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 stress, and the research on drought resistance mechanism of wheat is very important. 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, their CDS sequences were composed of 732 bp, 837 bp or 1035 bp, respectively, and one 712 bp intron was found in *DREB6*. Although AP2/EREBP domain of DREB2, DREB6 and Wdreb2 had 73.25% identity, they belong to different types of DREB transcription factor, and the expression of *Wdreb2* was significantly higher, yet was the lowest in *DREB2*. Under drought stress, the expression of *DREB2*, *DREB6* and *Wdreb2* could be induced, but had different trends along with the increase of stress time, and their expression had tissue specificity, was obviously higher in leaf. Promoter of *DREB2*, *DREB6* and *Wdreb2* in leaf was further studied, some elements related to adverse stress were found, and the promoter of *DREB2* and *Wdreb2* was slightly methylated, but *DREB6* promoter was moderately methylated. Compared with the control, the level of promoter methylation in *DREB2* and *DREB6* decreased as stressed for 2 h, then increased along with the increase of stress time, which was opposite in *Wdreb2* promoter, the status of promoter methylation in *DREB2*, *DREB6* and *Wdreb2* also had significant change under drought stress. Further analysis showed that promoter methylation of *DREB6* and *Wdreb2* was negatively correlated with their expression, especially was significant in *Wdreb2*.

Conclusions: *DREB2*, *DREB6* and *Wdreb2* might function differently in response to drought stress, and promoter methylation had more significant effects on gene expression of *Wdreb2* and *DREB6*.

Background

Drought stress is one of major abiotic stress factors, not only affects the growth and development of plants, but also severely restricts the sustainable production of agriculture [1]. In order to adapt or resist to adverse environment, plants usually make various responses in morphology, physiology and biochemistry, especially regulate the expression of some stress-resistant genes [2-5]. The resistant characters of plants are usually controlled by multiple genes, while one transcription factor can regulate the expression of multiple functionally related genes, there are many transcription factors related to resistant response of plants, such as DREB, bZIP, MYB and WRKY [6].

DREB transcription factor belongs to AP2/EREBP family, and contains one AP2/EREBP domain which is composed of about 60 amino acid residues with conserved element YRG and RAYD [7]. DREB transcription factor could specifically bind to dehydration responsive element/C-repeat (DRE/CRT) with core sequence of 5'-CCGAC-3' by AP2/EREBP domain, regulate the expression of genes related to abiotic stress response, such as high salt, low temperature and drought, and then would enhance the resistances

of plants to adverse stress [6]. For example, *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* can enhance the expression of downstream target genes, and drought tolerance of transgenic *Arabidopsis* would significantly increase [9]. At present, many genes encoding DREB transcription factor have been cloned from *Arabidopsis*, *Maize*, *Soybean*, *Sesame*, etc., its expression could be induced and would increase rapidly in a short time under abiotic stress [10], however the mechanism on expression regulation of DREB transcription factor is less studied, especially epigenetic regulation has not yet been reported.

DNA methylation is a major epigenetic modification, and plays vital function in the growth and development of plants [11], but DNA methylation of 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, for example, methylation level improved and methylation pattern was significantly different at different development stages of *Rice* [13], while methylation level of *Ryegrass* decreased and the expression of demethylated related genes was up-regulated [14]. Fan et al. also found that methylation level of *Dendrobium huoshanense* decreased, and methylation polymorphism gradually increased along with the increase of drought stress [15]. Some studies have shown that DNA methylation plays an important role in the response of plants to adverse stress, could regulate the expression of stress-resistant genes by changes of DNA methylation, and then would improve the resistance of plants to adverse stress [16]. For example, the physiological processes of *Rice* in response to drought stress were related to DNA methylation [13], the change of DNA methylation status was closely connected with drought resistance of trees [17], furthermore, methylation or demethylation of gene in plants would lead to the difference of gene expression under drought stress [18], and this changes of gene expression mediated by DNA methylation would make plants escape or endure drought stress [19].

Wheat (*Triticum aestivum* L.) belongs to Gramineae, is rich in starch, protein, sugar and other substances, and is one of main food crops. In recent years, the growth and development of wheat has been seriously influenced by drought stress which 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, stress-resistant genes and other studies [21], epigenetic regulation of wheat response to drought stress is rarely involved, especially the regulatory role of DNA methylation in DREB transcription factor response to drought stress. In this study, main members of DREB family in wheat were identified, the expression and promoter methylation of *DREB* gene were analyzed under drought stress, which would be helpful to reveal the regulatory mechanism of DNA methylation in the response of plants to drought stress.

