The promoters of *OsGLP* genes exhibited differentially methylated sites under drought and salt stress in rice cultivars

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Abstract DNA methylation at cytosine residues governs the regulation of stress responsive genes in plant for self-protection against various environmental abiotic stresses. Here, we analyzed the epigenetic consequences of drought and salinity on cytosine methylation dynamics of promoter regions of stress responsive Germin-like protein genes in rice (*Oryza sativa*). The bisulfite sequencing technique was employed to identify differential methylation status at cytosine residues in selected promoter regions of three *OsGLP* genes (*OsGLP4-1*, *OsGLP8-10* and *OsGLP8-12*) in leaves and roots of two elite *Indica* rice cultivars (tolerant KS282 and sensitive Super Basmati) under drought and salt stress. Our results identified cultivar, tissue and stress-dependent differentially methylated cytosine residues, however, the extent of methylation was found to be different depending upon CGN, CHG and CHH sequence contexts. Among all three *OsGLP* genes, the promoter region of *OsGLP8-12* was detected with the most heavily methylated and significantly differential methylated sites depending upon types of variety, tissue or stress conditions. However, no methylated site was detected in the promoter region of *OsGLP4-1*. Moreover, in the promoter regions of *OsGLP8-10* and *OsGLP8-12,* several differentially methylated sites in response to stress treatments were identified either near or within cis-regulatory elements (CREs) related to abiotic stress. This indicated the association between methylation in the promoter regions and regulation of *OsGLP* genes which might be a key mechanism associated with their regulation under abiotic stresses in contrasting rice cultivars.

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Introduction

Rice (*Oryza sativa*) is reported to be severely affected by drought and salt stresses (Reddy et al. 2017; Panda et al. 2021). However, the variation in the tolerance for these abiotic stresses was detected in different rice cultivars (Kumar and Dash 2019). Despite their genome similarities, the differential responses

to abiotic stresses might be attributed to the genetic and epigenetic mechanisms involved in regulation of stress responsive genes (Garg et al. 2015; Singroha 2019). Moreover, accumulated evidences indicate that rice plant is more sensitive towards drought and salinity at early seedling growth stage as compared to vegetative stage (Sokoto and Muhammad 2014; Hussain et al. 2018; Islam et al. 2018). Therefore, the analysis of epigenetic marks in stress responsive genes of rice at this early seedling stage can certainly provide the insightful information with the potential benefits for enhancement of stress tolerance in rice crop.

DNA methylation in cytosine nucleotides to 5-methylcytosine, is one of the most important epigenetic marks in genomes of eukaryotes that plays significant role in governing chromatin structure, replication and gene regulation (Sapna et al. 2020). In plants, cytosine methylation occurs at three different types including CGN, CHG and CHH (where N = A, C, T or G and H = A, C, T). The variation in methylation status of cytosine residues has been documented to be associated with suppression or activation of gene expression, however, this regulation is dependent upon tissue types, sequence context of cytosine residues and the genomic regions (Yaish et al. 2018; Skorupa et al. 2021). In this aspect, changes in DNA methylation status within or near CREs in the promoter region have also been suggested to regulate gene expression by directly interfering the interaction with transcription factors (Fei et al. 2017). Additionally, the role of DNA methylation in regulation of gene expression in plants under various abiotic stress conditions has also been reported (Garg et al. 2015; Probst and Mittelsten 2015; Rajkumar et al. 2020).

Earlier, studies have indicated the effect of drought and salinity in modulating cytosine methylation in various plant species. For example, in rice, 12.1% variation in methylation status was observed in different rice varieties, tissues and growth stages in response to drought stress (Singroha 2019). Another study highlighted the reduced level of methylated cytosine residues in salt treated roots of soybean plant as compared to control samples (Chen et al. 2019). In terms of gene expression, salt-tolerant rice variety Pokkali showed elevated expression of DNA demethylases whereas salt-sensitive IR29 exhibited activation of methyltransferases and demethylases in response to salt stress (Ferreira et al. 2015). Salt stress has been reported to decrease the level of methylated cytosine

residues in the promoter region of *OsMYB91* in rice, which may explain its elevated expression under salt stress leading to stress tolerance (Zhu et al. 2015).

Among various stress responsive genes in plants, germin-like protein (GLP) genes play a positive role in providing protection against abiotic stresses including drought and salt stresses. GLPs are plant glycoproteins which belong to cupin superfamily and play vital roles in plant germination, development and stress related mechanisms. GLPs can express in different tissues (like leaves and roots) and are reported to possess single or combination of enzyme activities such as oxalate oxidase (OXO) activity, super oxide dismutase (SOD) activity, nucleotide-sugar pyrophosphatase/phosphodiesterase activity and polyphenol oxidase activity (Ilyas et al. 2019; Zaynab et al. 2022). However, a majority of GLPs have been demonstrated to be superoxide dismutases, which facilitate the conversion of superoxide anion to H_2O_2 hence playing key role in plant stress response mechanism via ROS scavenging and cell wall reinforcement (Yasmin et al. 2015). In recent years, many reports have revealed the stress and tissue specific varied expression of rice GLP genes in response to drought and salt stresses (Banerjee et al. 2017; Anum et al. 2022).

The rice genome consists of 43 *OsGLP* genes distributed among different chromosomes (1, 2, 3, 4, 5, 8, 9, 11 and 12) with highest number on chromosome number 3, 8 and 12 in the form of clusters (Das et al. 2019). Several *OsGLP* genes particularly on chromosome 3, 4 and 8, for example *OsGLP4-1*, have been indicated to show varied expression in response to drought and salt stresses in different rice cultivars (Li et al. 2016; Anum et al. 2022). Currently no report is available regarding the DNA methylation analysis in promoter region of *OsGLP* genes in contrasting rice cultivars under drought and salt stress.

In this study, we explored the cytosine methylation pattern in the promoter region of 3 *OsGLP* genes (*OsGLP4-1*, *OsGLP8-10* and *OsGLP8-12*) from chromosome number 4 and 8. Methylation analysis was conducted in samples derived from leaves and roots of two important rice cultivars, tolerant KS282 and sensitive Super Basmati, under treatments of drought and salinity. To achieve this, we first analyzed the impact of drought and salt stresses on DNA methylation in promoter regions of selected *OsGLP* genes as well as compared the differential methylation status of these promoter regions in two

contrasting rice cultivars. Second, we investigated the tissue and stress specific potential role of differentially methylated sites in regulation of *OsGLP* genes by identifying CREs involved in gene regulation and abiotic stress response in selected promoter regions. We found several differentially methylated sites within and near identified CREs suggesting the association between promoter methylation and regulation of *OsGLP* genes under both stresses. This is the first study reporting the insightful information about the DNA methylation status of GLPs. The findings can pave the way for understanding the regulatory mechanisms of epigenetic marks in contrasting rice cultivars and can be utilized for enhancing the associated stress resistant traits in rice.

Materials and methods

Plant material

Two rice cultivars, tolerant KS282 and sensitive Super Basmati, (Ijaz et al. 2019) were utilized as plant material.

