Genome-Wide DNA Methylation Dynamics During Drought Responsiveness in Tibetan Hulless Barley

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Abstract

Differences in drought stress tolerance within diverse grass genotypes have been attributed to epigenetic modifications. DNA methylation is an important epigenetic alteration regulating responses to drought-stress. However, its effects on drought-tolerance are poorly understood in Tibetan hulless barley. Here, bisulfite sequencing was conducted to profile the DNA methylation patterns of drought-tolerant variety XL and drought-sensitive one DQ under drought and control conditions. A total of 5843 million reads were generated. We found the significant genome-wide changes in CHH methylation rates between XL and DQ, while CG or CHG methylation rates did not. Besides that, the two contrasting varieties do reveal distinct responses to drought-stress in differentially methylated region (DMR) numbers and antioxidant activities. Genes in drought-tolerant varieties XL are rapidly and significantly methylated to alleviate the drought stress. DMR related genes in XL might involve in defense response and response to stimuli, which are confirmed by gene ontology analysis. Then, we focused on 1003 transcription factors and identified 15 specific DMR related transcription factors exhibiting specific methylation changes under drought stimuli. Finally, we identified three DMR related TFs (*HVUL6H08680.2*, *HVUL4 h39100.2*, and *HVUL2H41931.2*) where Arabidopsis homologues involve in responses to drought conditions. Altogether, DNA methylation regulate responsiveness to environmental stimuli, which could be mediated by methylation of transcription factors in hulless barely.

Introduction

Hulless barley (*Hordeum vuglare* L. var. *nudum* Hook. f.), also called naked barley, is an important cereal crop in Tibet Plateau(Xu et al., 2016). It has been served as a healthy food for human consumption and animal feed for over thousands of years. Owing to high altitudes, the naked barley is cultivated in harsh environment such as valleys and higher land on Tibet(Liang et al., 2017). It is also affected by drought and low temperature in March every year when the weather is quite cold and dry(J. B. Du et al., 2011). In order to mitigate adversities such as drought, salinity, and low temperature, various strategies have been evolved in hulless barley(H. Li, Guo, Lan, Zhou, & Wei, 2014).

It is well-known that drought is the most serious environmental stress that affect crop growth and yield by causing a wide range of physiological and biochemical responses(Flowers, 1989; Iqbal, Murtaza, Saqib, & Ahmad, 2015). The influences of drought on the crop varies between varieties and developmental stages. Many genes responsible for drought tolerance have been reported for decades(Lenka, Katiyar, Chinnusamy, & Bansal, 2015; Moa, Liua, Lina, Xub, & Xianga, 2005). For example, the transcription factor AtHB13 has been reported to act as a positive regulator of drought tolerance in Arabidopsis. Genetically, drought tolerance is extremely complex trait involving in several genetic pathways such as polygenic control and complex morpho-physiological mechanisms(Xuekun Zhang et al., 2014). At the molecular level, drought stress could induce genome-wide changes in gene expression via epigenetic mechanisms like histone modification and DNA methylation in plants(Wang et al., 2016; Zong, Zhong, You, & Xiong, 2013). For example, the mutant allele (*met1*) DNA methyltransferase 1 locus could remove methylation at several genomic regions leading to specific expression of 31 stress response-related genes in tobacco(Ku, Lim, & Park, 2006; Zubko, Gentry, Kunova, & Meyer, 2012).

DNA methylation, an epigenetic modification, play crucial roles in plant growth and development as well as responses to various abiotic stresses(Arthur et al., 2018; Uthup, Ravindran, Bini, & Thakurdas, 2011). Actually, DNA methylation exists in all eukaryotes, and it frequently occurs at the 5-position of cytosine (5mC)(Kasai & Kawai, 2009). Under normal conditions, the proportion of methylcytosine in plants is 20–30% in plants, which usually occurs in three nucleotide sequences: CG, CHG and CHH (H indicates C, T or A)(Serre, Lee, & Ting, 2009). CG methylation is produced by the conserved DNA methyltransferase METHYLTRANSFERASE1 (MET1)(Zubko et al., 2012); CHG methylation is modified by the plant specific DNA methyltransferase CHROMOMETHYLASE3 (CMT3)(J. Du et al., 2012); and the de novo CHH is methylated by 24-nucleotide small interfering RNA dependent DNA methylation (RdDM) pathway(Ma et al., 2015). Interestingly, methylation levels contribute greatly to the process of adaptation to stress in plants. The hyper or hypo methylation changes in the hybrids could be an indicator of the expression levels of stress related gene under drought, when compared to their parents(Boyko et al., 2010; Hai & Zhang, 2009). This indicates the methylcytosine and its reversibility may regulate transgenerational response to stresses. An increasing number of studies revealed the transposon-rich heterochromatic regions show heavily methylation(Melamed-Bessudo & Levy, 2012). Over one-third of expressed genes are methylated within transcribed regions, while only 5% genes within their promoter regions in Arabidopsis(Xiaoyu Zhang et al., 2006). Thus, DNA methylation within genes is a common feature of eukaryotic genomes. Intriguingly, methylation of transcribed regions does not usually result in gene silencing(Xiaoyu Zhang et al., 2006). It seems to primarily occur at CG sites and appears to show

moderate correlation between the level of gene-body methylation and gene expression(Su, Wang, Xing, Liu, & Yong, 2014). DNA methylation shows the deposition of certain chromatin marks such as differentially modified histones because of the tight link between DNA methylation and histone modifications(Fuks, 2005). For instance, there is a relationship between the reduce in DNA methylation levels and the changes in level of H3K4me and H3K9me modifications in *DECREASE IN DNA METHYLATION1* (*DDM1*) mutant, which maintain cytosine DNA methylation within the heterochromatic locus regions(Sasaki, Kobayashi, Saze, & Kakutani, 2012; Zhou et al., 2013).