Results

Cloning and sequence analysis of *DREB*

As shown in Fig. S1, the CDS sequence of *DREB2*, *DREB6* and *Wdreb2* in wheat AK58 was 732 bp, 837 bp and 1035 bp respectively, *DREB2* and *Wdreb2* had no intron, but one 712 bp intron was found in *DREB6*. CD-search analysis indicated that the typical AP2/EREBP conserved domain was found in amino acid sequence of *DREB2*, *DREB6* or *Wdreb2* (Fig. S1), was composed of YRG and RAYD conserved modules with three β folds and one α helix, simultaneously, valine (V) and glutamate (E) were very conserved at 14 th or 19 th of AP2/EREBP domain (Fig. 1, a). The nucleotide sequences or amino acid sequences of *DREB2*, *DREB6* and *Wdreb2* were further compared by DNAMAN, the similarity among amino acid sequences was low with only 33.24% identity (Fig. 1, b), but AP2/EREBP domains had 73.25% identity, even reached to 83.93% between AP2/EREBP domains of DREB6 and Wdreb2 (Fig. 1, a).

Homologous sequences of DREB2, DREB6 and Wdreb2 from wheat AK58 were analyzed and compared (Table 1, Fig. S2), the similarity of wheat DREB2 was 95% with *Aegilops tauschii* ERF, and was about 60% with TINY from *Oryza sativa*, *Sorghum bicolor* or *Zea mays*, AP2/EREBP domain of DREB2 was the same to that of *Aegilops tauschii* ERF and *Zea mays* TINY (Fig. S2, a). As listed in Table 1, the similarity of wheat DREB6 and some sequences was higher and was 98% or so, such as *Thinopyrum elongatum* AP2/EREBP, *Aegilops biuncialis* DREB2, *Leymus multicaulis* DREB2, etc. AP2/EREBP domain of DREB6 was the same to that of *Thinopyrum elongatum* AP2/EREBP, *Aegilops biuncialis* DREB2 and *Agropyron mongolocum* AP2/EREBP (Fig. S2, b). In addition, the similarity of wheat Wdreb2 with *Aegilops tauschii* DREB2B reached up to 99%, was also higher and was about 95% with *Aegilops speltoides* DREB1, *Triticum turgidum* DRF or *Triticum dicoccoides* DREB (Table 1). Furthermore, AP2/EREBP domain of Wdreb2 was the same to that of *Aegilops tauschii* DREB2B and *Aegilops speltoides* DREB1 (Fig. S2, c).

Table 1 Homologous amino acid sequences of wheat DREB by BLASP

	Protein	Accession Number	Similarity
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 aestivum DREB2B	AAX132861	87%

The expression pattern of *DREB* in wheat

As shown in Fig. 2, under normal condition, the expression level of *DREB2*, *DREB6* and *Wdreb2* in leaf was obviously higher than that in root, which was especially significant in *Wdreb2* ($P<0.05$). Compared with that of *DREB6*, the expression level of *Wdreb2* was significantly higher, yet the expression level of *DREB2* was lower. Under drought stress, the expression level of *DREB2*, *DREB6* and *Wdreb2* in leaf was also higher than that in root ($P<0.05$), compared with the control, the expression of *DREB2*, *DREB6* and *Wdreb2* altered, but this change was different along with the increase of stress time.

Under drought stress, the expression level of *DREB2* increased, and reached to the highest level as stressed for 2 h, which was significantly higher than the control ($P<0.05$), however the expression level of *DREB2* decreased along with the increase of stress time, and was lower as stressed for 8-10 h, which was still higher than the control ($P<0.05$) (Fig. 2, a). The expression level of *DREB6* was also the highest as stressed for 2 h, and was significantly higher than the control ($P<0.05$). Subsequently, along with the increase of stress time, the expression level of *DREB6* gradually decreased, was significantly lower than the control as stressed for 10-12 h ($P<0.05$) (Fig. 2, b). As shown in Fig. 2 (c), the expression level of *Wdreb2* in root significantly increased under drought stress, was obviously higher the control as stressed

for 2 h, and also significantly increased in leaf when stressed for 6-8 h, especially stressed for 12 h ($P<0.05$).