Growth conditions and stress treatments

Seeds from rice varieties were de husked and surface sterilized (Anum et al. 2022). They were grown on Murashige and Skoog (MS) media in test tubes under 16 h photoperiod in a growth room (temperature 25 °C ± 1) for two weeks. Two weeks old seedlings were randomly grouped for control, salinity and drought conditions (3 replicates/group with 15 plant/replicate). Drought stress was given by placing seedlings on dried filter paper for 24 h and seedling were treated with salinity stress by keeping them in 200 mM NaCl solution for 24 h (Li et al. 2016). The leaves and roots for stress treated seedlings of both

Fig. 1 Analysis of methylation pattern in selected promoter regions of 3 *OsGLP* under control condition **a** Methylation dynamics in selected promoter regions of 3 *OsGLP* genes (*OsGLP4-1*, *OsGLP8-10* and *OsGLP8-12*) at CGN, CHG and CHH sequence contexts in KS282 and Super Basmati under control condition. The data indicates the average values for leaves and roots tissue samples. **b** Tissue specific comparison of methylation pattern in promoter region of *OsGLP8-12* at CGN, CHG and CHH sequence contexts in KS282 and Super Basmati under control condition
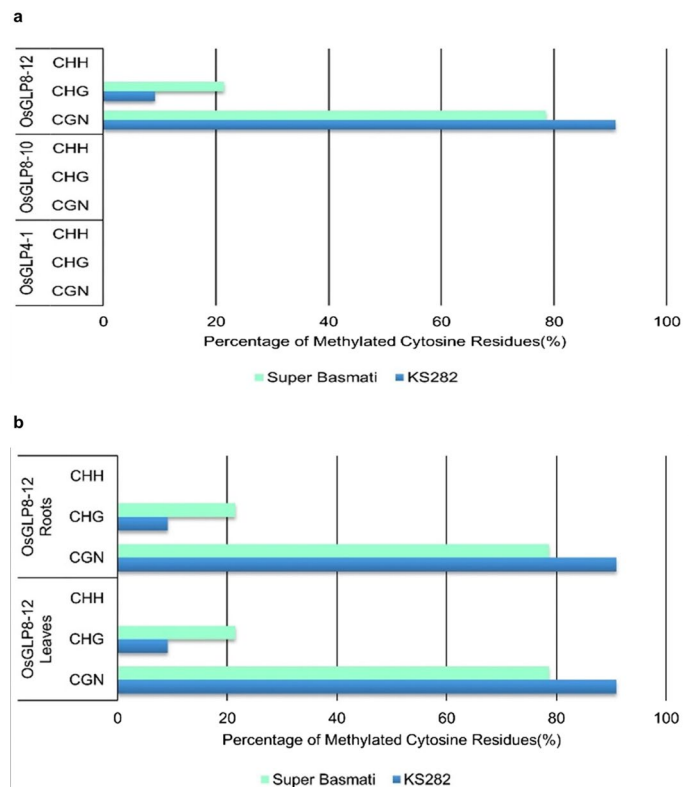
rice varieties were harvested, snap frozen and kept at -80 °C for extraction of genomic DNA.

Isolation and bisulfite conversion of genomic DNA

Genomic DNA was isolated from control and treated leaves and roots of KS282 and Super Basmati using DNeasy Plant Mini Kit (Qiagen, Germany) as per manufacturer's instructions. DNA concentration and quality was determined by using NanoDrop™ 1000 Spectrophotometer (Thermo Scientifc) and agrose gel electrophoresis. The extracted DNA was stored at -20 °C for downstream processes. Un-methylated cytosine bases in isolated genomic DNA from control and treated samples were modified to uracil by sodium bisulfite conversion using EpiTect Bisulfite Kit (Qiagen, Germany) following manufacturer's instructions.

Primer designing and optimization

To perform methylation analysis in the promoter region of three *OsGLP* genes (*OsGLP4-1*, *OsGLP8-10* and *OsGLP 8-12*), bisulfite sequencing was performed with bisulfite converted DNA from control and treated samples of leaves and roots of both varieties. For this purpose, bisulfite sequencing primers were designed for the selected promoter region for each *OsGLP* gene using MethPrimer software (Li and Dahiya 2002; Manning et al. 2006) (Online Resource 1). To obtain the best results for bisulfite sequencing, primers were selected with a size of 24 to 26 nucleotides and product length of less than 500 bp (303 bp for *OsGLP4-1*, 358 bp for *OsGLP8-10* and 259 bp for *OsGLP 8-12*). Designed primers were optimized with melting temperatures ranging between 52 to 58 °C.
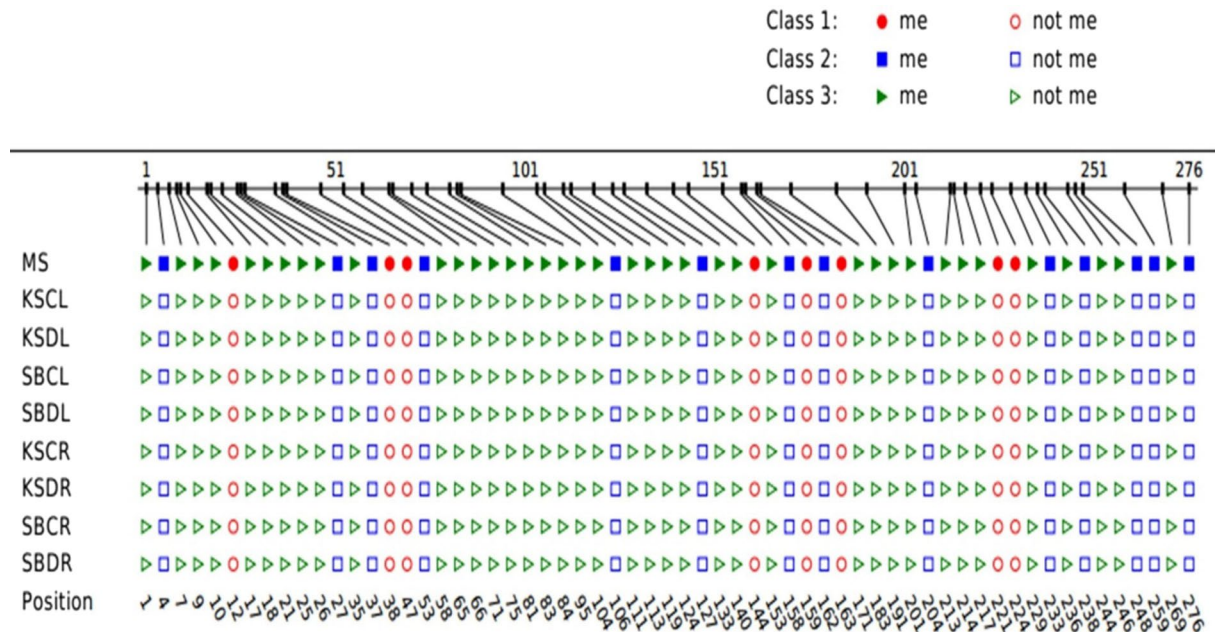