It is the first time that the epigenetic responses to drought between two contrasting varieties were revealed via bisulfite sequencing and RNA sequencing in Tibetan hulless barely. Here, we explored how DNA methylation is involved in drought responsiveness. Changes in the level of DNA methylation affect plant resistance to drought, especially the methylation changes of several transcription factor genes. Therefore, epigenetic changes in genome could be considered as an important regulatory mechanism for plants to adapt to drought and possibly other environmental stresses.

Materials And Methods

Plant materials and treatments

The Tibetan hulless barley (H. vulgare subsp. vulgare) varieties XL and DQ were used. The XL variety is highly resistant to drought stress, while the DQ is sensitive. Healthy seeds were sterilized by soaking in 2% H_2O_2 for 40 min, and rinsed in sterile water. Finally, seeds were germinated on moistened filter paper at 16–18℃ with 18-h light/10-h dark photoperiod and a relative humidity of 80% in a growth chamber. Artificial water stress was induced with 21% polyethylene glycol (PEG) 6000 solutions to achieve an osmotic potential. The roots and leaves were harvested from 10-day-old seedlings from two genotypes under normal and drought conditions at 0 h, 4 h, and 48 h, respectively; two plants from each pot were considered as biological replicates. All these tissue samples mentioned above were fast frozen in nitrogen and stored at -80 ℃ immediately.

Determination of the MDA content

The level of lipid peroxidation in plant tissues was measured by determination of MDA, which was measured using the thiobarbituric acid (TBA) method(Schmedes & H?lmer, 1989). About 0.3 g leaf sample of hulless barley seedling was homogenized with a mortar and pestle in 5mL 0.1% Trichloro acetic acid (TCA), then the homogenate was centrifuged at 10,000g for 15 min. About 5ml of 20% TCA containing 0.5% TBA was added to 1 ml supernatant aliquot. The mixture was heated at 95°C for 10 min, cooled immediately, and centrifuged at 10,000 g for 15 min. The reaction products of MDA and TBA show highest absorbance at 532 nm. Finally, MDA concentration was calculated using the following method.

Determination of Peroxidase (POD) and Catalase (CAT) Activity

To analyze the activities of plant antioxidant enzyme (POD,CAT), about 0.5 g frozen leaf samples were homogenized with a mortar and pestle in ice bath at a 1:10 ratio with 100 mM phosphate buffer saline containing 1 mM EDTA and 1% polyvinylpyrolidone (PVP). The crude homogenates were centrifuged at 10,000 g for 15 min at 4 ℃. The supernatant was used to determine the POD and CAT activities.

POD activity was measured by spectrophotometer following change of absorption at 420 nm due to guaiacol oxidation(Tamas, Mistrik, & Zelinova, 2016). Under these conditions, one unit of POD activity was defined as the amount of POD that was require for 50% inhibition of the enzymatic reaction in 1 ml enzyme extraction of per milligram of protein(Yu, Lv, & Shi, 2009). CAT activity was measured, which based on the fact that ammonium molybdate could rapidly terminate the H_2O_2 degradation reaction catalysed by CAT and generate a yellow complex. This complex is monitored by absorbance at 405 nm using the spectrophotometer. One unit of CAT activity was defined as 1 mmol H_2O_2 decomposed in one milligram of protein per second.

DNA extraction and BS-Seq

The DNA was isolated using a cetyltrimethylammonium bromide (CTAB) method form hulless barley leaves; the integrity was checked by agarose gel electrophoresis; the concentration was measured via a non-ultraviolet method(Allen, Flores-Vergara, Krasynanski, Kumar, & Thompson, 2006). Bisulfte treatment, library construction and sequencing were conducted by the company (Igenebook Co. Ltd). Un-methylated lambda DNA was spiked in to determine non-conversion rate, and conversion rate for all libraries

was higher than 99%. Finally, paired-end bisulfte-treated sample libraries were constructed and sequenced. In addition, a library was constructed from untreated DNA and sequenced as a control.

BS-seq data analysis

After removing low-quality reads, clean data were mapped to reference genome using BSMAP software (version: 2.9) which permits 8% mismatch per read(Xi & Li, 2009). Then, methylation levels were calculated based on the cytosine percentage in a given position by a custom Perl script(Macisaac, Bogutz, Morrissy, & Lefebvre, 2012). The methylation profiles for flanking 2kb regions and the CDS were plotted based on average methylation levels for each 100bp interval. Differentially methylation regions (DMRs) were identified using the tDMR package between three tissue samples with the following criteria: (a) at least five methylated cytosine sites in at least one sample; (b) coverage by more than ten reads; (c) a distance between adjacent methylated sites was less than or equal to 200bp; (d) a length of the region was between 40bp and 10,000bp; (e) a difference in methylation levels was at least two with a test value of $p \leqq 0.05$(Song et al., 2009).

Results

Physiological and biochemical differences between two contrasting hulless barley varieties

Reactive oxygen species (ROS) excessively accumulates in plant tissues exposed to drought stress. To analyze physiological and biochemical changes in drought-tolerant variety (XL) and drought-sensitive one (DQ), leaves were harvested to investigate changes of Malondialdehyde (MDA) concentration and enzymatic activity (Catalase, Peroxidase) after exposure to drought for 48 h. Firstly, concentration of MDA was tested in leaves, because MDA could serve as the biomarker for cellular and oxidative damage. MDA concentration significantly decreased in XL under drought stress conditions in leaves, while it enhanced sharply in DQ (Fig. 1A). The extremely slight cellular damage is observed in XL, which are consistent with the previous results(Q. Li et al., 2015). This suggests XL is more tolerable to drought stress when compared with the DQ variety.

Then, peroxidase (POD) activities and catalase (CAT) activities are analyzed in Tibetan hulless barley leaves. POD activities obviously reduced in XL under drought conditions, while it rose in DQ (Fig. 1B). On the contrary, activity of catalase (CAT) showed contrasting trends. CAT activities shew 1.64 folds rise in XL when compared to normal conditions, whereas it decreased by around 50% in DQ (Fig. 1C). Therefore, CTA might involve in improving drought tolerance in the drought-tolerant variety DQ, but POD activities might negatively correlate to the drought-tolerant genotypes in hulless barley. Also, we detected the MDA, POD, and CAT contents in roots to testify the change patterns in different plant tissues. A similar dynamical changes was observed in XL and DQ varieties, suggesting that the plant organs might exhibit identical responses to drought conditions.