Promoter analysis of wheat *DREB*

In this study, the promoter of *DREB2*, *DREB6* and *Wdreb2* was cloned, was respectively 1735 bp, 1792 bp or 649 bp, and was submitted to GenBank (MT974473, MT974471, MT974472). As shown in Fig. 3 and Table S1-S3, the promoter of *DREB2*, *DREB6* and *Wdreb2* contained basic regulatory element, such as TATA-box, CAAT-box, and there were 26, 18 and 5 TATA-boxes in the promoter of *DREB2*, *DREB6* or *Wdreb2*, respectively. Many elements related to adverse stress were also found in the promoter 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 found that there were some unique elements in the promoter of *DREB2*, *DREB6* or *Wdreb2*, for example, the promoter of *DREB2* had specially light response element MNF, leaf development element HD-ZIP and meristem specificity element OCT (Fig. 3, a; Table S1). A series of specific functional elements were also found in the promoter of *DREB6*, such as ethylene response element ERE, fungal elicitor response element Box-W1, MeJA regulatory element CGTCA-motif, and gibberellin response element P-box (Fig. 3, b; Table S2). Moreover, the promoter of *Wdreb2* had root specificity element as1, zein metabolism regulation element O2-site, light response element C-box, and CE3 element involved in ABA and VP1 reactions (Fig. 3, c; Table S3).

Methylation analysis of *DREB* promoter

The distribution of CpG island in the promoter of *DREB2*, *DREB6* and *Wdreb2* was predicated and analyzed by MethPrimer and EMBOSS CpG Plot, one CpG island with 234 bp was found in the promoter of *DREB2* (Fig. S3, a). As shown in Fig. S3 (b), four CpG islands located respectively in 507-644 bp, 826-960 bp, 1149-1584 bp or 1631-1735 bp of *DREB6* promoter, and one CpG island with 559 bp existed in the promoter of *Wdreb2* (Fig. S3, c). Furthermore, there were also functional elements in above CpG islands, such as abscisic acid response element, light response element, low temperature response element, and so on (Fig. 3, Table S1-S3).

Some CpG island regions predicted in *DREBs* promoter were further examined from wheat leaf by bisulfite sequencing PCR (BSP), and the CpG island region examined was respectively located in 1589-1904 bp (Fig. S3, a), 1135-1617 bp (Fig. S3, b) or 280-615 bp (Fig. S3, c) at the promoter of *DREB2*, *DREB6* or *Wdreb2*. As shown in Fig. 4 and Table 2, there were more CHH sites and less CHG sites in the promoter region of *DREB2*, *DREB6* and *Wdreb2*, but methylation rate of CG was the highest. In the promoter region of *DREB2*, CHH sites were not methylated, methylation rate of CG and CHH was 2.38% or 1.03%, and belonged to mild methylation (<20%) (Fig. 4, a; Table 2). As shown in Fig.4 (b) and Table 2, in the promoter region of *DREB6*, methylation rate of CG was 88.08% and was severely methylated (>60%), methylation rate of CHG was 51.36% and was moderately methylated (>20%), but methylation rate of

CHH was only 4.93% and belonged to mild methylation (< 20%). Furthermore, in the promoter region of *Wdreb2*, methylation rate of CG, CHG or CHH was 1.89%, 1.0% and 0.29%, respectively, which were all mildly methylated (Fig.4, c; Table 2).

Table 2 Methylation analysis of promoter region in wheat *DREB* gene

Gene	Pattern	Pattern frequency (%)	Methylation rate (%)	Total Methylation rate (%)
DREB2	CG	19.09	2.38	1.171
	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	1.08	0.48
	CHG	16.00	0.5	
	CHH	54.40	0.14	

Methylation level of *DREB* promoter under drought stress

Under drought stress, cytosine methylation altered in the promoter region of *DREB2*, *DREB6* and *Wdreb2* from wheat leaf (Fig. 5). Compared with the control, methylation rate of CG in the promoter region of *DREB2* decreased obviously (P<0.01), was 0.5% or 1.42% as stressed for 2 h and 10 h, but methylation rate of CHG and CHH increased significantly as stressed for 10 h (P<0.01). Further analysis showed that methylation level of *DREB2* promoter was obviously lower or higher than the control when stressed for 2 h or 10 h, and this difference was significant (P<0.05) (Fig. 6, a).