Fig. 2 Graphical representation of cytosine methylation map for comparative analysis of methylation pattern in promoter region of *OsGLP4-1* in leaves and roots of KS282 and Super Basmati based on the drought stress treatment and rice varieties using CyMATE. All three sequence contexts of cytosine residues CGN (class 1: red circles), CHG (class 2: blue squares) and CHH (class 3: green triangles) are represented in the map. Filled shapes are denoting cytosine (C) residues whereas empty shapes are representing thiamine (T) residues. The order of the individual sequences (from the multiple sequence alignment created by ClustalX) is listed on the left. Numbers at the top indicate the length of the alignment and numbers at the bottom represent potential methylation sites. Master Sequence, un-modified sequence derived from NCBI (MS); KS282 Control Leaves (KSCL); KS282 Drought Leaves (KSDL); Super Basmati Control Leaves (SBCL); Super Basmati Drought Leaves (SBDL); KS282 Control Roots (KSCR); KS282 Drought Roots (KSDR); Super Basmati Control Roots (SBCR); Super Basmati Drought Roots (SBDR)
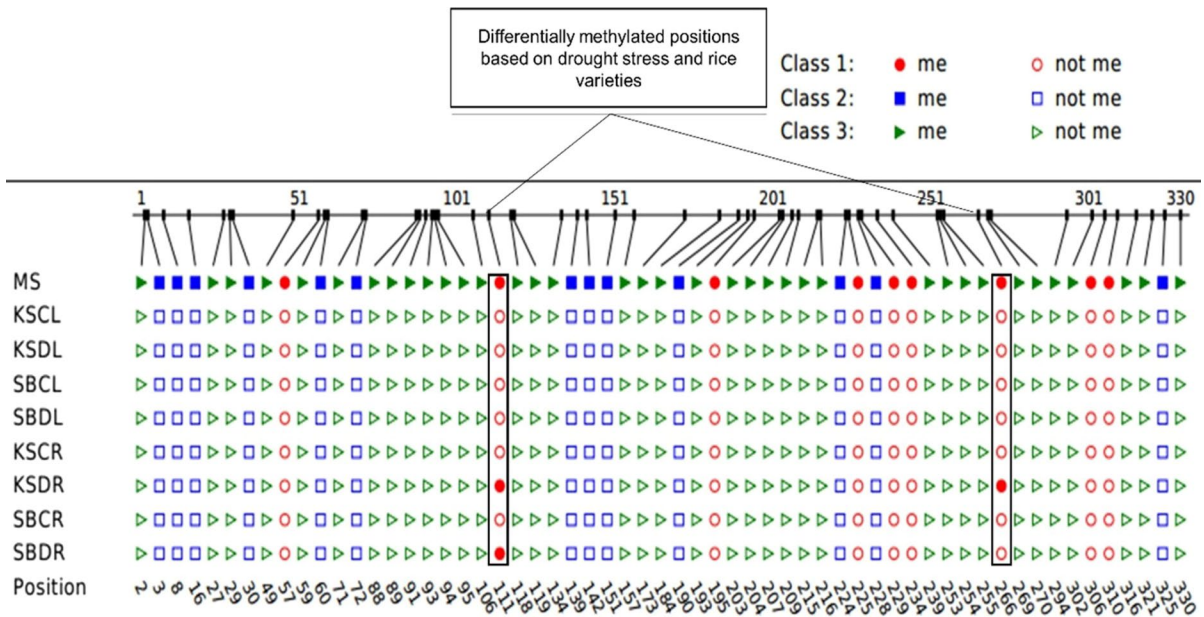
Fig. 3 Graphical representation of cytosine methylation map for comparative analysis of methylation pattern in promoter region of *OsGLP8-10* in leaves and roots of KS282 and Super Basmati based on the drought stress treatment and rice varieties using CyMATE. All three sequence contexts of cytosine residues CGN (class 1: red circles), CHG (class 2: blue squares) and CHH (class 3: green triangles) are represented in the map. Filled shapes are denoting cytosine (C) residues whereas empty shapes are representing thiamine (T) residues. The order of the individual sequences (from the multiple sequence alignment created by ClustalX) is listed on the left. Numbers at the top indicate the length of the alignment and numbers at the bottom represent potential methylation sites. Master Sequence, un-modified sequence derived from NCBI (MS); KS282 Control Leaves (KSCL); KS282 Drought Leaves (KSDL); Super Basmati Control Leaves (SBCL); Super Basmati Drought Leaves (SBDL); KS282 Control Roots (KSCR); KS282 Drought Roots (KSDR); Super Basmati Control Roots (SBCR); Super Basmati Drought Roots (SBDR)

PCR amplification and purification for bisulfite sequencing

Bisulfite converted DNA was utilized as template for PCR amplification of targeted regions with bisulfite sequencing primers using HotStar Taq Polymerase (Qiagen, Germany) on a GeneAmp PCR System 9700 (Applied Biosystems). Un-converted genomic DNA was used as negative control during PCR amplification. The PCR reaction mixture (for one reaction) was prepared as follows: 2.5 µl 10×PCR buffer, 0.5 µl dNTP mix, 1 µl of each specific bisulfite primer, 0.125 µl Hotstar Taq polymerase, 2 µl bisulfite converted DNA and H_2O to make final volume of 25 µl. Following temperature profile was used for amplification: initial activation step (15 min 95 °C), 35 cycles (1 min 94 °C; 1 min at optimal annealing temperature for each specific primer; 1 min 72 °C) and final extension step (10 min 72 °C). Purification of PCR products was performed by GeneJET PCR Purification Kit (Thermo Scientific) according to manufacturer's instructions and well-purified PCR products from all control and treated samples with each specific primer were sequenced commercially.