DNA methylomes of Tibetan hulless barley under drought conditions

In order to reveal the dynamics of genome-wide DNA methylation in contrasting Tibetan hulless barley varieties, we sequenced DNA methylomes of the hulless barley exposed to drought stresses for 0 h, 4 h and 72 h via bisulfite sequencing, respectively. Two replicates were conducted to assess biological and experimental reproducibility in DNA methylome analysis. Finally, a total of 5843 million 150bp paired-end reads were generated from sodium bisulfte treated DNA with 99% bisulfte conversion rate for each library. On an average, about 583 million 150bp sequencing reads was aligned to hulless barely genome for each sample with 25-fold coverage of the genome. An average of 52% clean read pairs were aligned to genome for each library (Table S1).

Genome-wide cytosine methylation at CG, CHG and CHH sequences was determined in all hulless barley samples. An average of 91.71% CG and 67.36% CHG was methylated in these samples, both of which shew higher methylation levels. In contrast, only 3.14% CHH was methylated, i.e. the lowest level of methylation (Table S2, Fig. 2A). In addition, we determined DNA methylation patterns in 2kb upstream of the transcription start site (TSS), gene body, and 2kb downstream of the transcription termination site (TTS). CG and CHG were methylated at gene bodies and their flanking regions with an obvious drop around TSS and TTS sites, which are consistent with the previous findings(Q. Li et al., 2015). In contrast, CHH methylation levels in both flanking regions were higher than that in gene bodies (Fig. S1).

We further determined and analyzed the CG, CHG, CHH methylation rates in these samples. The results revealed there were no signifcant genome-wide changes in CG or CHG methylation rates between the drought-tolerant variety (XL) and the drought-sensitive

one (DQ) at 4 h and 48 h, respectively (Fig. 2B). Nevertheless, CHH methylation rates decreased by 6% from 4 h to 48 h in the DQ variety, whereas it significantly increased by 48% in XL (Fig. 2C). This suggests CHH contents methylation contribute to the drought tolerance in Tibetan hulless barley, because CHH is specifically methylated by 24-nucleotide small interfering RNA dependent DNA methylation (RdDM) pathway.

Analysis of differentially methylation regions within same hulless barley varieties between normal and drought conditions

Differentially methylation regions (upstream of TSS, gene body, and downstream of TTS) were selected and identified, when we compared methylation regions within the same variety between drought and normal conditions at different periods. First of all, the CG-type, CHG-type and CHH-type DMR numbers were compared within drought-tolerant genotypes XL or drought-sensitive one DQ, respectively (Table 1). The results exhibited significant differences between XL and DQ. A total of 1840 and 141 DMR were identified in XL at 4 h and 48 h when compared between the two conditions, respectively. But, there were just about 50 DMR both at 4 h and 48 h in DQ. The results indicate that genes in drought-tolerant varieties XL are rapidly and significantly methylated to mitigate the drought stress, especially in 4 h after exposed to drought conditions. Then, we analyzed the number of CG-type, CHG-type and CHH-type DMR in XL and DQ at 4h. There were 1104 CG-type DMR, 660 CHG-type DMR and 76 CHH-type DMR in XL, but there were only 15 CG-type DMR, 18 CHG-type DMR and 29 CHH-type DMR in DQ. Considering the significant difference of DMR numbers between XL and DQ, the two contrasting varieties exhibits distinct responses to drought stress by methylating DNA. Thus, the DMR related genes in the three categories in XL might involve in response to drought stimuli. Then, these genes in XL or DQ were further analysed by gene ontology (GO). We found that CG-type related DMR genes in XL were markedly enriched for nucleotide binding, ADP binding, defense response, and response to stimulus (Fig. 3A).

Table 1
Statistics of the number of different methylation regions (DMR) within XL or DQ

Variety	Category / Combination	Total DMR	CG-type DMR	CHG-type DMR	CHH-type DMR
Drought-tolerant XL	DR_4h_vs_CK_4h	1840	1104	660	76
	DR_48h_vs_CK_48h	141	55	22	64
Drought-sensitive DQ	DR_4h_vs_CK_4h	62	15	18	29
	DR_48h_vs_CK_48h	50	7	17	26

Comparative differentially methylation regions between two contrasting varieties

In order to reveal molecular mechanism of drought tolerance, we compared the levels of methylation and gene expression between two contrasting varieties XL and DQ. Firstly, differentially methylation regions (DMR) were calculated and compared between XL and DQ under normal and drought conditions at 0 h, 4 h and 48 h, respectively. As shown in Table 2, the number of CG-type DMR slightly increased by 5% at 4 h when compared to 0 h between XL and DQ under normal conditions. However it sharply increased by 40% at 4 h under drought conditions. The similar trends were observed in the number of CHG-type and CHH-type DMR. The changes of DMR number between XL and DQ under drought conditions is more obvious than that under normal conditions, suggesting drought stress could induce DNA methylation to response to abiotic stimulus quickly. Interestingly, the number of DMR in CG, CHG, and CHH sequences between two varieties reduced from 4 h to 48 h under drought stress, but it increased under normal conditions. The contrasting DMR number changes between normal and drought conditions suggested that DNA methylation might regulate responsiveness to environmental stimuli. Furthermore, we conducted gene ontology (GO) analysis by using these DMR-related genes between XL and DQ exposed to drought stress. We found CG-type DMR-related genes were significantly enriched for cellular response to stimulus, response to stress, nucleotide binding, lyase activity, isomerase activity, hydrolase activity (Fig. 3B).

