Response of wheat DREB transcription factor to drought stress based on DNA methylation

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Abstract

Background: The growth and development of wheat are seriously influenced by drought, dehydration responsive element binding protein (DREB) plays an important role in the response of plant to drought stress, but epigenetic regulation for gene expression of DREB transcription factor is less studied, especially the regulatory role of DNA methylation has not been reported.

Results: In this research, *DREB2*, *DREB6* and *Wdreb2* were cloned from wheat AK58, and one 712-bp intron was identified in *DREB6*. Although AP2/EREBP domains of DREB2, DREB6 and Wdreb2 showed 73.25% identity, they belong to different types of DREB transcription factor. Under drought stress, different transcript expression patterns of *DREB2*, *DREB6* and *Wdreb2* were observed, and their expression had tissue specificity, was obviously higher in leaves. Promoters of *DREB2*, *DREB6* and *Wdreb2* were further studied, some elements related to stresses were found, and the promoters of *DREB2* and *Wdreb2* were slightly methylated, but *DREB6* promoter was moderately methylated. Compared with the control, the level of promoter methylation in *DREB2* and *DREB6* decreased after 2 h stress treatment, and then increased, which was opposite in *Wdreb2* promoter, the status of promoter methylation in *DREB2*, *DREB6* and *Wdreb2* also had significant changes under drought stress. Further analysis showed that promoter methylation of *DREB6* and *Wdreb2* was negatively correlated with their expression, especially in *Wdreb2*.

Conclusions: Our data suggest the different functions of *DREB2*, *DREB6* and *Wdreb2* in response to drought stress, and demonstrate the strong effects of promoter methylation on the regulation of *Wdreb2* and *DREB6* gene expression.

Background

Drought is one of major abiotic stresses, not only affects the growth and development of plants, but also severely restricts the sustainable production of agricultural biomass [1]. In order to adapt or resist to adverse environment, plants usually respond morphologically, physiologically and molecularlly, such as regulating the expression of stress-resistant genes [2-5]. Plant resistances are generally controlled by multiple genes, it is well known that one transcription factor can regulate the expression of multiple functionally related genes, and many transcription factors are related to resistant response of plants, such as DREB, bZIP, MYB and WRKY [6].

DREB transcription factor belongs to AP2/EREBP family, contains one AP2/EREBP domain which is composed of about 60 amino acid residues with the conserved element YRG and RAYD [7]. Through the AP2/EREBP domain, DREB transcription factor could specifically bind to dehydration responsive element/C-repeat (DRE/CRT) (core sequence: 5'-CCGAC-3'), this binding is involved in regulating the expression of genes related to abiotic stress response, such as high salt, low temperature and drought, which would enhance the resistances of plants to adverse stress [6]. *Arabidopsis* DREB1/CBF can regulate the expression of *rd29A*, *erd10*, *cor6.6*, *cor15a*, *rd17* and other stress-resistant genes to drought, low temperature and so on [8]. Overexpression of *DREB1A* in transgenic *Arabidopsis* also could enhance

the expression of its downstream target genes, which increased the drought tolerance of transgenic *Arabidopsis* significantly [9]. So far, many genes encoding DREB transcription factor have been identified from *Arabidopsis*, *Maize*, *Soybean*, *Sesame*, etc., their expression could be induced and would accumulate rapidly in a short time under abiotic stress [10]. However, the mechanisms on the regulation of *DREB* gene expression are less studied, especially on epigenetic regulations.

DNA methylation is a major epigenetic modification, and has a vital function in the growth and development of plants [11]. DNA methylation in plants is easily affected by physiological status, developmental stage and environmental factors [12]. Under drought stress, the state of DNA methylation would change in plants, methylation level and pattern were significantly different [13]. Fan et al. found that methylation level of *Dendrobium huoshanense* decreased, and methylation polymorphism gradually increased along with the increase of drought stress [14]. Methylation level of *Ryegrass* also decreased under drought stress, and the expression of demethylated related genes was up-regulated [15]. Some studies indicated that the improved resistance of plants to stresses was connected with the involvement of DNA methylation in regulating the expression of stress-resistant genes [16]. For example, the physiological processes of *Rice* were related to DNA methylation in response to drought stress [13], the modification of DNA methylation status is closely connected with drought resistance of *Fraxinus* hybrid trees [17]. Furthermore, methylation or demethylation of genes in plants can cause the difference of gene expression under drought stress [18], which would result in drought escape or tolerance [19].

Wheat (*Triticum aestivum* L.) belongs to Gramineae family, is rich in starch, protein, sugar and other substances, and is one of main food crops. In recent years, drought is the significant reason restricting the sustainable increase of wheat production [20]. However, studies on the response of wheat to drought stress are usually confined to phenotype, structure, physiology and biochemistry, or stress-resistant genes [21]. The epigenetic regulation of wheat response to drought stress is rarely involved, especially the regulatory role of DNA methylation in DREB transcription factor under drought stress. In this study, three members of DREB family in wheat AK58 were identified, the expression and promoter methylation of *DREB* genes were analyzed under drought stress. Thus, this investigation is beneficial to explore the regulatory mechanism of DNA methylation in the response of plants to drought stress.

Results

Cloning and sequence analysis of *DREBs*

As shown in Fig. S1, CDS sequences of *DREB2*, *DREB6* and *Wdreb2* in wheat AK58 comprise 732 bp, 837 bp and 1035 bp respectively, *DREB2* and *Wdreb2* have no intron, but one 712-bp intron was found in *DREB6*. A typical AP2/EREBP domain was identified in all deduced protein sequences of three *DREB* genes using the NCBI CD-search tool (Fig. S1). AP2/EREBP domain contains YRG and RAYD conserved modules with three β folds and one α helix, valine (V) and glutamate (E) are highly conserved at 14th or 19th residue of AP2/EREBP domain (Fig. 1, a). The amino acid sequences of DREB2, DREB6 and Wdreb2 were further compared and analyzed, despite the low overall sequence similarity among three DREBs

(33.24% identity) (Fig. 1, b), AP2/EREBP domains in three DREBs had 73.25% identity, even up to 83.93% between DREB6 and Wdreb2 (Fig. 1).