Data analysis

For investigation of methylation, analysis of bisulfite sequencing data was performed by determining cytosine methylation status for all three sequence contexts: symmetrical CGN and CHG as well as non-symmetrical CHH by using CyMATE software (Hetzl et al. 2007; Karan et al. 2012). Further, comparative analysis of all bisulfite sequences was accomplished by aligning the sequences for converted samples with reference sequence (for
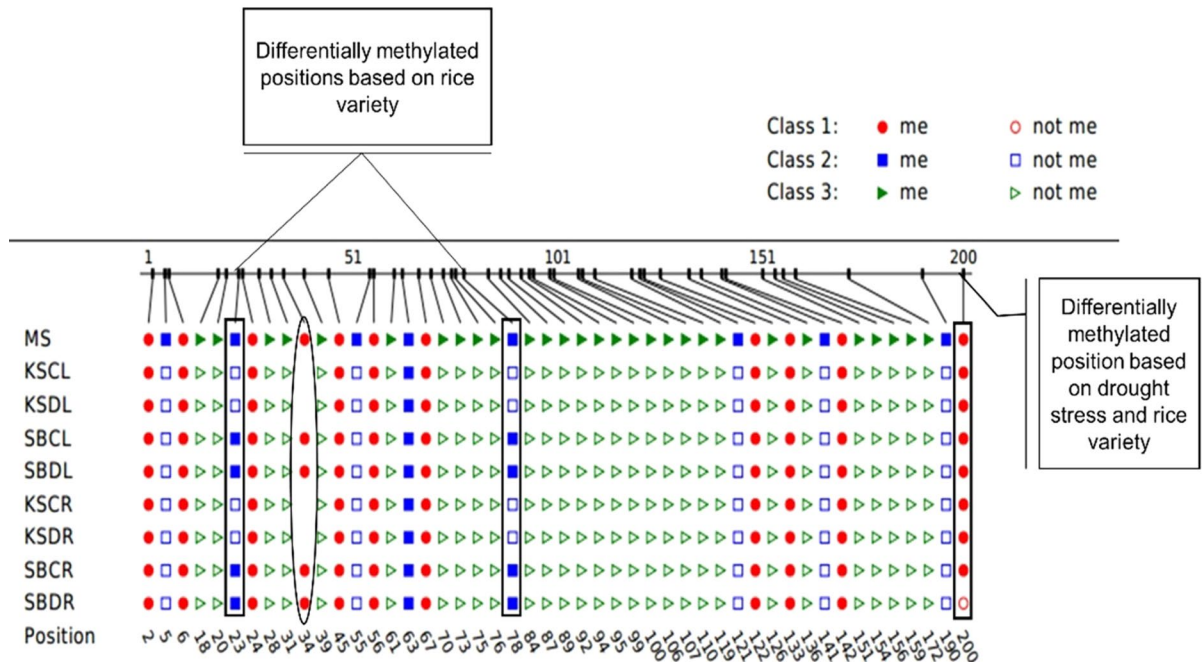
Fig. 4 Graphical representation of cytosine methylation map for comparative analysis of methylation pattern in promoter region of *OsGLP8-12* in leaves and roots of KS282 and Super Basmati based on the drought stress treatment and rice varieties using CyMATE. All three sequence contexts of cytosine residues CGN (class 1: red circles), CHG (class 2: blue squares) and CHH (class 3: green triangles) are represented in the map. Filled shapes are denoting cytosine (C) residues whereas empty shapes are representing thiamine (T) residues. The order of the individual sequences (from the multiple sequence alignment created by ClustalX) is listed on the left. Numbers at the top indicate the length of the alignment and numbers at the bottom represent potential methylation sites. Master Sequence, un-modified sequence derived from NCBI (MS); KS282 Control Leaves (KSCL); KS282 Drought Leaves (KSDL); Super Basmati Control Leaves (SBCL); Super Basmati Drought Leaves (SBDL); KS282 Control Roots (KSCR); KS282 Drought Roots (KSDR); Super Basmati Control Roots (SBCR); Super Basmati Drought Roots (SBDR)

each *OsGLP* gene targeted promoter region) by ClustalX and BioEdit (Kumar et al. 2018). PLACE and PlantCARE were utilized for identification and analysis of CREs in promoter regions of *OsGLP* genes (Fei et al. 2017).

Results

Methylation analysis of selected promoter regions of *OsGLP* genes under control condition

To analyze the cytosine methylation status in the promoter regions of *OsGLP* genes in control leaf and root samples of KS282 and Super Basmati, bisulfite sequencing was performed in selected promoter regions of the 3 *OsGLP* genes (*OsGLP4-1*, *OsGLP8-10* and *OsGLP8-12*). Promoter regions were selected within 1 kb upstream region of transcription start site for *OsGLP4-1* (from −153 to −455 bp), *OsGLP8-10* (from −436 to −794 bp) and *OsGLP8-12* (from −528 to −787 bp). As a result of methylation analysis in control samples using CyMATE software, among three *OsGLP* genes, the highest number of methylated cytosine residues was identified in *OsGLP8-12* whereas no methylated cytosine residue was detected in the promoter regions of *OsGLP4-1* and *OsGLP8-10* in both rice varieties (Fig. 1a). However, the level of methylation was different depending upon CGN, CHG and CHH sequence context as well as rice varieties. Overall, the level of methylation was found to be higher in CGN context than CHG and CHH contexts. In this aspect, in both leaves and roots, the promoter regions of *OsGLP8-12* exhibited 90.90% methylated CGN positions and 9.09% CHG positions in KS282 whereas 78.6% methylated CGN
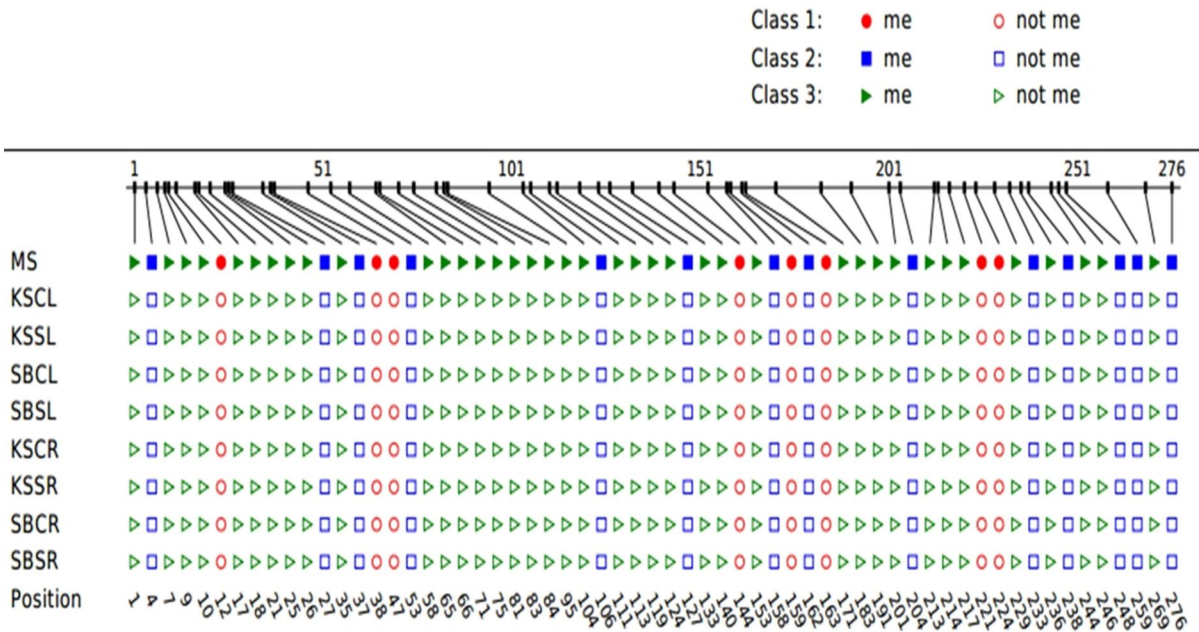
Fig. 5 Graphical representation of cytosine methylation map for comparative analysis of methylation pattern in promoter region of *OsGLP4-1* in leaves and roots of KS282 and Super Basmati based on the salt stress treatment and rice varieties using CyMATE. All three sequence contexts of cytosine residues CGN (class 1: red circles), CHG (class 2: blue squares) and CHH (class 3: green triangles) are represented in the map. Filled shapes are denoting cytosine (C) residues whereas empty shapes are representing thiamine (T) residues. The order of the individual sequences (from the multiple sequence align- ment created by ClustalX) is listed on the left. Numbers at the top indicate the length of the alignment and numbers at the bottom represent potential methylation sites. Master Sequence, un-modified sequence derived from NCBI (MS); KS282 Control Leaves (KSCL); KS282 Salt Leaves (KSSL); Super Basmati Control Leaves (SBCL); Super Basmati Salt Leaves (SBSL); KS282 Control Roots (KSCR); KS282 Salt Roots (KSSR); Super Basmati Control Roots (SBCR); Super Basmati Salt Roots (SBSR)

positions and 21.4% CHG positions were observed in Super Basmati (Fig. 1b). Interestingly, no methylated residue was observed at CHH position in any analyzed promoter of the *OsGLP* genes from control sample of both varieties.