Table 2
Statistics of the number of different methylation regions (DMR) between two contrasting varieties XL and DQ

Sample		CG-type DMR	CHG-type DMR	CHH-type DMR
Normal condotions	XL_CK_0h_vs_DQ_CK_0h	909	494	42
	XL_CK_4h_vs_DQ_CK_4h	958	542	115
	XL_CK_48h_vs_DQ_CK_48h	1354	858	109
Drought conditions	XL_DR_4h_vs_DQ_DR_4h	1276	812	181
	XL_DR_48h_vs_DQ_DR_48h	771	340	144

Then, CG-type, CHG-type and CHH-type related genes were identified and calculated between XL and DQ both in drought and normal conditions. As shown in Table S3, a number of 284 CG-type genes, 205 CHG-type genes and 51 CHH-type genes between two genotypes were identified at 4 h, while 152 CG-type genes, 97 CHG-type genes and 35 CHH-type genes were identified at 48 h. The results suggest that Tibetan hulless barley could quickly activate more genes to alleviate drought damage. Besides, we performed cluster analysis of these DMR related genes, of which more than 50% were transcribed. The results exhibit markedly opposite expression profiles of CG-type, CHG-type and CHH-type related genes between two contrasting varieties at 4 h and 48 h (Fig. 4).

Identification of differentially methylation regions related transcription factors in hulless barley exposed to drought stress

To identify differentially methylation regions related transcription factors (TF) in hulless barley after exposure to drought stress, we compared the bisulfite sequencing profiles of both between two contrasting varieties and within same genotypes under drought conditions and normal conditions at 0 h, 4 h, and 48 h, respectively. We mainly focused on the four groups of TF (*AP2/ EREB, bZIP, NAC* and *MYB*), which could involve in response to abiotic stresses in plants such as *Arabidopsis thaliana* and rice. A total of 659 TFs were identified according nucleotide sequence analysis of TFs from Tibetan hulless barley, which is homologous to these from rice (Table S4). Of the 659 TFs, 26 unique TFs (11 MYB, 9 NAC, 5 AP2/DREB, and 1 bZIP) appeared to be differentially methylated between different comparative combinations (Table S5). Intriguingly, 46 DMR related TFs were indentified in total, but there were as many as 20 repetitive DMR related TFs among different combinations (Table S6).

To identify DMR related TFs that respond to drought conditions, we compared the DMR related TFs selected from the combinations exposed to drought conditions with these from the combinations exposed to normal conditions. As a result, 15 specific DMR related TFs that specifically exhibit significant methylation changes under drought stimuli were identified, indicating the 15 TFs are induced by drought-stress (Table 3). Among these TFs, we found three hulless barley TFs whose gene homologue from *Arabidopsis thaliana* mediate responses to drought stresses(Rohit et al., 2016; Sultana, Khurram, Akihiro, Maho, & Motoaki, 2016). *RR12*, *HVUL6H08680.2* gene homologue from *Arabidopsis thaliana*, encoding type B cytokinin response regulators negatively regulate plant responses to drought; *RR2* that is homologous to *HVUL4 h39100.2* exhibits tolerance to drought and salt stresses; *CSP41B* with homology to *HVUL2H41931.2* is modulated by miR399f to mediate plant responses to salt, ABA, and drought. Besides that, 4 specific Arabidopsis TFs also could involve in abiotic and biotic stresses except for drought stress. *ANAC102* with homolog to both *HVUL7H38638.2* and *HVUL3H21712.2* mediates response to low oxygen stress; *AtTDR1* with homolog to *HVUL1H37933.2* enhances tolerance to salt stress via activation of ABA synthesis in Arabidopsis; *ATMYB71* with homolog to *HVUL4 h24905.2* might regulate plant defense via promoter-based integration. Of those remaining 8 specific DMR TFs, their homologous genes in Arabidopsis encode putative proteins with unknown functions.

Table 3
Drought stress responsive DMR transcription factors

Hulless barely_ID	Ath_ID	Gene function	Ath_Annotation	Rice_ID	Rice _Annotation
HVUL7H38638.2	AT5G63790.1	ANAC102 appears to have a role in mediating response to low oxygen stress (hypoxia) in germinating seedlings.	ANAC102,NAC domain containing protein 102	LOC_Os01g66120.1	No apical meristem protein, putative, expressed
HVUL6H08680.2	AT2G25180.1	Arabidopsis type B cytokinin response regulators ARR1, ARR10, and ARR12 negatively regulate plant responses to drought.	RR12, response regulator 12	LOC_Os02g08500.1	two-component response regulator, putative, expressed
HVUL6H52348.2	Na	Unknown	Unknown	LOC_Os11g03870.1	LTPL31 - Protease inhibitor/seed storage/ LTP family protein precursor
HVUL4H47728.2	Na	Unknown	Unknown	ChrUn.fgenesh.mRNA.49	expressed protein
HVUL4H39100.2	AT4G16110.1	Whereas, RR1, RR2, RR3, and RR4 transformants exhibited tolerance to drought and salt stresses but were found to be sensitive to the heat stress	RR2, response regulator 2	LOC_Os03g12350.4	two-component response regulator, putative, expressed
HVUL1H53471.2	AT5G05800.1	Unknown	Unknown protein	LOC_Os02g53020.1	transposon protein, putative, CACTA, En/Spm sub-class
HVUL7H17240.2	AT4G02210.1	Unknown	Unknown protein	LOC_Os06g39460.1	transposon protein, putative, CACTA, En/Spm sub-class, expressed
HVUL4H47192.2	AT1G66235.1	Unknown	Unknown protein	LOC_Os10g27370.1	transposon protein, putative, Pong sub-class, expressed