The deduced protein sequences of DREB2, DREB6 and Wdreb2 from wheat AK58 were also aligned with their homologous sequences, the sequence identity between wheat DREB2 and *Aegilops tauschii* ERF was 95%, but only around 60% identity was determined between wheat DREB2 and other homologous sequences (Table 1), and AP2/EREBP domain of wheat DREB2 was the same to that of *Aegilops tauschii* ERF (Fig. S2, a). As listed in Table 1, wheat DREB6 showed higher similarity to some homologous sequences (97% identity or so), its AP2/EREBP domain had higher identity with that of *Aegilops biuncialis* DREB2, *Agropyron mongolocum* AP2/EREBP, *Dasypyrum villosum* DREB and *Leymus multicaulis* DREB2 (Fig. S2, b). The higher sequence similarity (99% identity) was observed between wheat Wdreb2 and *Aegilops tauschii* DREB2B, wheat Wdreb2 also exhibited about 94% identity to *Aegilops speltoides* DREB1, *Triticum turgidum* DRF, *Triticum dicoccoides* DREB or *Triticum urartu* DREB2B (Table 1). Furthermore, AP2/EREBP domain of Wdreb2 was identical to that of *Aegilops tauschii* DREB2B and *Triticum turgidum* DRF 1 (Fig. S2, c).

Table 1 Homologous amino acid sequences of wheat DREBs by BLASP

	Protein	Accession Number	Identity
DREB2	*Aegilops tauschii* ERF	XP-020183719.1	95%
	Setaria italica ERF	XP-004968548.2	66%
	Oryza sativa TINY	XP-015644400.1	64%
	Brachypodium distachyon ERF	XP-010233006.1	64%
	Dichanthelium oligosanthes DREB3	OEL19602.1	61%
	Sorghum bicolor TINY	XP-002454993.1	61%
	Zea mays TINY	XP-020398183.1	59%
DREB6	*Thinopyrum elongatum* AP2/EREBP	AEI98920.1	98%
	Triticum aestivum DREBW73	AAY44604.1	98%
	Agropyron mongolocum AP2/EREBP	AJD80690.1	94%
	Aegilops biuncialis DREB2	CBX87024.1	97%
	Leymus multicaulis DREB2	AFO12475.1	97%
	Thinopyrum bessarabicum DREB	AIY22662.1	96%
	Dasypyrum villosum DREB	AIY22669.1	97%
Wdreb2	*Aegilops tauschii* DREB2B	XP-020156298.1	99%
	Triticum aestivum DREB5B	AAX13287.1	99%
	Aegilops speltoides DREB1	AC035588.1	96%
	Triticum turgidum DRF	AFO10996.1	95%
	Triticum aestivum DREB4B	AAX13283.1	94%
	Triticum dicoccoides DREB	ADM93284.1	93%
	Triticum urartu DREB2B	EMS45041.1	93%

The homologous sequences of wheat DREB2, DREB6 and Wdreb2 were retrieved with BLASTP algorithm, which are mainly from some species in Gramineae. The homologous sequences having higher identity with wheat DREBs were selected from some Genera in Gramineae, and were further analyzed.

The expression patterns of *DREBs* in wheat

As shown in Fig. 2, the expression levels of *DREB2*, *DREB6* and *Wdreb2* in leaves were significantly higher than that in roots except for the expression of *Wdreb2* in seedlings stressed for 2 h, in which more *Wdreb2* transcripts were accumulated in roots ($P<0.05$). Under drought stress, the expression of *DREB2*, *DREB6* and *Wdreb2* altered, and had its own unique expression profile (Fig. 2).

The expression of *DREB2* displayed similar trends in roots and leaves (Fig. 2, a), *DREB2* transcript abundance increased to higher level after stressed for 2 h ($P<0.01$), then decreased, and was lower as stressed for 8-10 h, which was still higher in leaves than the control ($P<0.05$). A significant rise in *DREB6* transcript level was also observed after stressed for 2 h ($P<0.01$), yet *DREB6* subsequently showed the declined expression, which was significantly lower in leaves than the control as stressed for 8-12 h (Fig. 2, b). As shown in Fig. 2 (c), under drought stress, *Wdreb2* was up-regulated in roots, especially after stressed for 2 h ($P<0.01$). Compared with the control, *Wdreb2* transcript level in leaves altered significantly, and increased as stressed for 6-8 h, especially stressed for 12 h ($P<0.01$).

Promoter analysis of wheat *DREB* genes

In this study, the promoters of *DREB2*, *DREB6* and *Wdreb2* were cloned, and submitted to GenBank (MT974473: 1735 bp, MT974471: 1792 bp, MT974472: 649 bp). As shown in Fig. 3 and Table S1-S3, the promoters of wheat *DREB* genes contain basic regulatory elements, such as TATA-box, CAAT-box, there are 13, 10 and 5 TATA-boxes in the promoters of *DREB2*, *DREB6* and *Wdreb2*, respectively. Many elements related to stresses were also found in the promoters of *DREB2*, *DREB6* and *Wdreb2*, such as drought response element DRE/CRT, low temperature response element LTR, abscisic acid response element ABRE, light response element GAG-motif, drought-induced element MYB binding sites, etc. (Fig. 3, Table S1-S3).

Further analysis showed that there were some unique elements in the promoters of *DREB2*, *DREB6* and *Wdreb2*. For example, light response element MNF, leaf development element HD-ZIP and meristem specificity element OCT are specifically present in the promoter of *DREB2* (Table S1). A series of specific functional elements were also identified in the promoter of *DREB6*, such as ethylene response element ERE, fungal elicitor response element W-box and MeJA regulatory element CGTCA-motif (Table S2). Moreover, root specificity elements as1, zein metabolism regulation element O2-site, light response element C-box, and CE3 element involved in ABA and VP1 reactions were detected in the promoter of *Wdreb2* (Table S3).

Promoter methylation analysis of *DREB* genes

The distribution of CpG island in the promoter regions of whet *DREBs* were predicated and analyzed using EMBOSS CpG Plot. One CpG island is present in the promoter of *DREB2*, *DREB6* or *Wdreb2*, its

length is 234 bp, 436 bp and 559 bp, respectively (Fig. S3). These putative CpG islands are preceded by some functional elements, such as abscisic acid responsive element, light responsive element, low-temperature responsive element and so on (Fig. 3, Table S1-S3).

Some CpG island regions with higher CG percent were further identified in wheat leaves using bisulfite sequencing PCR (BSP) (Fig. S3). As shown in Fig. 4 and Table 2, the majority of methylation sites were in CHH context among the three sequence contexts (CG, CHH and CHG, H = A, T, or C) in the promoter regions of *DREB2*, *DREB6* and *Wdreb2*, but DNA methylation had a strong preference to CG context. In the promoter region of *DREB2*, methylation in CHH site was not detected, methylation rates of CG and CHH sites were 2.38% and 1.03%, belonging to mild methylation (<20%) (Fig. 4, a; Table 2). Fig. 4 (b) and Table 2 showed dense methylation (>60%) at CG site (88.08%), moderate methylation (>20%) at CHG site (51.36%) and mild methylation at CHH site (4.93%) in the promoter region of *DREB6*. In the promoter region of *Wdreb2*, methylation rates of CG, CHG and CHH sites were 1.89%, 1.0% and 0.29%, respectively, which were all mildly methylated (Fig. 4, c; Table 2).