As shown in Fig. 6 (b), methylation level of *DREB6* promoter changed under drought stress, and was significantly higher than the control when stressed for 12 h (P<0.05). Compared with the control, methylation rate of CG and CHG was obviously lower or higher as stressed for 2 h and 12 h, although methylation rate of CG and CHG was significantly lower as stressed for 2 h, the promoter region of *DREB6* was still heavily CG cytosine methylated (>60%) and moderately CHG cytosine methylated (>20%). As stressed for 2 h or 12 h, methylation rate of CHH was higher than the control, but this change was less than that of CG and CHG (P<0.05).

Furthermore, methylation level of *Wdreb2* promoter also changed under drought stress, was significantly higher or lower than the control when stressed for 2 h and 12 h (P<0.01) (Fig. 6, c). Methylation rate of CG, CHG and CHH was respectively 2.16%, 1.5% or 1.02% as stressed for 2 h, and was obviously higher than the control (P<0.01), however was significantly lower than the control as stressed for 12 h (P<0.01).

Methylation status in *DREB* promoter under drought stress

As listed in Table 3, methylation status in the promoter region of *DREB2*, *DREB6* and *Wdreb2* had significant change under drought stress. Along with the increase of stress time, the number of hypermethylation sites significantly increased in *DREB2* promoter, for example, there were 1 CG site and 2 CHH sites in hypermethylation status as stressed for 2 h, but were 2 CG sites, 3 CHH sites and 1 CHG site as stressed for 10 h, furthermore, there were 3 CG sites and 1 CHH site in demethylation status under drought stress.

Table 3 Methylation pattern in promoter region of wheat *DREB* gene under drought stress

Gene	Type of cytosine	No. of cytosine	No. of methylation sites			
			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 represented the change of methylation status in the promoter region of wheat *DREB* gene under the stress of 15% PEG_{6000} as compared to the control (CK). T1 and T2 denoted 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.

Under drought stress, the number of hypermethylation and demethylation sites also changed in *DREB6* promoter (Table 3), as stressed for 2 h, 8 CHH sites and 1 CHG site were hypermethylated, 7 CHH sites and 1 CG site were demethylated, however there were 10 CHH sites, 1 CHG site and 1CG site in hypermethylation status, 8 CHH sites, 1CG site and 1CHG site were in demethylation status as stressed for 12 h (Table 3). Along with the increase of stress time, the number of hymethylation sites had hardly changed in *Wdreb2* promoter, but demethylation sites increased, and the change of methylation status was significant in CHH site, after stressed for 2 h, 2 CHH sites were respectively hypermethylated and demethylated, there were 1 CHH site in hypermethylation status and 2 CHH sites in demethylation status as stressed for 12 h (Table 3).

Correlation analysis between promoter methylation and expression of *DREB*

In order to explore the correlation between promoter methylation and expression of wheat *DREB2*, *DREB6* or *Wdreb2*, under drought stress for different times, their relative expression levels in wheat leaf and methylation rates of CG, CHG or CHH in their promoter regions were respectively analyzed by SPSS software. As listed in Table S4, Pearson coefficient r between expression of *Wdreb2* and methylation rate

of CG, CHG or CHH was respectively -0.986, -0.973 and -0.878, indicating that significant negative correlation existed between promoter methylation and gene expression of *Wdreb2*, similarly, promoter methylation and gene expression 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 factor plays an important role in the response of plant to drought stress, could specifically bind to DRE/CRT element in the promoter of stress-responsive gene and then would enhance the response or tolerance of plant to adverse stress [6]. AP2/EREBP domain of DREB transcription factor is composed of about 60 amino acid residues, has two conserved regions of YRG and RAYD [7]. In this study, *DREB2*, *DREB6* and *Wdreb2* were cloned from wheat AK58, one 712 bp intron was found in *DREB6*, AP2/EREBP domain of DREB2, DREB6 and Wdreb2 had 73.25% identity, the amino acid at 14 th or 19 th of AP2/EREBP domain was V and E, respectively. However, the similarity was lower among nucleotide sequences or amino acid sequences of *DREB2*, *DREB6* and *Wdreb2*, BLASTP results further showed that *DREB2*, *DREB6* and *Wdreb2* were different types of DREB transcription factor and might respectively belong to DREBA-4 class, DREB-2 class or DREB-1 class, which was also found in other research [22, 23].