Hulless barely_ID	Ath_ID	Gene function	Ath_Annotation	Rice_ID	Rice _Annotation
HVUL1H37933.2	AT3G23230.1	AtTDR1 enhances tolerance to salt through the transcriptional activation of ascorbic acid synthesis in Arabidopsis.	AtTDR1; Integrase-type DNA-binding superfamily protein	LOC_Os04g18650.1	AP2 domain containing protein, expressed
HVUL4H45104.2	AT1G71450.1	Unknown	Integrase-type DNA-binding superfamily protein	LOC_Os11g13840.1	AP2 domain containing protein, expressed
HVUL1H36756.2	AT1G61110.1	Unknown	NAC025, NAC domain containing protein 25	LOC_Os07g48450.1	no apical meristem protein, putative, expressed
HVUL3H56913.2	Na	Unknown	Na	Na	Na
HVUL3H21712.2	AT5G63790.1	ANAC102 appears to have a role in mediating response to low oxygen stress (hypoxia) in germinating seedlings.	NAC102, NAC domain containing protein 102	LOC_Os01g60020.1	NAC domain transcription factor, putative, expressed
HVUL2H41931.2	AT1G09340.1	CSP41B was significantly repressed upon water stress. miR399f might also modulates plant responses to salt, ABA, and drought,by regulating the expression of ABF3 and CSP41b.	CSP41B, chloroplast RNA binding	LOC_Os12g23180.1	3-beta hydroxysteroid dehydrogenase/isomerase family protein, putative, expressed
HVUL4H24905.2	AT3G24310.1	Promoter-based integration in plant defense regulation.	ATMYB71, myb domain protein 305	LOC_Os03g04900.1	MYB family transcription factor

To reveal DNA methylation status of the above 15 specific DMR TFs that appear to be differentially methylated by the imposition of drought stress, the sequence corresponding to upstream of TSS, gene body, and downstream of TSS was subjected to bisulfate sequencing by using the modified version of the previous SNV-calling method. We mainly focused on the three TFs

(*HVUL6H08680.2*, *HVUL4 h39100.2*, and *HVUL2H41931.2*) described above. In gene *HVUL6H08680.2*, the significantly differential methylation locates in the gene body, which means the cytosines were demethylated after exposure to salinity stress for 48 h in XL (Fig. 5A). In gene *HVUL4 h39100.2*, the significantly differential methylation exhibits in upstream of TSS, and the methylation levels in XL are notably lower than that in DQ after exposed to drought conditions for 4 h (Fig. 5B). In gene *HVUL2H41931.2*, the significantly differential methylation locates in downstream of the TSS, which means it is hypermethylated in stressed XL seedlings (Fig. S2). Clearly, DNA methylation in *HVUL6H08680.2*, *HVUL4 h39100.2*, and *HVUL2H41931.2* could be affected by drought-stress.

Discussion

Reactive oxygen species (ROS) significantly accumulates in plant tissues exposed to drought conditions. We investigated changes of Malondialdehyde (MDA) contents and enzymatic activity (Catalase, Peroxidase) both in leaves and roots after exposure to drought for 48 h in drought-tolerant variety XL and sensitive one DQ, respectively. Interestingly, similar dynamical changes was observed both in leaves and roots in XL and DQ. We only sequenced DNA methylomes of the leaves in hulless barley in this study. It is necessary to analyze and compare the DNA methylomes between leaves and roots, which could be considered as a potential method to uncover the response pathway to drought conditions.

We further determined and analyzed the CG, CHG, CHH methylation rates in XL and DQ. The results revealed that there were no signifcant genome-wide changes in CG or CHG methylation rates between the two varieties, while CHH methylation rates did not. Then, we analyzed the number of CG-type, CHG-type and CHH-type DMR in XL and DQ at 4h. There were 1104 CG-type DMR, 660 CHG-type DMR and 76 CHH-type DMR in XL, but there were only 15 CG-type DMR, 18 CHG-type DMR and 29 CHH-type DMR in DQ. However, the CG-type DMR and CHG-type DMR sharply decline in drought-sensitive variety DQ when compared to XL, while the CHH-type does not. It is possible that the extremely low CHH methylation rates result in the less CHH-type number. CHH methylation is specifically perpetuated by the combined action of siRNAs and DRM2. Thus, it indicates the siRNAs and DRM2 could play important roles in response to drought stress.

In order to identify differentially methylation regions related transcription factors (TF) in hulless barley after exposure to drought stress, 1003 TFs were identified according from Tibetan hulless barley, which is homologous to these from *Arabidopsis thaliana* and rice(Rohit et al., 2016). Finally, 15 specific DMR related TFs were identified, which exhibit significant methylation changes under drought stimuli. The Arabidopsis homologues of 3 TFs (*HVUL6H08680.2, HVUL4 h39100.2, HVUL2H41931.2*) have been proved to be involved in response to drought conditions. Therefore, DNA methylation regulate responsiveness to environmental stimuli by methylating transcription factors in hulless barely.

Altogether, it is the first time that the roles of DNA methylation in drought responsiveness were revealed in Tibetan hulless barley. Significant genome-wide changes were found in CHH methylation rates between two contrasting varieties (XL and DQ). They showed obviously different response to drought stress both in DMR numbers and antioxidant activities. Gene ontology analysis confirm DMR related genes in XL could involve in defense response and response to stimulus. Of the 1003 TFs, 26 unique TFs appeared to be differentially methylated. Intriguingly, 15 specific DMR related TFs exhibiting special methylation changes exposed to drought stress were identified, and 7 TFs of those might involve in response to drought stress or other abiotic stresses. Finally, we found methylation levels of some transcription factors have changed significantly under drought-stress treatment such as *HVUL6H08680.2*, *HVUL4 h39100.2*, and *HVUL2H41931.2* whose gene homologue from *Arabidopsis thaliana* involve in responses to drought stresses. Therefore, our results proved that hulless barely might respond to drought stress via methylation on transcription factors.

Declarations

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Author contribution

Mingzhai Yu and Yuzhen Basang conceived and supervised the work. Qijun Xu performed the DNA methylation analyses. Haizhen Yang perfomed the biochemical experiments. Haizhen Yang and Wang Mu planted the hulless barley. DunZhu Jabu wrote the article with contributions from all other authors.

Conflict of Interest Statement

The authors have declared that there is no conflict of interest.