Table 2 Methylation analysis of promoter regions in wheat *DREB* genes

Gene	Pattern	Pattern frequency (%)	Methylation rate (%)	Total Methylation rate (%)
DREB2	CG	19.09	2.38	1.17
	CHG	11.82	0.00	
	CHH	69.09	1.03	
DREB6	CG	25.93	88.08	31.89
	CHG	11.11	51.36	
	CHH	62.96	4.93	
Wdreb2	CG	29.60	1.89	0.88
	CHG	16.00	1.00	
	CHH	54.40	0.29	

Methylation level of *DREB* promoters under drought stress

Under drought stress, cytosine methylation altered in the promoter regions of *DREB2*, *DREB6* and *Wdreb2* from wheat leaves (Fig. 5). Compared with the control, methylation rate at CG site in the promoter region of *DREB2* decreased obviously as stressed for 2 h (0.5%) and 10 h (1.42%) (P<0.01), but methylation rates at CHG and CHH sites increased significantly after stressed for 10 h (P<0.01). The overall methylation level of *DREB2* promoter was significantly lower as stressed for 2 h but higher as stressed for 10 h than that in untreated leaves (Fig. 6, a).

As shown in Fig. 6 (b), after stressed for 2 h, methylation rates at CG and CHG sites in the promoter of *DREB6* decreased significantly, but the promoter region of *DREB6* was still heavily CG cytosine methylated (>60%) and moderately CHG cytosine methylated (>20%). The significantly increased methylation level of *DREB6* promoter was observed as stressed for 12 h, which occurred in all three contexts (CG, CHG and CHH). Fig. 6 (c) displayed the changes in promoter methylation of *Wdreb2* under

drought stress. Compared with the control, the overall methylation level was significantly higher as stressed for 2 h (P<0.01), and methylation rates at CG, CHG and CHH sites were respectively 2.16%, 1.5% and 1.02% , however longer duration of treatment (12 h) led to the decreased methylation level (P<0.01).

Methylation status in *DREB* promoters under drought stress

As listed in Table 3, methylation status in the promoter regions of *DREB2*, *DREB6* and *Wdreb2* were significantly changed under drought stress. The demethylation at 3 CG sites and 1 CHH site in the promoter of *DREB2* were detected, and longer duration of stress treatment resulted in enhanced hypermethylation of *DREB2* promoter at CG, CHG and CHH sites

Table 3 Methylation patterns in promoter regions of wheat *DREB*s under drought stress

Gene	Type of cytosine	No. of cytosine	No. of methylation site			
			Hypermethylation site		Demethylation site	
			CK-T1	CK-T2	CK-T1	CK-T2
DREB2	CG	21	1	2	3	3
	CHG	13	0	1	0	0
	CHH	76	2	3	1	1
DREB6	CG	34	0	1	1	1
	CHG	15	1	1	0	1
	CHH	84	8	10	7	8
Wdreb2	CG	37	0	1	1	2
	CHG	20	0	0	1	1
	CHH	68	2	1	2	2

CK-T1 and CK-T2 represent the change of methylation status in the promoter regions of wheat *DREB* genes under the stress of 15% PEG_{6000} solution as compared to the control (CK). T1 and T2 denote methylation status in the promoter region of *DREB6* or *Wdreb2* after wheat seedlings had been stressed for 2 h and 12 h, respectively, or represented methylation status in the promoter region of *DREB2* as stressed for 2 h and10 h.

After stressed for 2 h, 8 CHH sites and 1 CHG site were hypermethylated in the promoter of *DREB6*, 7 CHH sites and 1 CG site were demethylated. 12 h later, the increase in hypermethylation of *DREB6* promoter was obseved at CG and CHH sites, and the increase in demethylation was observed at CHG and CHH sites (Table 3). Compared to CG and CHG sites, methylation status of CHH site in *Wdreb2* promoter was affected more strongly by drought stress (Table 3). For example, 2 CHH sites were hypermethylated and demethylated after stressed for 2 h, respectively, 1 CHH site in hypermethylation status and 2 CHH sites in demethylation status as stressed for 12 h.

Correlation analysis between promoter methylation and expression of *DREB*

In order to explore the correlation between promoter methylation and expression of wheat *DREB2*, *DREB6* and *Wdreb2* under drought stress, the relative expression levels of *DREB*s in wheat leaves and methylation rates at CG, CHG or CHH sites in their promoter regions were respectively analyzed

by Pearson correlation coefficient. As listed in Table S4, Pearson coefficient r between expression level of *Wdreb2* and methylation rates at CG, CHG and CHH sites was respectively -0.986, -0.973 and -0.878, indicating that the significant negative correlation existed between promoter methylation and gene expression of *Wdreb2*. Similarly, promoter methylation and expression level of *DREB6* was negatively correlated (Table S4). Although the significant negative correlation existed between expression of *DREB2* and methylation rate of CG or CHG (Table S4), but promoter methylation of *DREB2* had no negative correlation with its expression as stressed for 10 h (Fig. 2, a; Fig. 6, a).

Discussion

DREB transcription factors play an important role in the response of plants to drought stress, could specifically bind to DRE/CRT element in the promoter of stress-responsive genes, and then would enhance the response or tolerance of plants to adverse environmental conditions [6]. DREB transcription factor is characterized by AP2/EREBP domain, which is composed of about 60 amino acid residues and contains two conserved regions of YRG and RAYD [7]. In this study, *DREB2*, *DREB6* and *Wdreb2* were cloned from wheat AK58, and one 712-bp intron was found in *DREB6*, AP2/EREBP domains of DREB2, DREB6 and Wdreb2 had 73.25% identity, the amino acids at 14 th and 19 th of AP2/EREBP domain were conservatively V and E, respectively. However, the similarity was lower among the full-length nucleotide sequences or amino acid sequences of *DREB2*, *DREB6* and *Wdreb2*. BLASTP analysis further revealed that DREB2, DREB6 and Wdreb2 were different types of DREB transcription factors and might respectively belong to DREBA-4 class, DREB-2 class and DREB-1 class [22, 23].

