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Assessment of DNA methylation pattern under drought stress using methylation-sensitive randomly amplified polymorphism analysis in rice

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Abstract

DNA methylation is known to regulate gene expression when plants are exposed to abiotic stress such as drought. Therefore, insight into DNA methylation pattern would be useful for a better understanding of the expression profile of genes associated with drought adaptation. In the present study, we attempted to analyse the DNA methylation pattern at the whole-genome level and the expression of a few drought-responsive genes in rice under different regimes of soil water status, i.e. puddled, 100 and 60% field capacities (FC). The methylation-sensitive randomly amplified polymorphic DNA analysis was employed to identify DNA methylation pattern. We observed an increase in DNA methylation at 60% FC, and reduced methylation under 100% FC compared to puddled condition. The genes such as protein phosphatases (PP2C) and phenylalanine ammonia-lyase (PAL) having CpG islands in their promoter region had lower expression level under 100 and 60% FC compared to puddled conditions. Heat shock protein 70 (HSP70) and RNA helicase 25 (RH25), with no CpG islands in their promoter region, exhibited enhanced expression compared to puddled plants. In rice, increased DNA methylation seems to be an important mechanism associated with drought responses, which probably regulates the methylation-sensitive gene expression. The drought-induced changes in DNA methylation would contribute for epigenetic mechanism. The study provided evidence to argue that drought-induced increased methylation might be one of the major mechanisms associated with acclimation responses in field crops like rice.

Keywords: CpG island, DNA methylation, drought stress, gene expression, MS-RAPD, rice

Introduction

Rice, a staple food crop, is conventionally cultivated under flooded anaerobic conditions (Castaneda *et al.*, 2002; Chan *et al.*, 2012). Efforts are being made to grow rice under semi-irrigated (aerobic) conditions (Dharmappa *et al.*, 2019). Irrigation practices like furrow and multiple inlet irrigation, alternate wetting and drying and overhead sprinklers have gained interest to optimize water use (Bouman *et al.*, 2007; Girsang *et al.*, 2019; Graham-Acquaah *et al.*, 2019).

Semi-irrigated rice experiences desiccation stress between irrigations which affects its growth and productivity (Borrell *et al.*, 1997; Dharmappa *et al.*, 2019). Thorough knowledge of stress tolerance mechanism can help in crop improvement and management. Divergent genes and pathways associated with desiccation stress are reported (Li *et al.*, 2016; Parvathi and Nataraja, 2017; Priya *et al.*, 2019; Sharma *et al.*, 2019; Azzouz-Olden *et al.*, 2020). In transgenic plants, stress-inducible genes such as LEA, HSPs, AREB/ABFs, bZIPs contributed to enhanced

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stress tolerance (Ali *et al.*, 2017). In model plants, epigenetic mechanisms such as DNA methylation, and histone modifications, involved in stress responses are reported (Munshi *et al.*, 2015; Mozgova *et al.*, 2019; Godwin and Farrona, 2020); however, studies in field crops are limited (Wang *et al.*, 2016).

DNA methylation is one of the important epigenetic mechanisms in mammals and plants (Meyer, 2015; Schübeler, 2015). In higher eukaryotes, DNA methylation determines chromatin structure, regulates replication and gene expression (Tirado-Magallanes *et al.*, 2017; Varotto *et al.*, 2020). DNA methylation is generally associated with suppression of gene expression while demethylation results in gene activation (Zemach *et al.*, 2010). In plants, DNA methylation regulates gene expression under stress (Garg *et al.*, 2015). The CpG dinucleotide is the predominant site for DNA methylation in vertebrates (Bird, 1987), and CpG clusters are referred to as CpG islands (CGI) (Deaton and Bird, 2011). In plants, promoter methylation suppresses gene expression in a tissue-specific manner (Finnegan *et al.*, 1998; Zilberman *et al.*, 2007). A correlation between promoter CGI and transcription initiation in vertebrates exists (Deaton and Bird, 2011). Our study, using 5′-Azacitidine (methylation inhibitor) and rice genotypes (AC39020 and BPT5204), contrasting for drought tolerance indicated that CGI could be associated with the methylation pattern in the promoter region, and methylation might modulate gene expression under salinity stress (Sapna and Nataraja, 2016).