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Figures

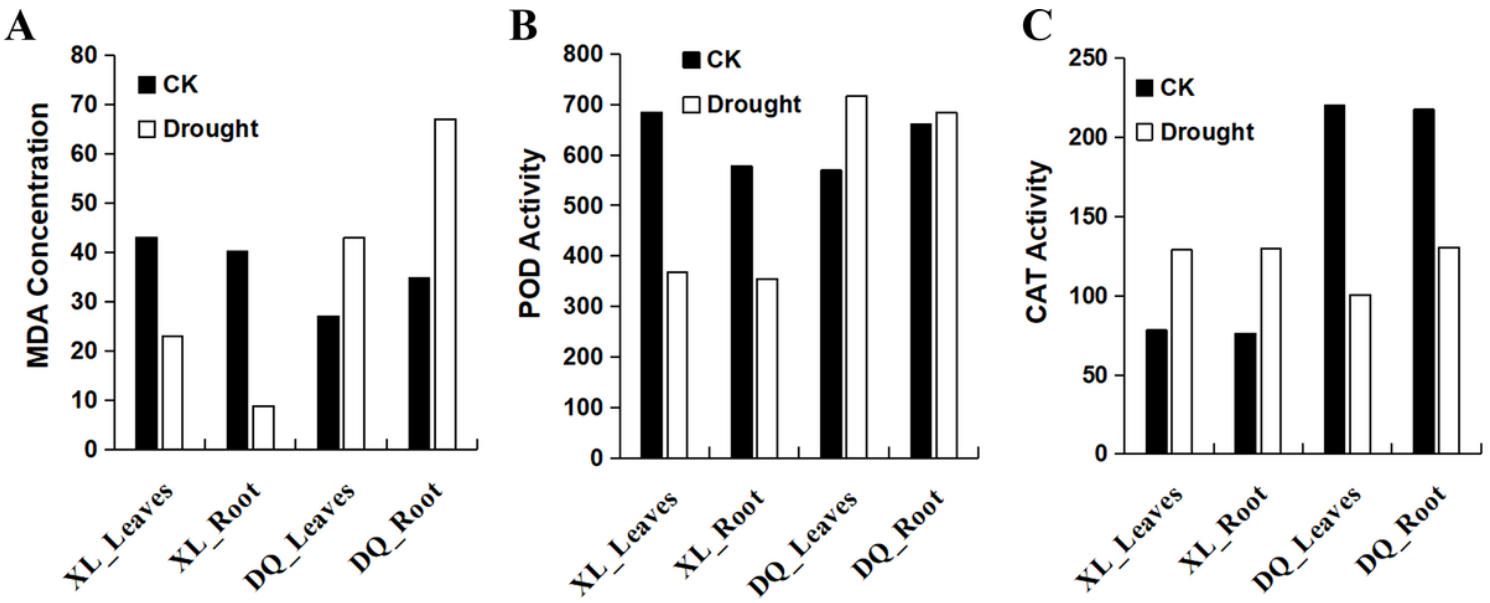

Figure 1

The physiological change in two contrasting hulless barely varieties. MDA, malondialdehyde; POD, peroxidase; CAT, catalase; XL, the drought-tolerant hulless barely; DQ, the drought-sensitive hulless barely; Drought, the hulless barely varieties were treated with 21% polyethylene glycol (PEG) 6000 solutions to achieve an osmotic potential; CK, the hulless barely grew in normal conditions.

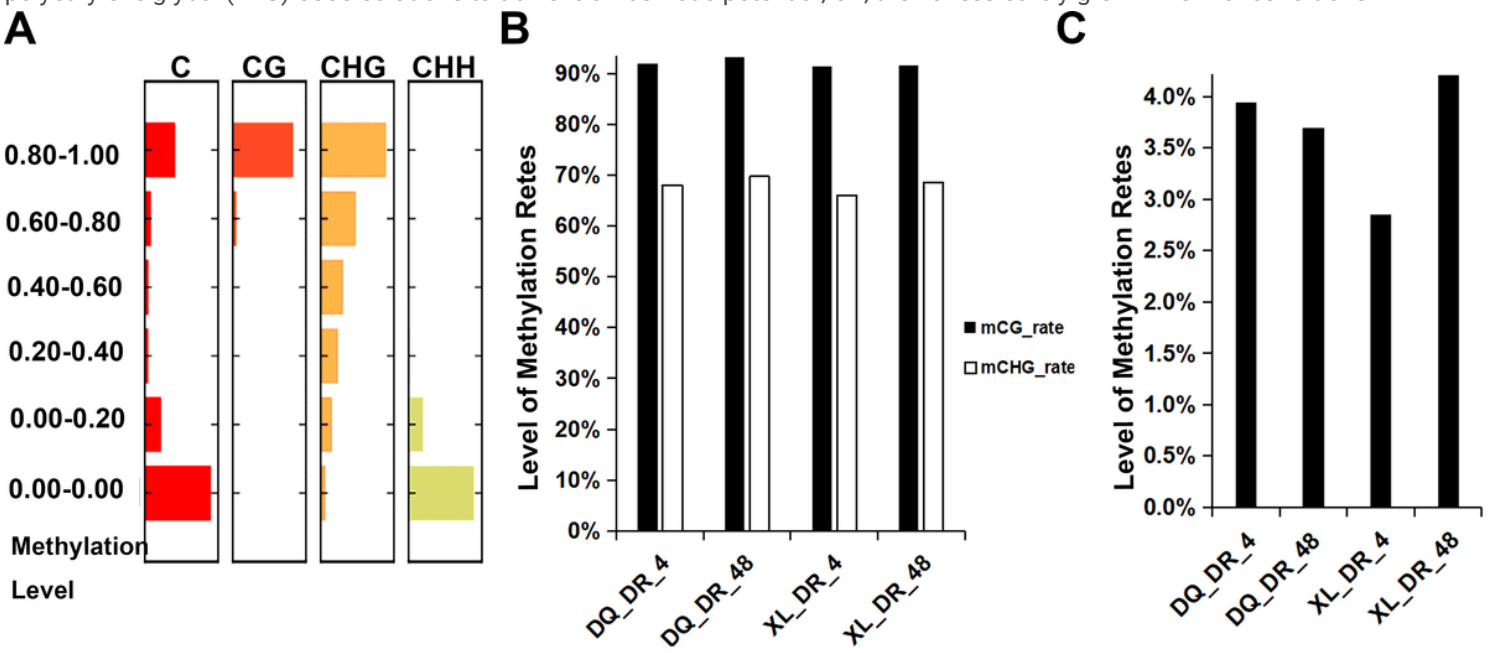

Figure 2

The methylation rates between the drought-tolerant variety (XL) and the drought-sensitive one (DQ). (A). Methylation levels in CG, CHG, and CHH contents; (B). The CG and CHG methylation rates; (C).The CHH methylation rates.