Under abiotic stresses, such as drought, low temperature, high salt, etc., the expression of DREB transcription factor would change [24, 25]. In this study, the expression of *DREB2*, *DREB6* and *Wdreb2* also altered under drought stress and generally accumulated to the higher level after stressed for 2 h, but they showed different expression trends along with the increase of stress time. The expression levels of *DREB2*, *DREB6* and *Wdreb2* were also different, which was similar to other research [26]. Further analysis showed that the expression of *DREB2*, *DREB6* and *Wdreb2* had tissue specificity, the accumulation of *DREBs* transcripts was obviously higher in leaves than that in roots, which was also found in other research [27]. The expression of *DREB* in *Daucus carota* showed tissue specificity too, the main role of *DcDREB-A1-1* and *DcDREB-A1-2* was in leaves and roots, respectively [28].

It is well known, the *cis*-acting regulatory elements in the promoters provide the possibility for the transcription and expression of genes [29]. There are some *cis*-acting elements related to stresses in plant promoter, such as DRE/CRT, ERE, ABRE, LTR and so on [30]. Except typical regulatory elements, TATA-box and CAAT-box, the promoters of *DREB2*, *DREB6* and *Wdreb2* in wheat AK58 contain DRE/CRT, LTR, ABRE, drought-induced MYB binding site, etc, which demonstrates that the expression of *DREB2*, *DREB6* and *Wdreb2* may be influenced by adverse environmental factors. Furthermore, some studies found that DNA methylation could regulate the expression of stress-responsive genes, and play an important role in the response of plants to adverse stress [16], especially promoter methylation had more significant effects on gene expression [31]. In the promoter regions of *DREB2*, *DREB6* and *Wdreb2*, CpG islands with a variety of

cis-acting elements were detected, BSP analysis showed that there were more CHH sites and less CHG sites in the promoter regions of *DREB2*, *DREB6* and *Wdreb2*, but the methylation rates at CG sites were the highest.

Many studies have found that the degree and state of DNA methylation in plants would change under drought stress, low temperature, high salt and other conditions [32, 33], especially the change of methylation state in the promoter of genes [34]. Under drought stress, methylation level altered in the promoter regions of *DREB2*, *DREB6* and *Wdreb2*. Compared with the control, methylation level in *DREB2* and *DREB6* promoters decreased after stressed for 2 h, then increased along with the increase of stress time, which was opposite in *Wdreb2* promoter. Furthermore, methylation status in the promoter regions of *DREB2*, *DREB6* and *Wdreb2* had significant change under drought stress, such as demethylation and hypermethylation. Zilberman also found that gene expression could be promoted or inhibited by the demethylation and hypermethylation of promoter, respectively [35].

Further analysis showed that promoter methylation of *DREB6* and *Wdreb2* was negatively correlated with their expression by Pearson coefficient, especially in *Wdreb2*, which was also found in other studies [35, 36]. Although the promoters of *DREB2* and *Wdreb2* with low methylation level was both slightly methylated, the expression of *Wdreb2* was significantly higher than that of *DREB2*, indicating that promoter methylation might have little effect on gene expression of *DREB2* and its promoter possibly belongs to low CpG-contain promoter. Similarly, the promoters of *z1B4* and *z1B6* in *Zea mays* were almost not methylated [37], DNA methylation was not also found in the promoters of some genes in *Arabidopsis* or tomato and only occurred in their coding regions [38, 39]. In addition, one CpG island was also predicted in the coding region of *DREB2*, *DREB6* and *Wdreb2*, and the CpG island almost covered the whole coding region of *DREB2*. However, it is unclear about the relationship between DNA methylation in the coding region and gene expression of wheat *DREB* genes, the mechanism of DNA methylation regulating the expression of wheat *DREB* genes needs to be further studied.

Conclusions

In this study, *DREB2*, *DREB6* and *Wdreb2* were identified in wheat AK58, and one 712-bp intron was found in *DREB6*. Under drought stress, the expression patterns of *DREB2*, *DREB6* and *Wdreb2* were different, and their expression level was obviously higher in leaves. Some elements related to stresses were also found in the promoter regions of *DREB2*, *DREB6* and *Wdreb2*, further analysis showed that promoter methylation of *DREB6* or *Wdreb2* was negatively correlated with their expression, especially was significant in *Wdreb2*. Therefore, *DREB2*, *DREB6* and *Wdreb2* in wheat might function differently in response to drought stress, and promoter methylation had more significant effects on gene expression of *Wdreb2* and *DREB6*, which would be helpful to reveal the regulatory mechanism of DNA methylation in plant response to drought stress.

Methods

Cultivation and treatment of wheat seedlings

In this study, seeds of wheat AK58 were kindly provided by Xinxiang Academy of Agricultural Science, Henan, China. Cultivation of wheat seedlings was performed according to Duan et al. [33], wheat seeds were firstly surface-sterilized for 10 min by 0.1% $HgCl_2$, and then were washed for 50 min in sterile water. Subsequently, sterilized seeds were sown in pots (with a diameter of 15 cm) containing nutrition soil and vermiculite (1:1), were cultured at 24 ± 1 ℃ with 45% relative humidity in 14 h photoperiod of 50 μmol m^{-2} s^{-1} light intensity, and were irrigated with 5 ml distilled water every two days.

At the three-leaf stage, wheat seedlings were exposed to 15% PEG_{6000} solution for 2 h, 6 h, 8 h, 10 h and 12 h. Roots and leaves of PEG_{6000}-treated and untreated wheat seedlings were collected, immediately freezed with liquid nitrogen and then stored at -80 ℃.

Extraction of genomic DNA

Genomic DNA was extracted from roots or leaves of wheat seedlings by cetyltriethyl ammonium bromide (CTAB) method [40]. The yield and purity of genomic DNA were determined at 260 nm with micro-spectrophotometry, the integrity of genomic DNA was detected by 0.8% agarose gel electrophoresis. Subsequently, genomic DNA from wheat seedlings was stored at -20 °C.

Isolation and reverse transcription of RNA

Total RNA from roots or leaves of wheat seedlings was extracted using RNAiso Plus kit (TaKaRa, Japan) according to the instructions, DNase treatment and phenol-chloroform extraction were performed to remove DNA, and RNA samples were dissolved in RNase-free dH_2O. The integrity of total RNA was verified by 1.0% agarose gel electrophoresis, the yield and purity of total RNA was determined with UV spectrophotometer. RNA samples were used for the generation of cDNA or stored at -80 °C. First strand cDNA was synthesized with the HiScript II Q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, China).