In rice under drought, several genes corresponding to differentially methylated regions are altered (Wang *et al.*, 2016). Drought induces alterations in DNA methylation locus and differential patterns in plants with variety, tissue and stress specificity (Wang *et al.*, 2011; Kinoshita and Seki, 2014). In *Arabidopsis*, drought during vegetative stage induces phenotypic and DNA methylation plasticity (Van-Dooren *et al.*, 2020). Stress-responsive transcription factors of Zinc-finger family showed a correlation between methylation and gene expression pattern under stress (Ahmad *et al.*, 2019).

In this study, we examined DNA methylation pattern using methylation-sensitive randomly amplified polymorphic DNA (MS-RAPD) approach, and expression of four genes in rice genotype, IR64 (Lenka *et al.*, 2011) grown under different soil water status. We observed drought-induced methylation, which might be one of the major mechanisms associated with drought responses in rice.

Materials and methods

Plant material and stress treatment

Two days old rice seedlings (*Oryza sativa* subsp. *indica*), variety, IR64, a lowland cultivar developed by the International Rice Research Institute in the Philippines, were raised in polybags in a containment facility under controlled conditions for 15 d. Healthy seedlings were then transplanted to pots (approximately 30 kg capacity) containing garden soil and maintained under greenhouse conditions. One set of plants were grown under puddled condition while other sets were maintained at 100% soil field capacity (FC) by gravimetric approaches (Karaba *et al.*, 2007). A subset of plants maintained at 100% FC was exposed to drought stress (60% FC), 30 d post transplanting (at vegetative stage) using the gravimetric approach. The three sets of plants (puddled, 100 and 60% FC) were grown under greenhouse conditions. Physiological and biochemical parameters were recorded to assess the stress effect, and the fourth leaf of every tiller was collected 14 d post stress imposition for further studies.

Estimation of plant water status

The degree of stress was assessed using relative water content (RWC) which was measured in 50 d old plants using a standard protocol (Barrs and Weatherley, 1962). Leaf discs collected were floated in water for 5 h at 28°C after recording the fresh weight and then the turgid weight was determined. The dry weight was recorded after drying the samples for 72 h at 80°C in a hot air oven.

Estimation of total chlorophyll

About 100 mg leaf tissue was soaked in 10 ml mixture of 80% (v/v) acetone and dimethyl sulfoxide (1:1) to extract chlorophyll. Total chlorophyll was estimated according to Hiscox and Israelstam, (1979). The absorbance of the extract was measured at 652 nm using UV visible spectrophotometer (UV-VIS 2450, Shimadzu Corporation, Kyoto, Japan). Percentage reduction in chlorophyll content over puddled conditions was calculated.

Measurement of gas exchange parameters

Gas exchange parameters were recorded by a portable photosynthesis system, infrared gas analyser-IRGA (LICOR Inc., Lincoln, Nebraska, USA) (Nataraja and Jacob, 1999). Three readings were taken in the same leaf (4th leaf). All the gas exchange assessment was made at a light intensity of 1200 µmol/m^2/s, ambient CO_2 concentration of 420 µmol/mol and 32°C temperature. The relative air humidity in the leaf chamber was maintained at 70–80%.

Genomic DNA extraction and MS-RAPD

DNA was extracted by the cetyltrimethylammonium bromide method as described by Doyle and Doyle (1987).

