0.05 level of significance

** Correlation is significant at the 0.01 level of significance

heterosis, indicating the ability of hybrids to unleash the activity of drought specific genes. This suggest that hybrids of drought tolerant and drought susceptible genotypes with desirable background can be successfully utilized for breeding program to utilize epigenetic heterosis with increased drought tolerance in drought affected areas.

Correlation matrix for both drought and control plants revealed that there is existence of a significant negative relationship with methylation percentage to number of seed per panicle, panicle weight and yield per plant, which indicates that the hyper-methylation leads to suppression of genes responsible for different traits contributing to grain yield. A significant positive correlation exhibited between spikelet sterility and methylation which further confirm the fact that hyper-methylation leads to suppression of beneficial genes like grain filling. Radchuk et al. (2005) also reported similar finding in barley that expression of genes responsible for storage protein (prolamine) is repressed due to presence of

higher level CpG methylation. They found that different cytosine methylating enzymes' expression increased during starch accumulation phase in endosperm, an indication of transcriptional regulation via DNA methylation.

Many years of the study on cytosine DNA methylation in different types of experimental material indicate that bothsuppression and expressionof genes takes place due to methylation, but suppression due to stress environment is more common among genes which are expressed only in ambient environment and luxurious in nature (Steward et al. 2002; Madlung and Comai 2004 and Suzuki et al. 2006). In the drought condition there may be expression and suppression of some genes, which are responsive to drought (Steward et al. 2002).

Here we conclude that cytosine methylation polymorphism studies in genomic DNA can be well used in breeding programs to find best parents and their hybrid to utilize epigenetic heterosis effect for tolerance to drought conditions and also for drought responsive novel gene isolation.

Table 6 Correlation matrix for mean values of different agronomic traits and methylation percent of rice plants under drought stress condition

	DFF	PH	TN	PL	NSP	SP	PW	SW	Y	MP
DF	1									
PH	.154	1								
TN	.266	-.323	1							
PL	-.436	.568	.052	1						
NSP	-.227	.307	.382	.661	1					
SP	-.157	-.664	-.069	-.471	-.327	1				
PW	-.124	.694	.031	.847**	.707	-.778*	1			
SW	-.197	.559	-.704	.278	-.370	-.211	.175	1		
Y	-.229	.630	.176	.766*	.500	-.758*	.765*	.367	1	
MP	.029	-.421	.020	-.444	-.176	.626	-.590	-.502	-.743*	1

DFF days to 50 % flowering, *PH* plant height (cm), *TN* tiller number/plant, *PL* panicle length (cm), *NSP* number of seeds/panicle, *SP* spikelet sterility %, *PW* panicle weight (g), *SW* 100 seed weight (g), *Y* yield/plant (g), *MP* methylation %

Fig. 5 Comparative bar chart representations of some of the agronomic traits recorded and methylation percent for the parents and hybrids in drought and control conditions. X-axis is common for all the charts shows the genotypes and Y-axis shows scale values for different parameters

Optimization of this method for detection of methylation in wider array of rice germplasm and their hybrids will help in developing superior parental and hybrid combination over variegated environment. This MSAP approach is also well applicable to any other crop for studying drought response, as it does not need any prior genome sequence information and the cytosine DNA methylation is common in all the crop plants.

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