Cloning and *in silco* analysis of *DREB* genes

In order to clone *DREB* genes from wheat AK58, specific primers were designed according to the sequences of wheat *DREB2* (GU785008), *DREB6* (AY781361) and *Wdreb2* (AB193608), and were listed in Table S5. Genomic DNA and cDNA of wheat AK58 were used as the amplification templates to obtain DNA or cDNA sequence of *DREB* genes.

In this experiment, PCR reaction system was composed of 2.0 μl DNA template, 1.0 μl each primer (10 μM), 10.0 μl 2x Taq Mix and 6.0 μl ddH_2O. The following PCR procedure was used for the amplification: 95° C for 5 min, followed by 35 cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min), finally extended at 72 °C for 5 min. After PCR amplification products were detected by 1.0% agarose gel electrophoresis, target fragments were obtained by gel extraction and recycling, and then were sequenced in Vazyme (Nanjing, China).

*In silic*o analysis of target sequences was performed as described below, extrons, introns and ORFs of wheat *DREB* genes were analyzed with ProtParam, the conserved domains of amino acid sequences encoded by wheat *DREB* genes were analyzed by CD-search in NCBI, homologous sequences were retrieved with BLASTP algorithm from GenBank® (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the alignments on amino acid sequences or homologous sequences of wheat DREBs were carried out using DNAMAN.

Fluorescence quantitative real-time PCR

The expression of *DREB* genes in wheat was studied by fluorescence quantitative real-time PCR (qRT-PCR), the internal reference gene was *β-Actin*, and these primers for qRT-PCR were listed in Table S5. qRT-PCR was performed on LightCycler 96 Real-time PCR instrument, cDNA synthesized by reverse transcription of total RNA was used as the template in qRT-PCR.

According to the instructions of AceQ qPCR SYBR Green Master Mix kit (Vazyme, China), qRT-PCR reaction system consisted of 1.0 μl AceQ qPCR SYBR Green Master Mix, 0.5 μl each primer (10 μM), 2.0 μl cDNA template and 16.0 μl ddH_2O. qRT-PCR was performed as the following procedures: pre-denaturation for 5 min at 95 °C, followed by 40 cycles of 95° C for 10 s and 60 °C for 30 s.

The relative expression level of wheat *DREB* genes under drought stress was normalized and analyzed by the comparative Ct ($^{2\text{-}\Delta\Delta ct}$) method [41]. The calculation formula was as follows: Relative expression level $=2^{-\Delta\Delta Ct}$, ΔΔCt (target gene) =ΔCt (treatment group) -ΔCt (control group), ΔCt (target gene) =Ct (target gene) -Ct (reference gene). Furthermore, data were obtained from three biological replicates, and each qRT-PCR experiment was repeated three times.

Isolation and analysis of promoter sequence

The promoter regions were cloned to further analyze expression pattern of *DREB* genes in wheat AK58, specific primers were designed according to the promoter sequences of wheat *DREB2* (GU785008), *DREB6* (HG670306.1) or *Wdreb2* (KF731666), and were listed in Table S5.

The target promoter sequences were amplified in 20-μl PCR reaction mixture consisting of 2.0 μl DNA template, 10.0 μl 2x Taq Mix, 1.0 μl each primer (10 μM) and 6.0 μl ddH_2O. The reaction condition of PCR procedure was 95 °C for 5 min, followed by 40 cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 1.5 min), finally extended at 72 °C for 5 min. PCR amplification products were separated by 1.0% agarose gel electrophoresis, the target fragments were obtained by gel extraction and recycling, then were sequenced in Vazyme (Nanjing, China). PlantCARE and PLACE were used to analyze *cis*-acting elements in the promoter sequences of wheat *DREB* genes.

Methylation analysis of promoter

CpG islands (Island size > 100, GC Percent > 50.0, Obs/Exp > 0.6) in the promoter regions of wheat *DREBs* were predicted and analyzed using EMBOSS CpG Plot. According to the analysis of CpG islands,

amplification primers for bisulfite sequencing PCR (BSP) were designed using MethPrimer, Methyl Primer Expressv1.0 and Primer Premier5.0 (Table S5). The CpG island of *DREB6* promoter was amplified in two parts (region I and region II) because of the limited length of BSP amplification.

In this study, genomic DNA from wheat leaves was firstly treated with EZ DNA Methylation-Lightning^{TM} Kit (Zymo Research, America), and then used as the template in BSP amplification of *DREB* promoters. BSP reaction system was 30.0 μl, and composed of 2.0 μl bisulfite-treated DNA, 1.0 μl each primer (10 μM), 3.0 μl 10×buffer (Mg^{2+}), 1.0 μl dNTP, 1.0 μl Relia™ hot-start Taq polymerized aes and 21.0 μl ddH_2O. PCR amplification procedure was as follows: pre-denaturation at 95 °C for 4 min, followed by 40 cycles (94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s), and final extension at 72 °C for 5 min. PCR amplification products were detected by 1.0% agarose gel electrophoresis, it was found that only target fragments were amplificated, the target fragments were obtained by gel extraction and recycling, and were sequenced by GENEray (Shanghai, China).

In addition, at least 10 clones were sequenced for each target fragment and three biological replicates were set up, the analysis on methylation site, methylation type and methylation rate were performed with CyMATE and Kismeth.

Statistical analysis

Statistical analysis of data was performed in this study, expression level of genes and methylation ratio of promoters were tested by significance level, ANOVA and multiple comparisons of Duncan's multiple range, the correlation between gene expression and promoter methylation was analyzed by Pearson correlation coefficient r of SPSS software.

Abbreviations

BSP: bisulfite sequencing PCR; CTAB: Cetyltriethyl ammonium bromide; DRE/CRT: dehydration responsive element/C-repeat; DREB: dehydration responsive element binding protein; E: glutamate; qRT-PCR: quantitative real-time PCR; V: valine.