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MS-RAPD analysis was performed by overnight digestion of 2 µg genomic DNA with methylation-insensitive restriction enzyme *MspI* (SibEnzyme) and methylation-sensitive restriction enzyme *HpaII* (SibEnzyme). The digested DNA was further subjected to PCR using random primers. Out of 40 primers, 10 primers that gave reproducible results for two replications were selected for further analysis. The sequences (5′→3′) of the primers utilized were GGAAGCCAAC (P15), CACAGCTGCC (R2), GTCTACGGCA (R6), GGACAACCAG (R15), ACGGCAAGGA (R20), GGCAGGCTGT (T7), GG AGCCTCAG (X11), GGGCCAATGT (Y16), GACGTGGTGA (Y17), and GTGGAGTCAG (Y18).

The RAPD-PCR was performed in a 10 µl reaction mixture containing 100 ng of DNA template, 1X buffer, 0.2 mM dNTP each, 1.5 mM $MgCl_2$ and 1U *Taq* DNA Polymerase (Invitrogen by Life Technologies, California, USA). The amplifications were performed in a thermal cycler (ProFlex PCR systems by Applied Biosystems, California, USA) programmed as follows: 3 min at 95°C, followed by 45 cycles of 95°C (1 min), 35°C (1 min) and 72°C (90 s), and a final extension of 10 min at 72°C. The PCR products were resolved on 1.5% (w/v) agarose gel and the amplified product was detected by ethidium bromide staining. Based on the procedure described by Erturk *et al*. (2014), polymorphism in the MS-RAPD profile was identified as the disappearance of a normal band and appearance of a new band in the treated sample compared to control.

CpG island detection

The gene IDs were obtained from the Rice Annotation Project database (RAP-DB). The promoter sequences (2 kb) were obtained from the plant promoter database (http://ppdb.agr.gifu-u.ac.jp/ppdb/cgi-bin/index.cgi). These sequences were submitted to the tool EMBOSS (https://www.ebi.ac.uk/Tools/seqstats/emboss_newcpgreport) and CpG islands from 2 kb upstream region of the gene were extracted.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from leaf tissues by a modified phenol chloroform protocol (Sajeevan *et al*., 2014). About 2 µg RNA was converted to cDNA (Invitrogen Super Script™ First-Strand Synthesis, California, USA). The qRT-PCR analysis was performed using a few drought-responsive genes (DRGs) with or without CpG islands, identified using *in silico* analysis. qRT-PCR was carried out using a SYBR green supermix (iTaq™ Universal SYBR® Green Supermix, BIO-RAD, California, USA) in a CFX 96 real-time touch system (BIO-RAD). The reaction was performed in a 20 µl volume and the conditions were as follows: 95°C for 3 min, followed by 35 cycles (95°C for 10 s, 60°C for 15 s, 72°C for 30 s). A no template control was included in each reaction. Primers used in the expression studies are presented in Table 2. The qRT-PCR results were normalized to *Ubiquitin* from the respective sample and fold change calculated by using $2^{(-\Delta\Delta C(T))}$ method (Livak and Schmittgen, 2001). The transcript levels in 100 and 60% FC plants were expressed in relation to puddled plants.

Statistical analysis

The results obtained from the physiological experiments and qRT-PCR were represented as mean ± standard error (SEM). The effects of drought on the physiological parameters were determined using one-factor ANOVA at $P < 0.05$. The significant difference for the expression data was tested using Student's *t*-test with minimal significance at 0.05 level.

Results

Assessing the effect of drought stress

The rice plants exposed to drought stress showed leaf rolling and wilting phenotype 14 d post stress (Fig. 1). The stress effect on the plants subjected to different regimes of moisture stress was evaluated by analysing RWC, photosynthetic rate, stomatal conductance and chlorophyll content. There was a significant reduction ($P < 0.05$) in RWC in plants maintained at 100% (77.2%) and 60% (42.4%) FC, compared to puddled (89%) plants (Fig. 2(a)). The photosynthetic rates were significantly reduced ($P < 0.05$) from 14 µmol/m^2/s in puddled to 12 and 10 µmol/m^2/s under 100 and 60% FC, respectively (Fig. 2(b)). The stomatal conductance also showed significant reductions in 100% FC (0.35 mol/m^2/s) and 60% FC (0.31 mol/m^2/s) compared to puddled (0.43 mol/m^2/s) (Fig. 2(c)) plants. As expected, there was a reduction in chlorophyll content under stress. At 100 and 60% FC, a significant reduction ($P < 0.05$) of 20 and 51%, respectively, was noticed in the chlorophyll content (Fig. 2(d)). These physiological parameters indicated that the rice plants grown under 100 and 60% FC were experiencing desiccation stress.