Drought induced differential methylation patterns in promoter regions of *OsGLP* in leaves and roots of rice varieties

To investigate the location, class and methylation status of cytosine residue in the selected promoter regions of *OsGLP* genes in response to drought stress, comparative methylation analysis of bisulfite sequences was performed in promoter regions of 3 *OsGLP* genes. For promoter region of *OsGLP4-1*, overall analysis indicated no differentially methylated sites in any of the bisulfite converted sequences from control and drought treated leaves and roots of KS282 and Super Basmati (Fig. 2, Online Resource 2). However, the analyzed promoter segment of *OsGLP 8–10* was observed with differentially methylated sites in roots of KS282 and Super Basmati under drought stress with KS282 exhibiting higher methylation rate (22.22%) at CGN positions as compared to Super Basmati (11.11%) (Fig. 3, Online Resource 3). Among 3 *OsGLP* genes, bisulfite sequence of *OsGLP8-12* promoter that was observed with most methylated sites in both KS282 and Super Basmati exhibited differentially methylated sites with respect to a particular variety, tissue and stress type. For example, in control samples, 23.40% methylated cytosine residues in KS282 and 29.17% in Super Basmati were observed with differential methylation status at CHG position whereas single CGN site was found with differential methylation status (de-methylation) in roots of Super Basmati under drought stress (Fig. 4, Online Resource 4).
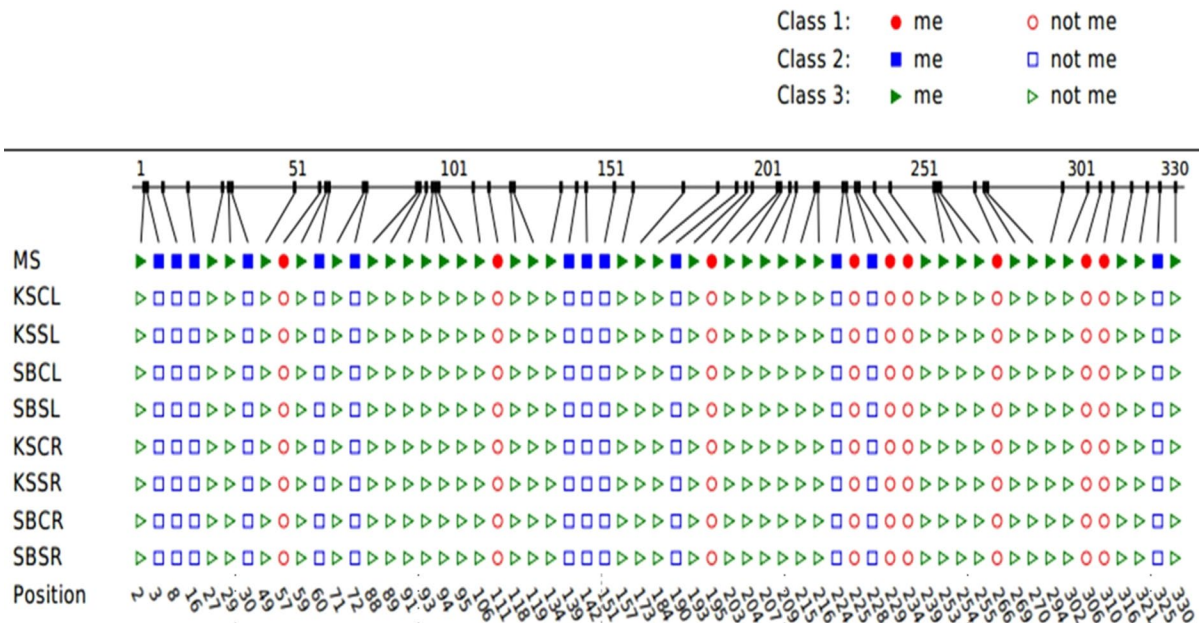
Fig. 6 Graphical representation of cytosine methylation map for comparative analysis of methylation pattern in promoter region of *OsGLP8-10* in leaves and roots of KS282 and Super Basmati based on the salt stress treatment and rice varieties using CyMATE. All three sequence contexts of cytosine residues CGN (class 1: red circles), CHG (class 2: blue squares) and CHH (class 3: green triangles) are represented in the map. Filled shapes are denoting cytosine (C) residues whereas empty shapes are representing thiamine (T) residues. The order of the individual sequences (from the multiple sequence align- ment created by ClustalX) is listed on the left. Numbers at the top indicate the length of the alignment and numbers at the bottom represent potential methylation sites. Master Sequence, un-modified sequence derived from NCBI (MS); KS282 Control Leaves (KSCL); KS282 Salt Leaves (KSSL); Super Basmati Control Leaves (SBCL); Super Basmati Salt Leaves (SBSL); KS282 Control Roots (KSCR); KS282 Salt Roots (KSSR); Super Basmati Control Roots (SBCR); Super Basmati Salt Roots (SBSR)

Salt induced differential methylation patterns in promoter regions of *OsGLP* in leaves and roots of rice varieties