Declarations

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Availability of data and material

The datasets generated during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

HYD and YQZ conceived this experiments. YQZ obtained and analyzed experiment data. HHW and WJJ analyzed experiment data and wrote this paper. ZKD, XYW, and QTQ participated in text editing. LNJ, YQZ and HYD revised this manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

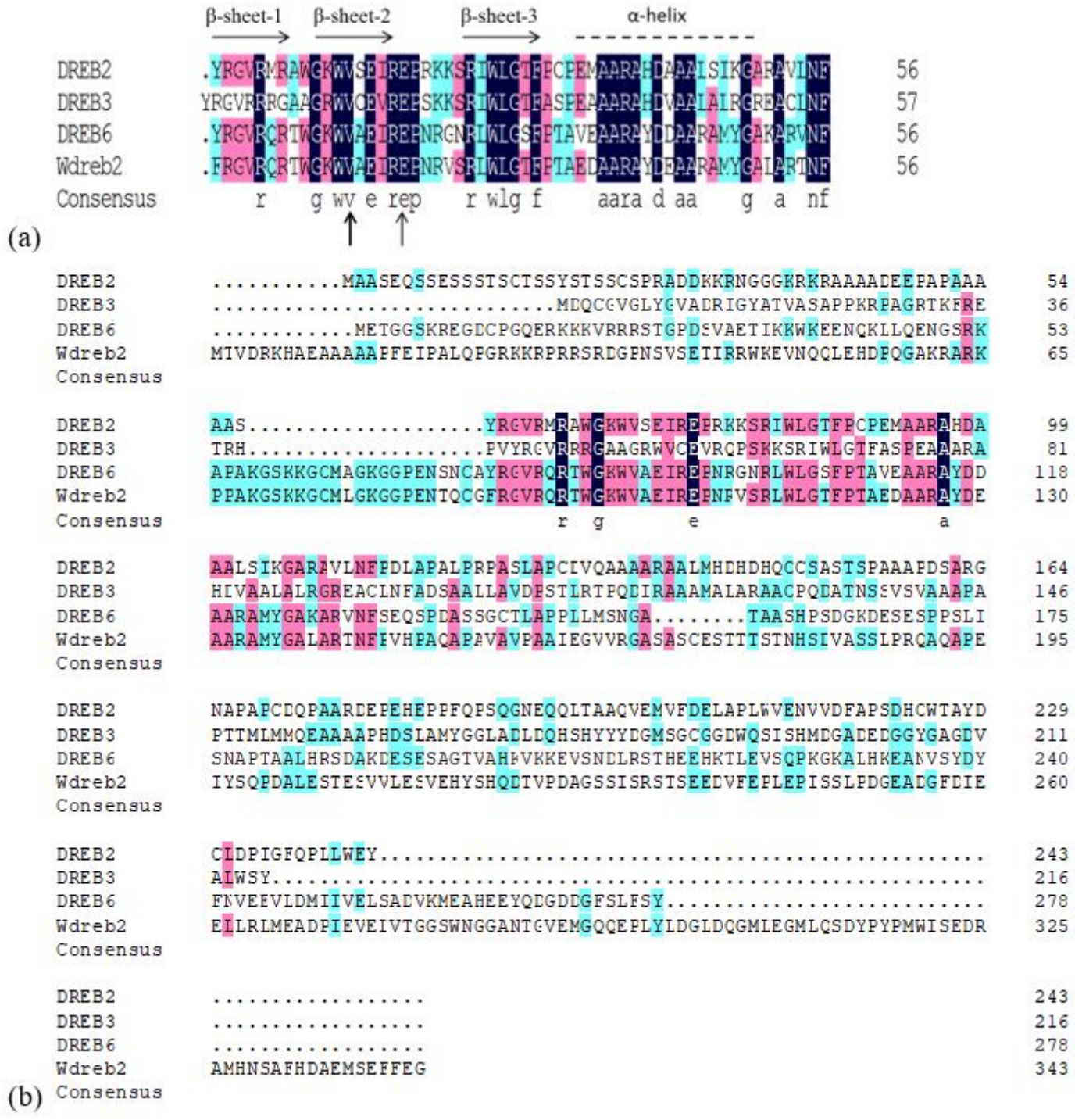

Figure 1

Comparison analysis of wheat DREBs. (a) and (b) represented the alignment on AP2/EREBP domain or amino acid sequence of DREB2, DREB6 and Wdreb2, β fold and α helix were indicated with the arrow or dotted line, respectively.