DNA methylation patterns

MS-RAPD PCR with primer Y18 revealed a similar number of fragments in DNA digested with *HpaII* and *MspI* under puddled and 60% soil FC, with an increase in the number of fragments in 60% FC compared to puddled plants (Fig. 3). However, DNA samples from plants subjected to 100% FC, digested with *HpaII* showed a reduced number of fragments compared to *MspI* digested DNA. This

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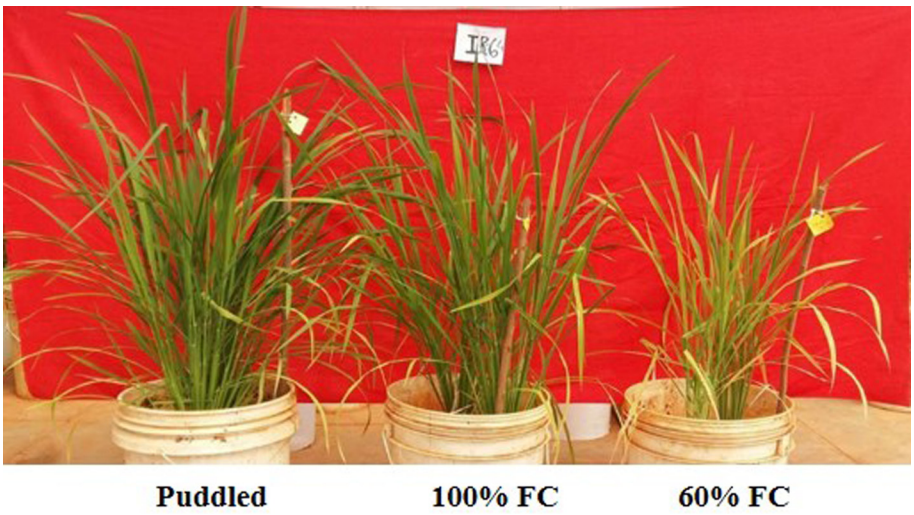

Fig. 1. Phenotype of rice genotype IR64 under different soil water status. Photographs were taken 14 d after drought stress imposition.