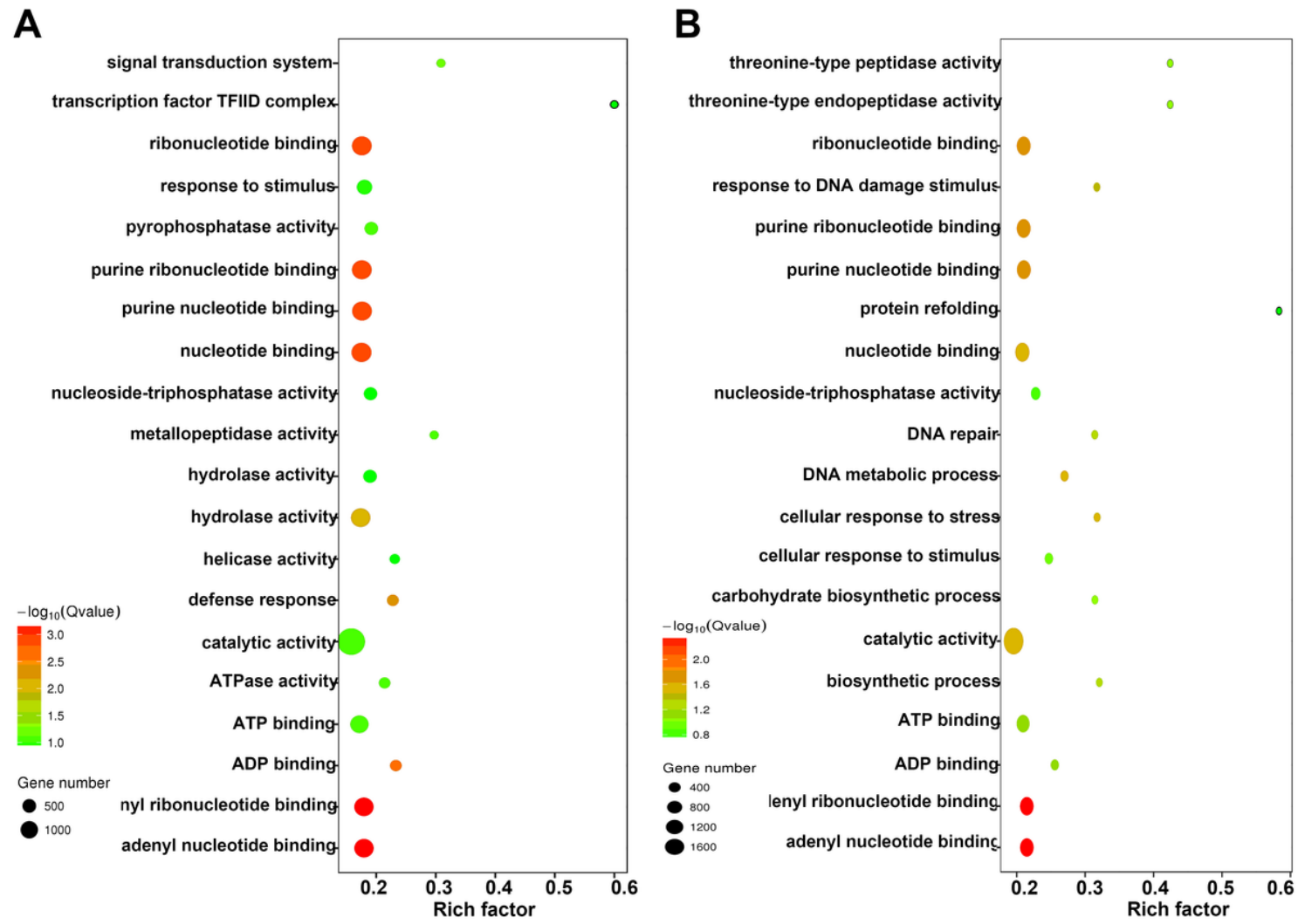

Figure 3

Gene ontology analysis of CG-type related DMR genes within XL in category of DR_48_vs_DR_4(A); gene ontology analysis by CG-type related DMR genes between XL and DQ exposed to drought stress.