Under abiotic stresses, such as drought, low temperature, high salt, etc., the expression of DREB transcription factor would alter [24, 25]. In this study, the expression of *DREB2*, *DREB6* and *Wdreb2* could be induced under drought stress, and generally reached to the highest level after stressed for 2 h, but showed different trends along with the increase of stress time. The expression levels of *DREB2*, *DREB6* and *Wdreb2* were also different, as stressed for 2 h, the expression of *Wdreb2* was significantly higher, but was the lowest in *DREB2*, Lopato et al also found that the expression of *DREB2* was very low [26]. Further analysis showed that the expression of *DREB2*, *DREB6* and *Wdreb2* had tissue specificity, and was obviously higher in leaf than that in root, which was similar in other research [27], the expression of *DREB* in *Daucus carota* also showed tissue specificity, *DcDREB-A1-1* and *DcDREB-A1-2* had main role in leaf or root, respectively [28].

It is well known, the cis-acting regulatory elements in the promoter provide the possibility for transcription and expression of gene [29], there are some cis-acting elements related to adverse stress in plant promoter, such as DRE/CRT, EREH, ABRE, LTR and so on [30]. Except typical regulatory element TATA-box and CAAT-box, the promoter of *DREB2*, *DREB6* and *Wdreb2* in wheat AK58 contained DRE/CRT, LTR, ABRE, and drought-induced MYB binding site, etc, confirming that the expression of *DREB2*, *DREB6* and *Wdreb2* may be influenced by adverse stress. Furthermore, in the promoter of *DREB2*, *DREB6* and *Wdreb2*, CpG island with a variety of cis-acting elements was detected by MethPrimer and EMBOSS CpG Plot, some studies found that DNA methylation could regulate the expression of stress-responsive genes, and play an important role in the response of plant to adverse stress [16], especially promoter methylation had more significant effect on gene expression [31]. BSP analysis showed that there were more CHH sites and

less CHG sites in the promoter region of *DREB2*, *DREB6* and *Wdreb2*, but the methylation rate of CG sites was the highest.

Many studies have found that degree and state of DNA methylation in plant would change under drought stress, low temperature, high salt and other conditions [32, 33], especially the change of methylation state in the promoter of gene [34]. Under drought stress, methylation level altered in the promoter region of *DREB2*, *DREB6* and *Wdreb2*, compared with the control, methylation level in *DREB2* and *DREB6* promoter 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 region of *DREB2*, *DREB6* and *Wdreb2* had significant change under drought stress, such as demethylation and hypermethylation, Zilberman also found that gene expression could be respectively promoted or inhibited by demethylation and hypermethylation of promoter [35].

Further analysis showed that promoter methylation of *DREB6* and *Wdreb2* was negatively correlated with their expression by Pearson coefficient, especially was significant in *Wdreb2*, this negative correlation was also found in other studies [35, 36]. Although the promoter 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 promoter of *z1B4* and *z1B6* in *Zea mays* was almost not methylated [37], DNA methylation was not found in the promoter 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 to the relation between DNA methylation in the coding region and gene expression of wheat *DREB*, the mechanism of DNA methylation regulating the expression of wheat *DREB* needs to be further studied.

Conclusions

In this study, *DREB2*, *DREB6* and *Wdreb2* were cloned and identified from wheat, and one 712 bp intron was found in *DREB6*. Under drought stress, the expression of *DREB2*, *DREB6* and *Wdreb2* would be induced, was obviously higher in leaf, but had different trends along with the increase of stress time. In the promoter region of *DREB2*, *DREB6* and *Wdreb2*, some elements related to adverse stress were also found, 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

Experimental materials

In this study, seeds of wheat AK58 were kindly provided by Xinxiang Academy of Agricultural Science, Henan, China. The tolerance of wheat AK58 is strong to drought stress, and its yield is generally high and stable. Primers and their sequences used in this study were listed in Table S5, and all primers were synthesized by Yingjie Ji Trade Co., Ltd. (Shanghai, China).