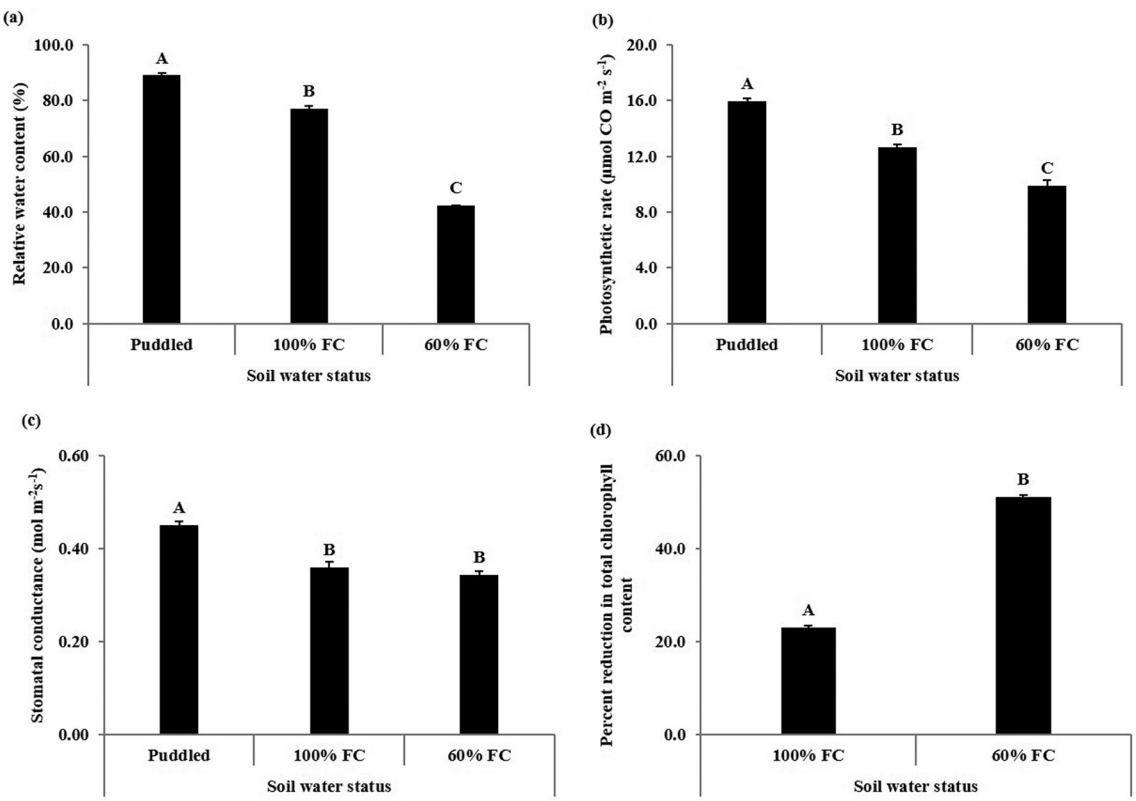

Fig. 2. Evaluation of drought stress by assessment of (a) relative water content, (b) photosynthetic rate, and (c) stomatal conductance, (d) per cent reduction in total chlorophyll content of rice plants grown under different levels of soil water status. The significance at levels $P < 0.05$ is indicated as alphabets.

suggests that the plants subjected to 60% FC have increased methylation. Most of the primers used for this analysis showed a similar banding pattern under severe stress. The polymorphic bands observed under moisture stress with each primer are represented in Table 1. The increase in the number of bands in *HpaII* digested DNA suggests

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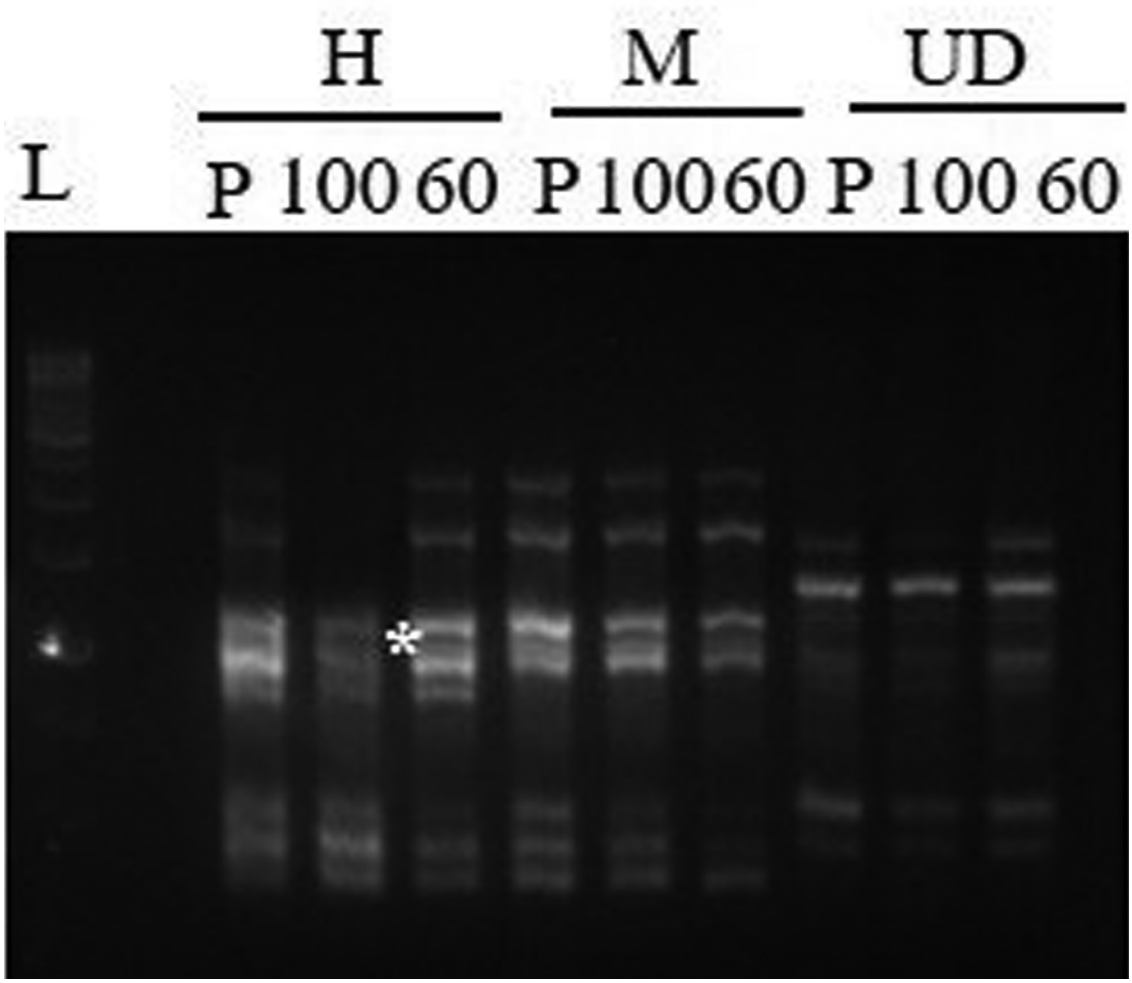