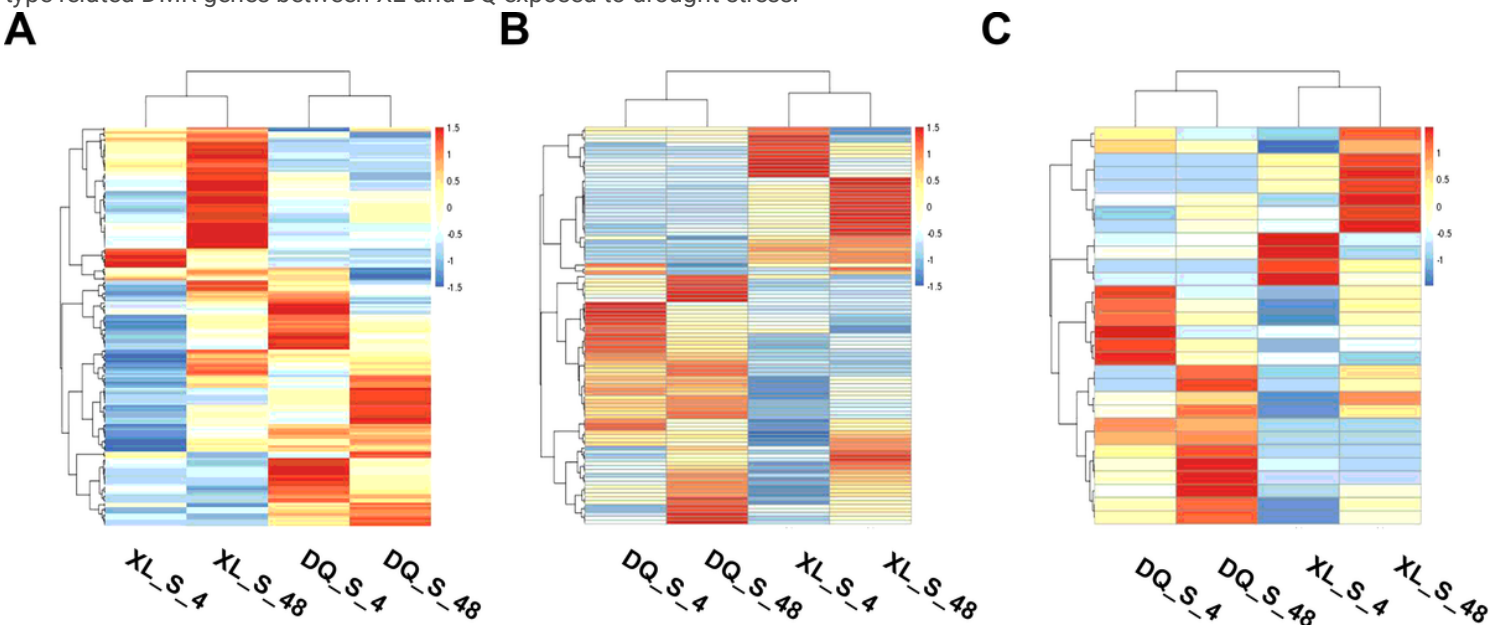

Figure 4

The expression profiles of CG-type (A), CHG-type (B) and CHH-type (C) related genes between drought-tolerant varieties XL and drought-sensitive one DQ. XL:drought-tolerant variety hulless barely; DQ: the drought-sensitive hulless barely; CK: hulless barely were exposed to normal conditions; S: hulless barely were exposed to drought conditions.

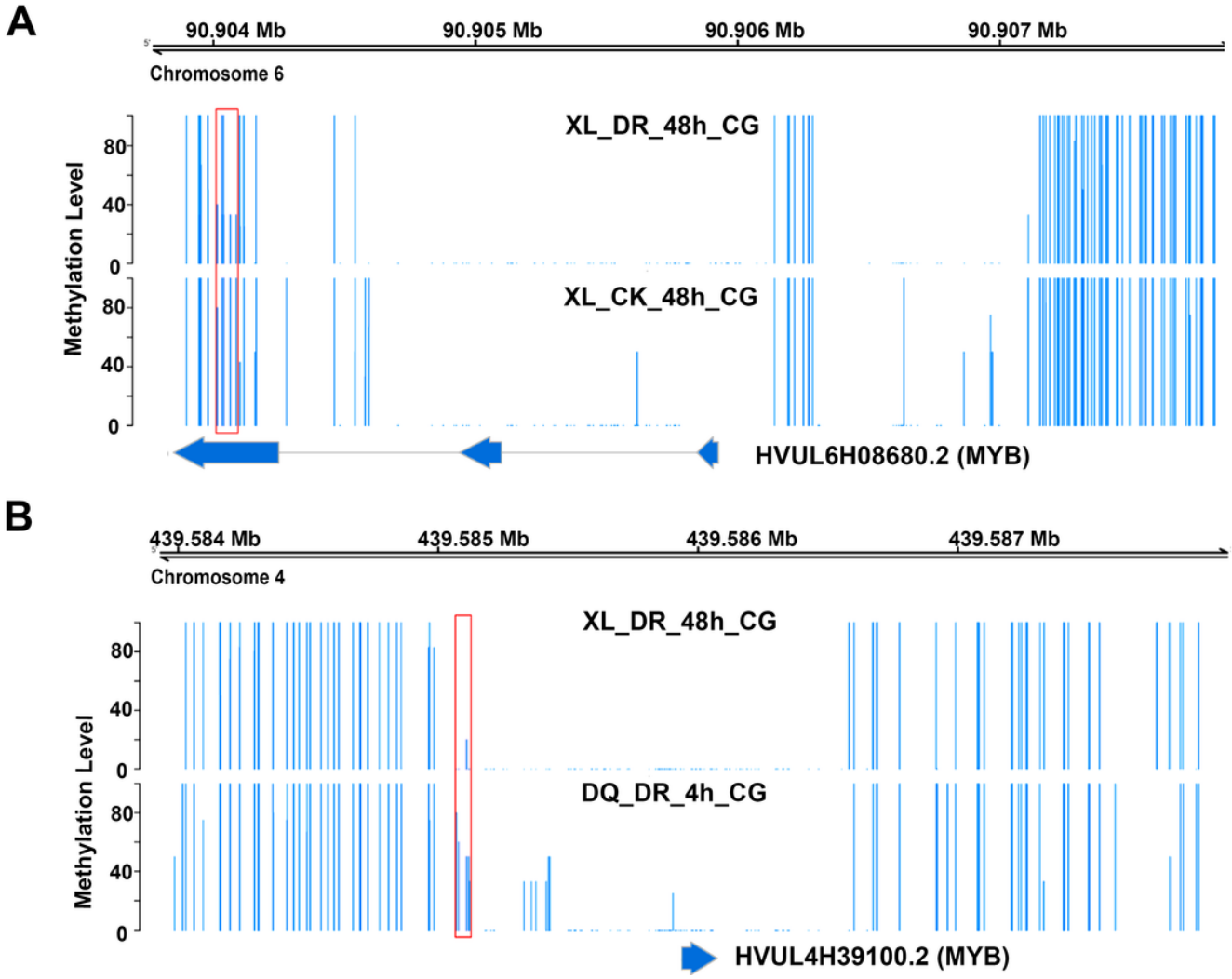

Figure 5

Methylation status of the specific DMR transcription factors. (A) Methylation status of HVUL6H08680.2; (B). Methylation status of HVUL4 h39100.2; XL:drought-tolerant variety hulless barely; DQ: the drought-sensitive hulless barely; CK: hulless barely were exposed to normal conditions; DR: hulless barely were exposed to drought conditions.

Supplementary Files

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