To understand the impact of salt stress on methylation pattern in promoter regions of *OsGLP* genes, bisulfite sequences of promoter regions of 3 *OsGLP* genes (*OsGLP4-1*, *OsGLP8-10* and *OsGLP8-12*) were analyzed in control and salt treated leaves and roots of KS282 and Super Basmati by utilizing cytosine methylation map generated by CyMATE and multiple sequence alignment by CLUSTALX/BioEdit. Similar to drought stress, the comparative methylation map and multiple sequence alignment of control and salt treated samples from both rice varieties showed no differentially methylated positions in the bisulfite converted selected promoter region of *OsGLP4-1* (Fig. 5, Online Resource 5). However, in contrast to drought stress, salt stress induced no change in methylation status of cytosine residues in the selected promoter segment of *OsGLP8-10* and all of the possible methylation sites were found to be un-methylated in leaves and roots of both rice varieties under control and salt stress conditions (Fig. 6, Online Resource 6). The comparative analysis of bisulfite converted sequences of *OsGLP8-12* promoter from control and salt treated samples revealed the methylation patterns similar to drought treated samples with single but same salt stress induced de-methylated CGN site in roots of Super Basmati. Interestingly, in addition to differentially methylated sites, *OsGLP8-12* promoter sequence from KS282 and Super Basmati showed difference in their nucleotide sequence at two different sites where cytosine was replaced with adenine at one mutated site and guanine was replaced with adenine at the other site in KS282 while master sequence and sequences from Super Basmati shared same sequence (Fig. 7, Online Resource 7).
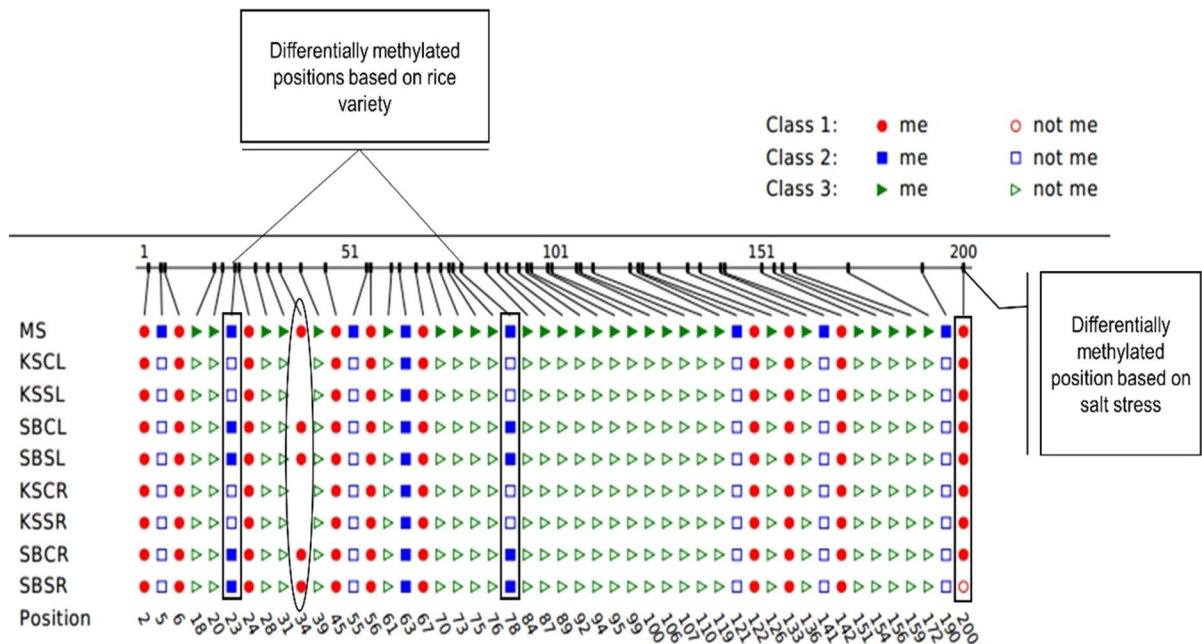
Fig. 7 Graphical representation of cytosine methylation map for comparative analysis of methylation pattern in promoter region of *OsGLP8-12* in leaves and roots of KS282 and Super Basmati based on the salt stress treatment and rice varieties using CyMATE. All three sequence contexts of cytosine residues CGN (class 1: red circles), CHG (class 2: blue squares) and CHH (class 3: green triangles) are represented in the map. Filled shapes are denoting cytosine (C) residues whereas empty shapes are representing thiamine (T) residues. The order of the individual sequences (from the multiple sequence align-ment created by ClustalX) is listed on the left. Numbers at the top indicate the length of the alignment and numbers at the bottom represent potential methylation sites. Master Sequence, un-modified sequence derived from NCBI (MS); KS282 Control Leaves (KSCL); KS282 Salt Leaves (KSSL); Super Basmati Control Leaves (SBCL); Super Basmati Salt Leaves (SBSL); KS282 Control Roots (KSCR); KS282 Salt Roots (KSSR); Super Basmati Control Roots (SBCR); Super Basmati Salt Roots (SBSR)

Stress induced cytosine methylation changes around CREs in promoter regions of *OsGLP* genes

To better understand the importance of modulated methylation patterns at specific cytosine residues on the functionality of selected genes with respect to a particular stress treatment, the location and meth-ylation status, analysis of CREs was performed in the promoter regions of *OsGLP* genes. Selected promoter sequences for *OsGLP4-1*, *OsGLP8-10* and *OsGLP8-12* were analyzed in detail by PLACE and PlantCare, and significant CREs for abiotic stress and transcription regulation were identified. For instance, the analyzed sequence of *OsGLP4-1* consisted of various CREs mainly including MYB, MYC, W-box, CTCC-motif, TATA-Box and CAAT-box. However, all the possible sites of methylation were found to be un-methylated within or around these CREs (Fig. 8). Furthermore, several important CREs (MYB, W-box, GT1-motif, ABRE/ACGT, CTCC-motif, TATA-Box and CAAT-box) were also determined in promoter region of *OsGLP8-10* and one of the two differentially methylated sites (identified in bisulfite sequence analysis) was found in close proximity with W-box CTCC, MYB and GT1 motif while the other site was observed near GT1 motif and CAAT-box (Fig. 9). Characteriza-tion of CREs in the promoter region of *OsGLP8-12* also indicated the presence of MYB, W-box, LTR, CTCC-motif, CAAT-box, TATA-box and ABRE/ACGT-motif and several methylated and differen-tially methylated sites were observed near or within these CREs. Investigation of differentially methyl-ated sites with respect to CREs showed that one of the differentially methylated sites at CHG position was present nearly after CTCC-motif, MYB and
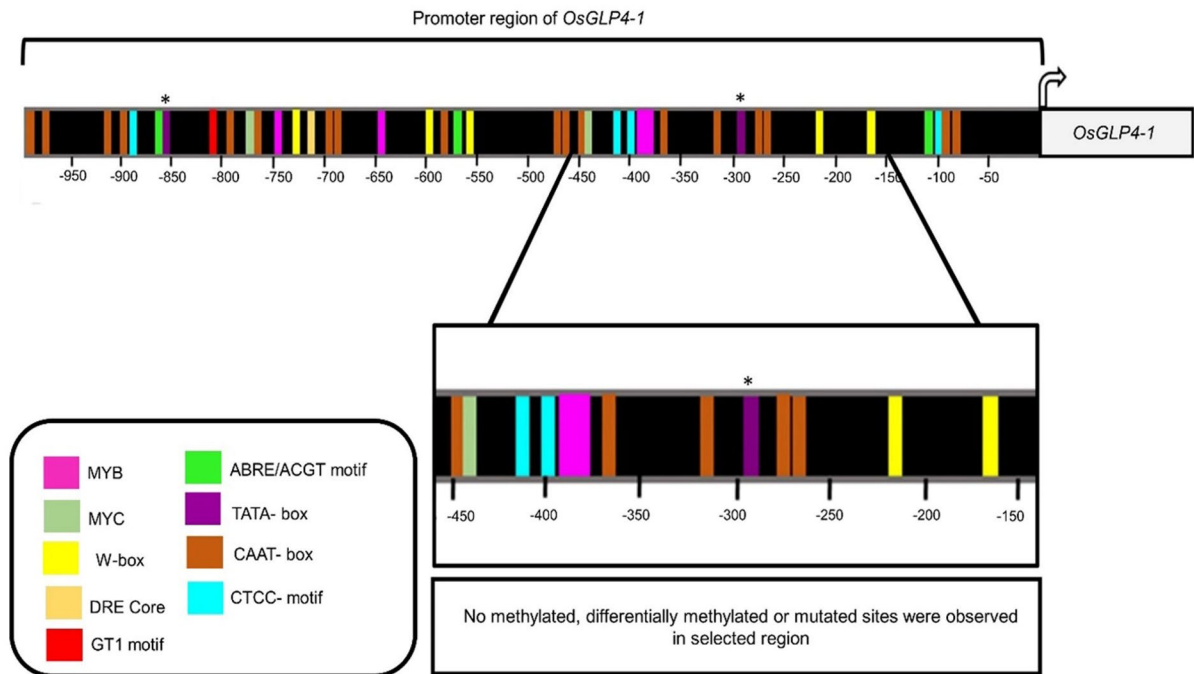
Fig. 8 Schematic diagram denoting cis-regulatory elements and their methylation status in the promoter region of *OsGLP4-1*
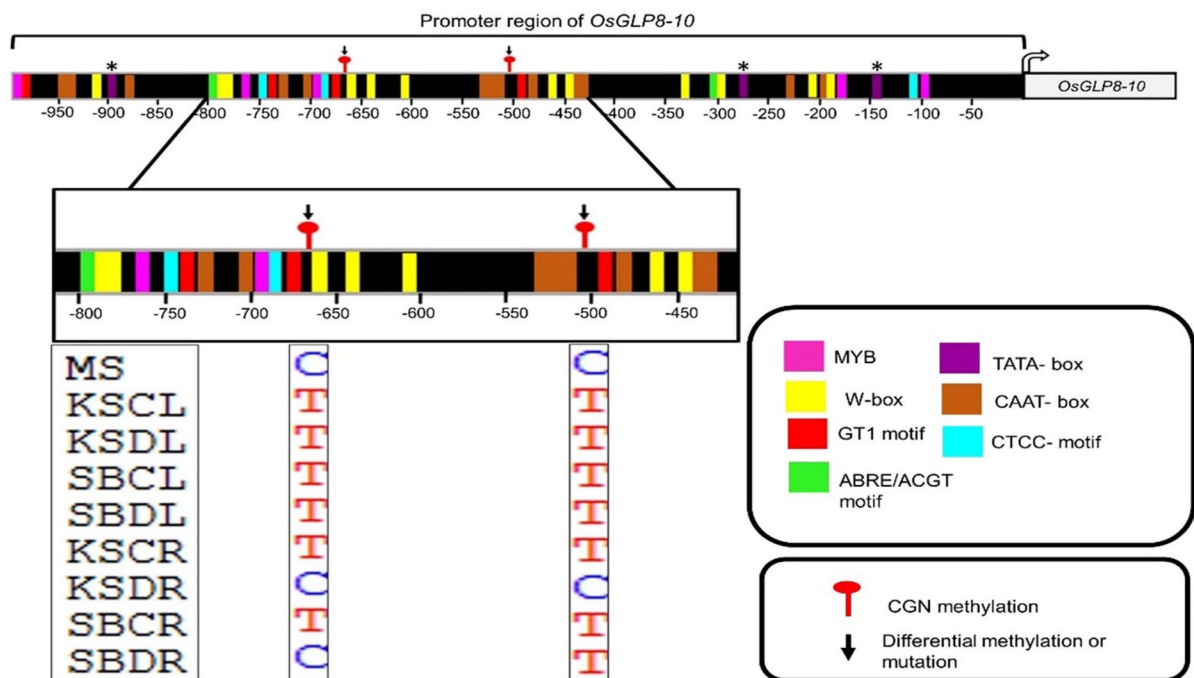