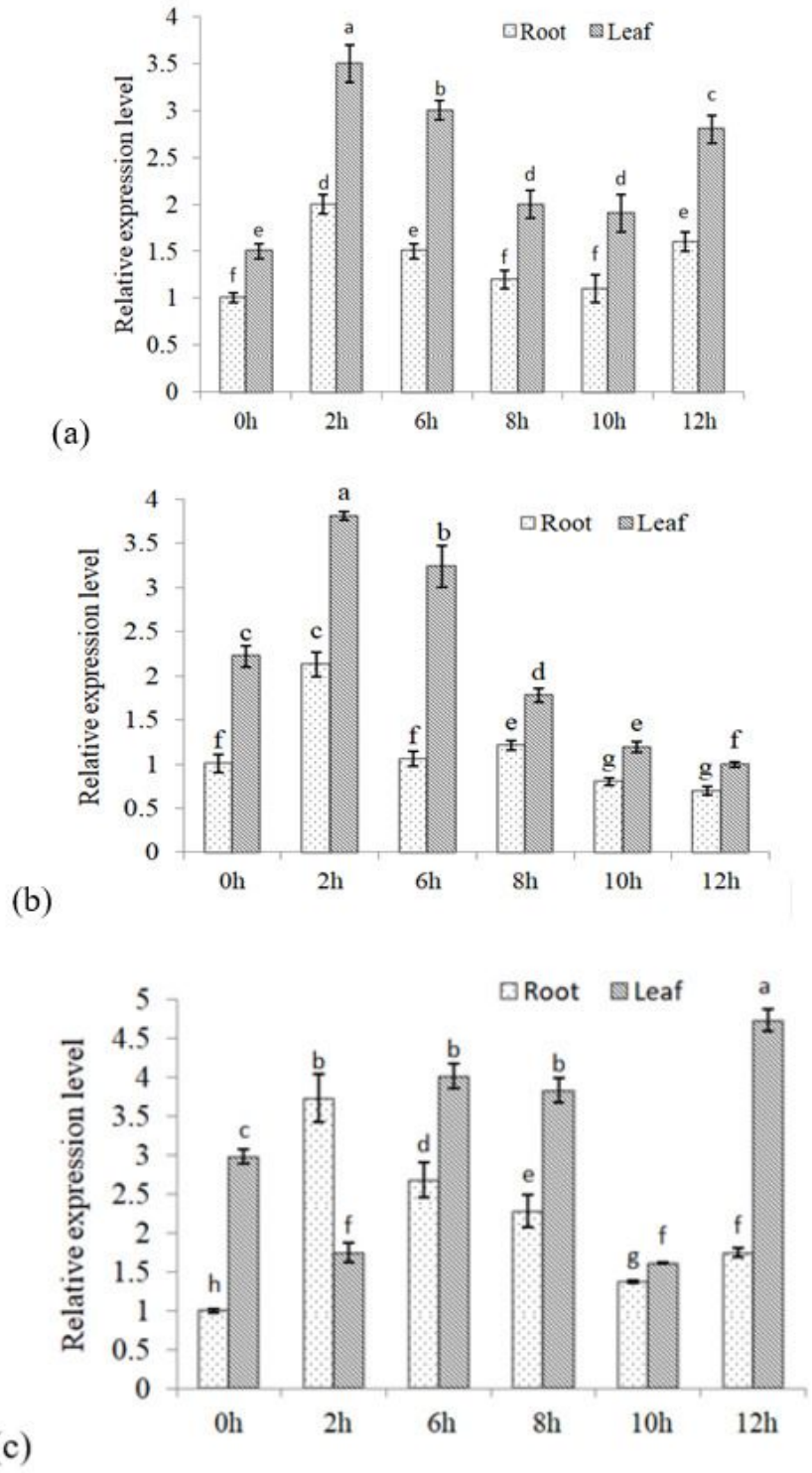

Figure 2

The expression of wheat DREB gene under drought stress. (a), (b) and (c) represented the expression of DREB2, DREB6 and Wdreb2 in wheat seedlings treated with 15% PEG6000 solution, 0h, 2h, 6h, 8h, 10h and 12h indicated the number of hours after treatment. Furthermore, three biological replicates were set up, the error bar was standard error of mean, and the different lowercase letters above bars represented significant different among treatments of drought stress ($P < 0.05$).

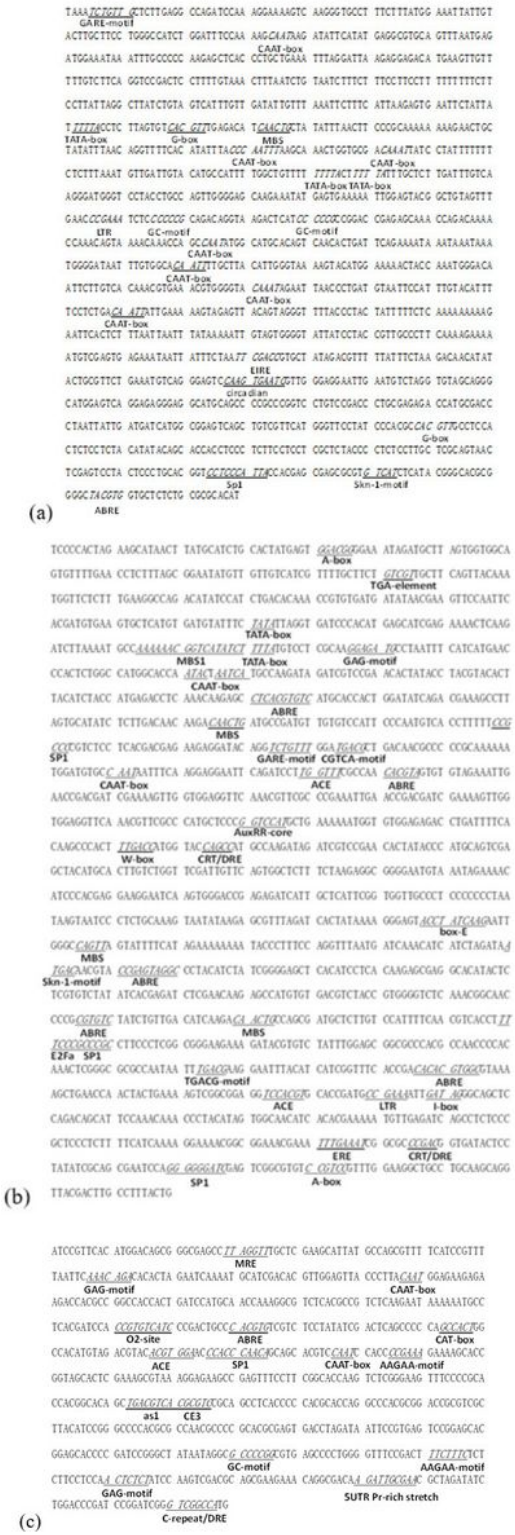

Figure 3

Promoter information of wheat DREB gene. (a), (b) and (c) represented the promoter sequence and partial cis-acting elements of DREB2, DREB6 or Wdreb2, respectively.