Fig. 3. MS-RAPD using RAPD primer, Y18 in the leaf tissue of rice plants grown under different water status. The samples were digested with *HpaII* and *MspI* and subjected to RAPD-PCR. 'L' is for ladder. The variation in methylation pattern as described in the text is marked with by asterisks.

Table 1. MS-RAPD profile showing the total number of polymorphic bands in rice plants grown under different levels of soil water status

Primer	Total number of bands						Total polymorphic bands			
	Puddled		*100*		*60*		*100*		*60*	
IR64	H	M	H	M	H	M	H	M	H	M
P 15	4	7	6	4	6	6	2	3	2	1
R 2	3	3	1	3	6	4	2	0	3	1
R 6	1	1	1	1	2	1	0	0	1	0
R 15	2	2	2	2	4	4	0	0	2	2
R 20	1	4	1	1	3	1	0	3	2	3
T 7	7	5	3	5	7	5	4	0	0	0
X 11	1	5	5	6	1	6	4	1	0	1
Y 16	5	6	5	4	7	3	0	2	2	3
Y 17	4	6	5	6	4	5	1	0	0	1
Y 18	7	7	6	8	8	8	1	1	1	1

100 and 60 refer to 100 and 60% FC, respectively; H and M refer to digestion with *HpaII* and *MspI*, respectively.

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Table 2. Primers used for gene expression studies in rice plants

RAP Os-IDs	Gene name	Primer sequences (5′-3′)		No. of CpG islands
		Forward	Reverse	
OS01G0618400	DEAD-box ATP-dependent RNA helicase 25 (RH25)	GGCTACGGTACCTTCTGTTGATTCAAGC	GAATTTGCTTGGGTATAGCTGGCGG	0
Os02g0626100	Phenylalanine ammonia-lyase (PAL)	GAGGAGGAGCTCCGCTCTGCG	GCCGAGGAACACCTTGTTGCACTCC	3
Os05g0457200	Protein Phosphatase 2C (PP2C)	GAGCTGGAGCGCATCAAGGC	GCCATCTCGTTGGTCACCACGTC	1
Os03g0277300	Heat Shock protein 70 (HSP70)	CGTGACGTTCGACATCGACGCG	GTTGTACGCGTAGTTCTCCAGCG	0

that there was an increase in methylation at 60% FC, whereas the reduced number of bands in 100% FC and puddled indicated less methylation.