Fig. 9 Schematic diagram denoting cis-regulatory elements and their methylation status in the promoter region of *OsGLP8-10*. Methylated Cytosine (C); Un-methylated Cytosine (T). Master Sequence, un-modified sequence derived from NCBI (MS); KS282 Control Leaves (KSCL); KS282 Drought Leaves (KSDL); Super Basmati Control Leaves (SBCL); Super Basmati Drought Leaves (SBDL); KS282 Control Roots (KSCR); KS282 Drought Roots (KSDR); Super Basmati Control Roots (SBCR); Super Basmati Drought Roots (SBDR)
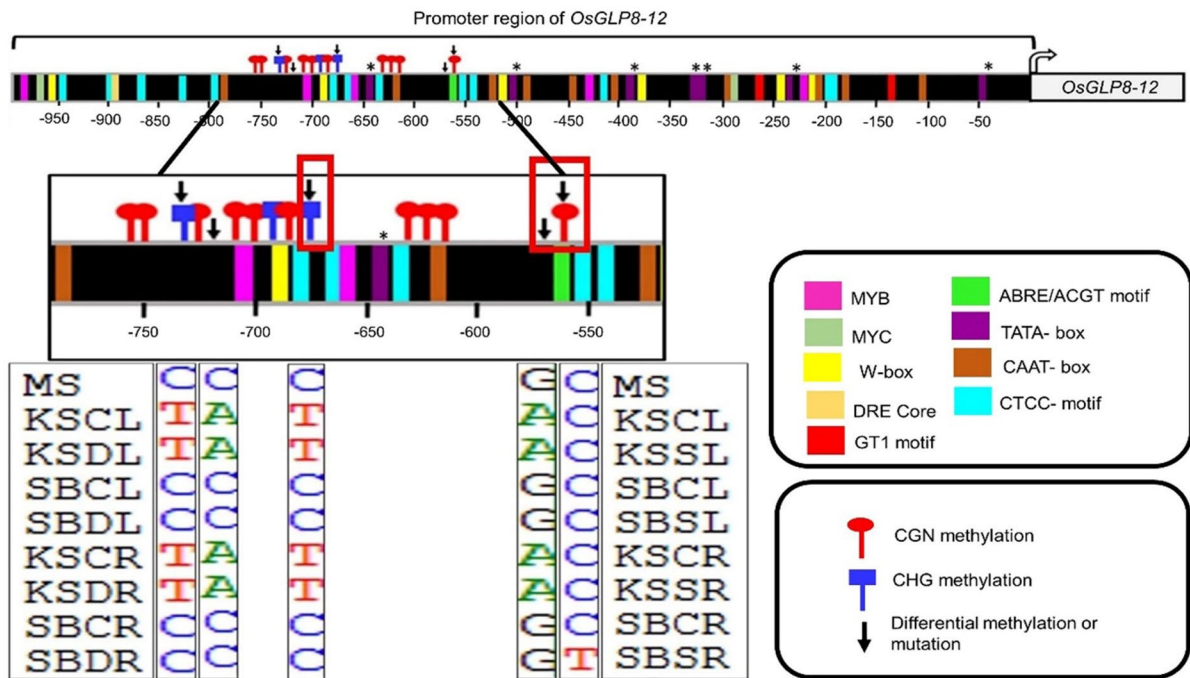
Fig. 10 Schematic diagram denoting cis-regulatory elements and their methylation status in the promoter region of *OsGLP8-12*. Methylated Cytosine (C); Un-modified Cytosine (T); Adenine (A); Guanine (G). Master Sequence, un-modified sequence derived from NCBI (MS); KS282 Control Leaves (KSCL); KS282 Drought Leaves (KSDL); Super Basmati Control Leaves (SBCL); Super Basmati Drought Leaves (SBDL); KS282 Control Roots (KSCR); KS282 Drought Roots (KSDR); Super Basmati Control Roots (SBCR); Super Basmati Drought Roots (SBDR); KS282 Salt Leaves (KSSL); Super Basmati Salt Leaves (SBSL); KS282 Salt Roots (KSSR); Super Basmati Salt Roots (SBSR)

W-box motifs indicating that these motifs were followed by un-methylated cytosine in KS282 while methylated cytosine in Super Basmati in all samples. In addition to this, single site that was differentially methylated in response to salt and drought stress (de-methylated in roots of Super Basmati under salt and drought stress) was identified within ABRE/ACGT-motif. Interestingly, one of the two mutated sites observed in *OsGLP8-12* promoter sequence in KS282 was determined near this differentially methylated CRE motif (Fig. 10).