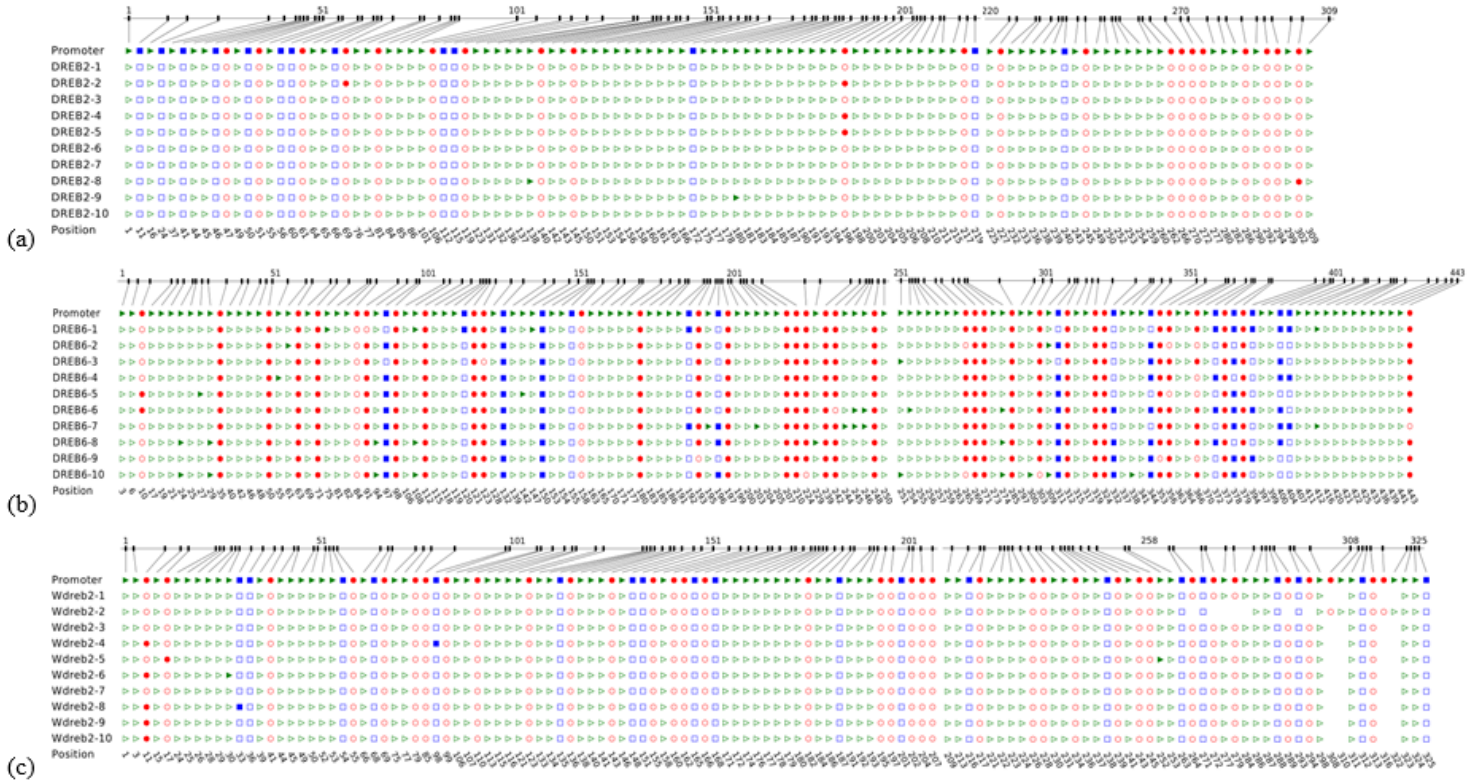

Figure 4

Bisulfite sequencing result for the promoter region of wheat DREB gene using CyMATE. (a), (b) and (c) represented methylation sites in the promoter region of DREB2, DREB6 and Wdreb2 from wheat leaf. Red circles, blue squares and green triangles represented CG, CHG or CHH, filled and hollow circles denoted methylated and unmethylated cytosine, respectively, and each row represented the sequencing result of one positive clone. In addition, numbers along the top show the length of CpG island sequence, and numbers along the bottom indicate individual potential methylation sites.

Figure 5

Methylation profile in the promoter region of wheat DREB gene under drought stress. (a), (b) and (c) represented cytosine methylation maps for the promoter sequence of DREB2, DREB6 and Wdreb2 in wheat seedlings stressed with 15%PEG6000 solution. Red, blue and green circles represented CG, CHG or CHH, filled and hollow circles denoted methylated and unmethylated cytosine, respectively. Each row represented the sequencing result of one positive clone.

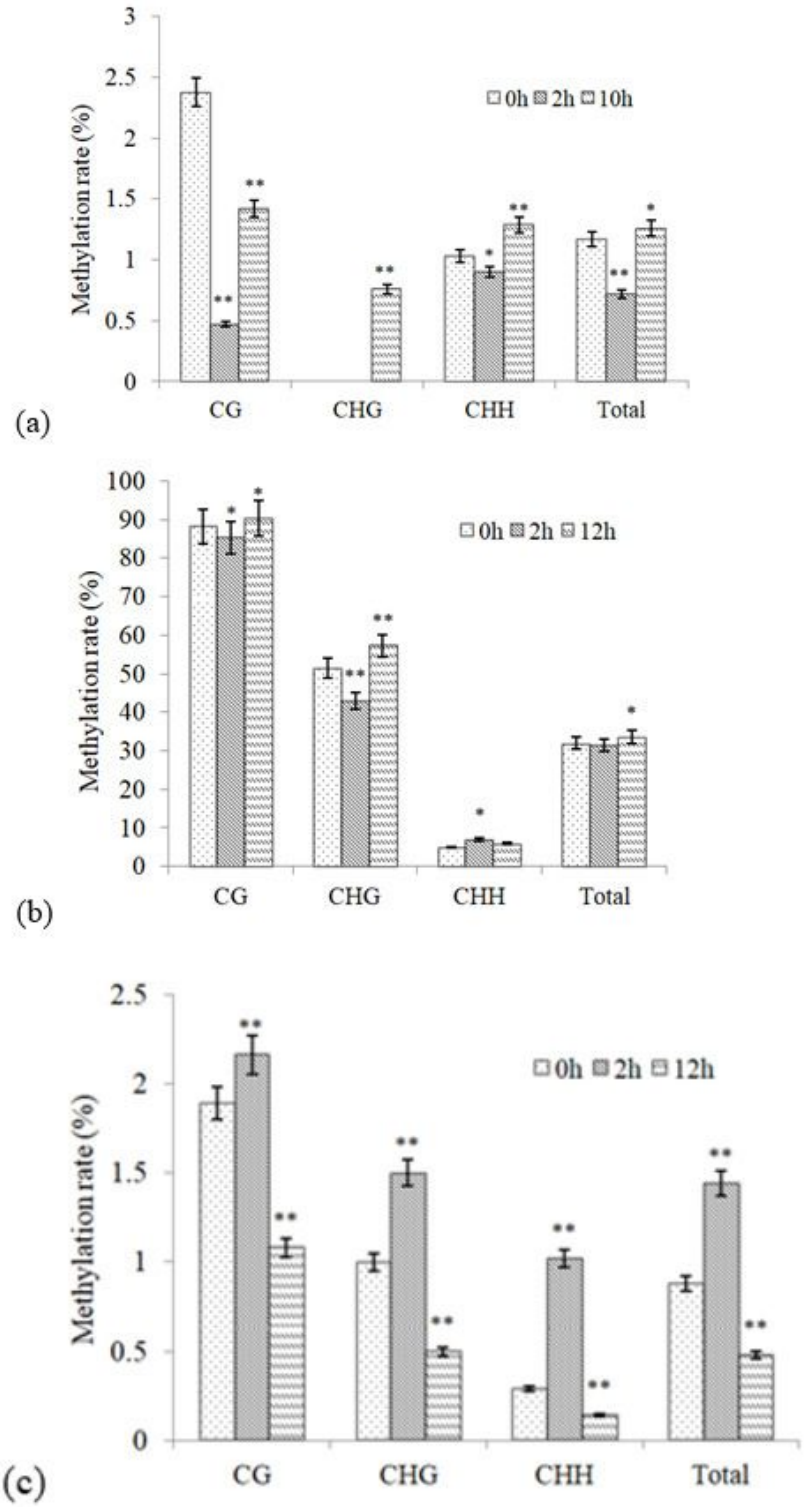

Figure 6

Effect of drought stress on methylation level of DREB promoter in wheat. (a), (b) and (c) represented methylation rate in the promoter region of DREB2, DREB6 and Wdreb2 after wheat seedlings were stressed with 15% PEG6000 solution. In addition, three biological replicates were set up, the error bar was standard error of mean, * and ** represented significant difference ($P<0.05$) or highly significant difference ($P<0.01$).

Supplementary Files

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