Gene expression

Based on the *in-silico* analysis using the existing microarray datasets on rice genotypes subjected to drought stress, a few differentially regulated genes having a cut-off value of 1.5 were identified. The expression levels of four different genes with or without CpG islands (Table 2) were analysed using qRT-PCR (Fig. 4). Analysis of the promoter region of protein phosphatase 2C (PP2C) gene indicated the presence of one CpG island. The expression of PP2C was low (<1.5-fold) under 100 and 60% FC. The phenylalanine ammonia-lyase (PAL) with three CpG islands showed lower expression in 60% FC (<1-fold) and 100% FC (<1-fold). Heat Shock protein 70 (HSP70) and DEAD-box ATP-dependent RNA helicase 25 (RH25) do not possess any CpG island in their promoter region. The expression of HSP70 under 60% FC (>2-fold) was higher than plants maintained at 100% FC (<1-fold). The RH25 gene showed a significant increase in the expression under 100% (0.45-fold) and 60% (6.91-fold) FC. The study provided a lead but not conclusive evidence, on the relevance of CpG islands and its association with gene expression in rice.

Discussion

DNA methylation is one of the epigenetic modifications that take place in the nucleosome at different levels through reversible biochemical reactions (Mozgova *et al.*, 2019). It plays a key role in regulating gene expression required for modulating plant growth and development, and response to different environmental factors. We used rice, one of the most drought-sensitive and an important food crop traditionally grown under well-irrigated conditions (Wang *et al.*, 2016), for DNA methylation analysis. There are reports on DNA methylation and epigenetic mechanism in *indica* rice under drought stress to understand the complex regulatory mechanisms involved in drought responses (Garg *et al.*, 2015; Wang *et al.*, 2016). We used MS-RAPD approach to examine DNA methylation pattern in the leaf tissue of rice genotype, IR64 grown under different soil moisture status. As reported by Erturk *et al.* (2014), we used methylation-sensitive (*HpaII*) and insensitive (*MspI*) restriction enzymes for the methylation analysis. This technique has been successfully used in crop plants such as cotton and maize to study DNA methylation (Erturk *et al.*, 2014; Karaca *et al.*, 2019). A similar approach was also used to understand the epigenetic effects of salinity on barley (Demirkiran *et al.*, 2013). Our analysis indicated

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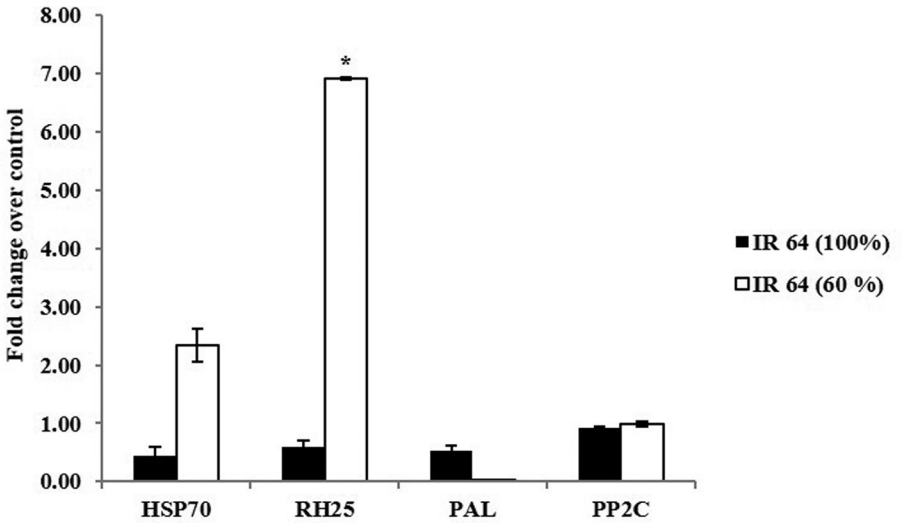