Discussion

Evidences are growing in favor of DNA methylation at cytosine residues influencing the regulation of stress responsive genes to generate plant response against various environmental abiotic stress conditions including drought and salinity. However, the role of modulated methylation status of cytosine residues in regulating the expression of stress responsive GLP genes under drought and salt conditions has not been documented. In current study, epigenetic consequences of salt and drought stress on cytosine methylation dynamics of promoter regions of *OsGLP* genes have been assessed in two contrasting rice varieties.

Among 3 *OsGLP* genes, comparison of cytosine DNA methylation sites (CGN, CHG and CHH) in control leaves and roots samples indicated higher methylation density in the promoter region of *OsGLP8-12* in both rice varieties. Importantly, the bisulfite sequence of *OsGLP8-12* exhibited site-dependent differential methylation level at three sequence contexts (CGN > CHG > CHH) in KS282 and Super Basmati. Similar to this study, higher methylation levels in CGN context have also been reported in earlier studies performed on different genes in rice and other plants (Cokus et al. 2008; Garg et al. 2015; Hossain et al. 2017). One of the major reasons for high level of methylation at CGN

and CHG sites may be due to the maintenance of methylation status in these sequence contexts which leads to the methylation of newly replicated DNA strands (Rajkumar et al. 2020). Additionally, although same methylation pattern was observed in KS282 and Super Basmati, however, the level of methylation at each sequence context was found to be dependent upon a particular rice variety indicating the differential role of DNA methylation in two different rice cultivars.

Drought stress has been reported to cause major variations in expression of abiotic stress responsive genes including *OsGLP* genes. In our recent work, *OsGLP4-1*, *OsGLP8-10* and *OsGLP8-12* have been shown to exhibit modulated expression under drought stress in leaves and roots of KS282 and Super Basmati (Anum et al. 2022). Interestingly, in current study, analysis of promoters of these *OsGLP* genes for altered DNA methylation pattern against drought stress indicated few differentially methylated sites in the analyzed regions depending upon a particular tissue and rice variety. *OsGLP8-10* that was least expressed in drought treated roots of KS282 and Super Basmati was observed with methylated sites in its promoter region in roots under drought stress with varied level of methylation in both cultivars. Furthermore, *OsGLP8-12* that was previously reported to be up-regulated only in roots of Super Basmati under drought stress (Anum et al. 2022) was observed with un-methylated CGN site in its promoter region in drought treated roots. A study on whole genome methylome showed that promoter methylation seemed to be associated with tissue specific gene expression in plants (Surdonja et al. 2017). Moreover, in accordance with our study, drought stress has been documented to induce cultivar and site specific alteration in methylation status of DNA in two contrasting rice varieties with an increased differential methylation in promoters of abiotic stress responsive genes including drought stress (Wang et al. 2011; Zheng et al. 2013; Sapna et al. 2020). In view of previous reports as well as current study it can be suggested that differentially methylated sites in the promoter regions of *OsGLP* genes may play a role in modulating their cultivar and tissue specific expression in response to drought stress.

All three *OsGLP* genes analyzed in this study for their DNA methylation analysis have also previously

been identified as salt stress responsive in the course of expression analysis (Anum et al. 2022). Analyzing methylation variation revealed minor but cultivar, tissue and site specific changes in the methylation status in the selected promoter regions of some *OsGLP* genes. *OsGLP8-12* that was reported with elevated expression during transcriptomic analysis only in salt stress treated roots of Super Basmati was detected with de-methylation at CGN sequence context in the same sample in present study indicating the importance of differential methylation at CGN site in salt stress response. Similar to this, a previous report on differential methylation in seedlings of contrasting rice cultivars has also indicated variety and site specific variation in methylation status with an effect on gene expression suggesting the role of cytosine methylation in salt stress response. However, in contrast to our analysis, major differences in methylation status were reported in CHH context in association with altered expression of stress responsive genes in rice seedling under salt stress (Rajkumar et al. 2020). This may suggest the significance of site specific differential methylation in generating tissue specific abiotic stress response. In addition to this, several other studies have also revealed the association of salt stress induced removal of cytosine methylation in promoter region of stress related genes with their increased expression in various plant species including rice indicating its role in regulating gene expression under stress conditions (Zhu et al. 2015; Zhang et al. 2020).

Furthermore, the role of CREs present in the promoter regions of stress responsive genes in regulating gene expression by interacting with transcription factors under abiotic stress conditions is well recognized (Bjornson et al. 2016). In this study, in silico analysis of selected promoter regions of *OsGLP* genes revealed the presence of CREs involved in gene regulation and abiotic stress response including salt and drought stress. Also, the results identified cultivar, tissue and stress-dependent differentially methylated cytosine sites within or in close proximity of several identified CREs mainly including MYB, W-box and ABRE/ACGT in the promoter regions of *OsGLP8-10* and *OsGLP8-12*. Previously, expression and promoter methylation study of *TaGAPC1* gene in wheat also reported its differential expression and differential methylation of cytosine residues on CREs in two wheat cultivars with contrasting drought tolerance (Fei et al. 2017).

Although, it has been documented that the binding of a transcription factor can be blocked by the DNA methylation at specific sites in the promoter sequence, however, there is also a possibility that methylation in the promoter sequence may not affect the binding of transcription factor (Kulakovskiy and Makeev 2009; Skorupa et al. 2021). Overall, differential methylation status of specific cytosine residues within or near CREs can be a crucial regulatory mechanism in modulating the expression of *OsGLP* genes under drought and salt stress in two contrasting rice varieties. These DNA methylation variations may contribute to breeding by providing useful markers and epigenetically-controlled adaptive traits with potential applications in improvement of rice crop under abiotic stresses.

Conclusion

Present study revealed the differential methylation patterns in promoter regions of *OsGLP* genes in two contrasting rice cultivars under drought and salinity. Among all three *OsGLP* genes selected for methylation analysis, promoter region of *OsGLP8-12* exhibited most methylated and differentially methylated residues depending upon a particular variety, tissue or stress condition whereas no methylated residues were observed in promoter region of *OsGLP4-1* in all samples. Also, several differentially methylated sites in response to stress treatments in selected promoter regions of *OsGLP8-10* and *OsGLP8-12* were identified either near or within stress related cis-regulatory elements indicating the potential role of site specific alterations in cytosine methylation in regulating response of *OsGLP* genes to these stress conditions. Overall results suggested that the stress, tissue and cultivars specific methylation changes in promoter regions may be a key alternative regulatory mechanism in modifying the expression of *OsGLP* genes for generating drought and salt stress response in rice cultivars.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest relevant to the content of this article.

Consent to participate A consent has been taken from all participants of the study.

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