Fig. 4. qRT-PCR expression analysis of four different genes in the leaf tissue of rice plants grown under different soil water status. *Ubiquitin* was used as the internal control and the expression in plants subjected to 100 and 60% FC was indicated as fold change over control (puddled). '*' indicates significance at $P < 0.05$.

increased methylation under severe drought stress (60% FC) compared to the plants grown under puddled conditions, and the data presented here are similar to the previous reports which state that abiotic stresses tend to modify the methylation status of genomic DNA (Correia *et al.*, 2016; Wang *et al.*, 2016).

Global methylation or demethylation may be correlated to gene expression under abiotic stresses (Wang *et al.*, 2016). Zemach *et al.* (2010) suggested that the genes which are least expressed are most likely to be methylated and highly expressed genes are less methylated. Whole-genome methylome analysis revealed that the methylation, especially at the CpG islands in the promoter region, appeared to be related to tissue-specific expression in plants (Surdonja *et al.*, 2017). Surdonja *et al.* (2017) showed increased DNA methylation in the promoter region of *HvCKX2.1* under severe drought stress. Drought induces cultivar-specific transgenerational and site-specific variations in DNA methylation in two contrasting rice genotypes, and the differential methylation was higher in the promoter regions of the genes responsive to different abiotic stress (Wang *et al.*, 2011; Zheng *et al.*, 2013). Our previous study indicated that CpG islands might be associated with methylation under drought stress (Sapna and Nataraja, 2016). Promoter CpG methylation of genes may negatively regulate the binding of transcriptional factors or may bind methyl-CpG-binding domain proteins to regulate the gene expression (Su *et al.*, 2011). It is likely that methylation events affect the expression pattern of such genes associated with drought response.

We analysed the expression of four genes in leaf tissues collected from the plant grown under puddled, 100 and 60% FC. Since CpG island is the predicted target for methylation, we selected the genes with or without CpG islands in the promoter region. These genes included the enzymes involved in ABA signalling, protein phosphatase 2C(PP2C), PAL involved in the biosynthesis of phenolic compounds (Boudet, 2007; Yang *et al.*, 2018), a chaperone (HSP70) and a helicase involved in RNA metabolism (RH25). At 60% FC, PP2C and PAL which have CpG islands in their promoter region showed lower levels of expression than HSP70 and RH25 which do not contain CpG island. A recent study on pineapple suggested that, DNA methylation at CpG islands in the promoter regions of a gene promoting somatic embryogenesis, *SERK1* represses its expression (Luan *et al.*, 2020). Another study on transgenic flax crop showed that the gene chalcone synthase was more accessible to DNA methylation when located within CpG islands and the expression of the gene depended on the methylation status in these regions (Dzialo *et al.*, 2017). The promoter of succinate dehydrogenase gene (*sdh2-1*) showed increased methylation but highly reduced gene expression on the eighth day of germination in the scutellum of maize seeds (Eprintsev *et al.*, 2016). These reports as well as the present study provided a lead to argue that CpG islands may play a role in modulating the gene expression in plants. Since CpG islands are predicted targets for methylation, it would be good to examine their relevance using global analysis approach. There is a positive correlation between DNA methylation and gene expression under stress in rice (Rajkumar *et al.*, 2020), which suggests the relevance of DNA methylation in abiotic stress responses. This study provided only a lead and indicated the need for exploring the importance of CpG islands in the

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promoter region in regulating DRGs especially under severe stress. The correlation between the methylation status of the CpG islands and gene expression is to be established using contrasting rice genotypes under varied soil moisture regimes. The technique used in this study provided general information on genome-wide methylation, but site-specific methylation using whole-genome bisulfite sequencing and a global transcriptome analysis is needed to establish a strong relationship between DNA methylation and gene expression under stressful conditions.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S1479262120000234.

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