Cultivation and treatment of wheat seedlings

Cultivation of wheat seedlings was performed according to methods and conditions used by Duan et al. [33], wheat seeds were firstly surface-sterilized for 10 min by 0.1% $HgCl_2$, then were washed for 50 min by sterile water. Subsequently, sterilized seed were sown in pots (diameter of 15 cm) containing nutrition soil and vermiculite (1:1), were cultured at 24 ± 1 ℃ with 45% relative humidity and 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 irrigated with 15% PEG_{6000} solution, roots and leaves of wheat seedlings were collected at 0 h (just before drought stress), and 2 h, 6 h, 8 h, 10 h or 12 h after subjected to drought stress in light condition, and immediately freezed with liquid nitrogen and then store at -80 ℃. In addition, there were three biological replicates for each experiment group in this study.

Extraction of genomic DNA

Genomic DNA was extracted from root or leaf of wheat seedlings by cetyltriethyl ammonium bromide (CTAB) method [40], the yield and purity of genomic DNA were determined at 260 nm by micro-spectrophotometry, and 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 in root and leaf of wheat seedlings was respectively extracted by RNAiso Plus (TaKaRa, Japan) according to the instructions. In order to remove DNA, DNase/RNase-free treatment and phenol-chloroform extraction were performed in this research, RNA was dissolved in RNase-free dH_2O and was stored at -80 °C. Furthermore, the integrity of total RNA was verified by 1.0% agarose gel electrophoresis, the yield and purity of total RNA was determined by UV spectrophotometer.

In addition, cDNA was synthesized by reverse transcription of the extracted total RNA from wheat seedlings, the method of reverse transcription was referred to the introduction of HiScript II Q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, China).

Cloning and analysis of *DREB* gene

In order to clone *DREB* genes from wheat AK58, specific primers were designed according to the sequence of wheat *DREB2* (GU785008), *DREB6* (AY781361) and *Wdreb2* (AB193608), and were listed in Table S5, furthermore, genomic DNA and cDNA of wheat AK58 were respectively used as the amplification template 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 ddH2O. PCR procedure was at 95° C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and 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 addition, analysis of target sequences was performed in the following, the extron, intron and ORF of *DREB* gene in wheat was analyzed with ProtParam, the conserved domain and amino acid sequence encoded by wheat *DREB* gene were analyzed by CD-search in NCBI, BLASTP was used to search similar amino acid sequences of wheat DREB, the domain or homologous sequences of wheat DREB were respectively compared with DNAMAN.

Fluorescence quantitative real-time PCR

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

According to the instruction 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 dH2O, and qRT-PCR procedure was pre-denaturation for 5 min at 95 °C followed by 40 cycles of 95° C for 10 s and 60 °C for 30 s.

In addition, the relative expression level of wheat *DREB* 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, three biological replicates were set up, and each qRT-PCR experiment was repeated three times.

Isolation and analysis of promoter sequence

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

PCR reaction system of *DREB* promoter was 20 μl, consisted of 2.0 μl DNA template, 10.0 μl 2x Taq Mix, 1.0 μl each primer (10 μM) and 6.0 μl ddH2O. The reaction conditions of PCR procedure was at 95 °C for 5 min, followed by 40 cycles of 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 with 1.0% agarose gel electrophoresis, and the target fragments were obtained by gel extraction and recycling, then were sequenced in Vazyme (Nanjing,

China). Furthermore, PlantCARE and PLACE were used to analyze cis-acting elements in the promoter sequence of wheat *DREB*.

Methylation analysis of promoter

CpG island (Island size > 100, GC Percent > 50.0, Obs/Exp > 0.6) in the promoter of *DREB* was predicted and analyzed by MethPrimer and EMBOSS CpG Plot. According to the analysis of CpG island, amplification primers of bisulfite sequencing PCR (BSP) were designed by MethPrimer, Methyl Primer Expressv1.0 and Primer Premier5.0 (Table S5), and 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 leaf of wheat seedlings was firstly treated with EZ DNA Methylation-Lightning^{TM} Kit (Zymo Research, America), then was used as template in BSP amplification of *DREB* promoter. BSP reaction system was 30.0 μl, and composed of 2.0 μl bisulfite-treated DNA, 1.0μ l 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 dH2O. PCR amplification procedure was 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 with 1.0% agarose gel electrophoresis, found that only target fragments were amplificated, subsequently the target fragments were obtained by gel extraction and recycling, and were sequenced in GENEray (Shanghai, China).

In addition, at least 10 clones of per target fragment were sequenced and three biological replicates were set up in this study, statistics analysis on methylation site, methylation type and methylation rate was performed with CyMATE and Kismeth.

Statistical analysis

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

Acknowledgements

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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

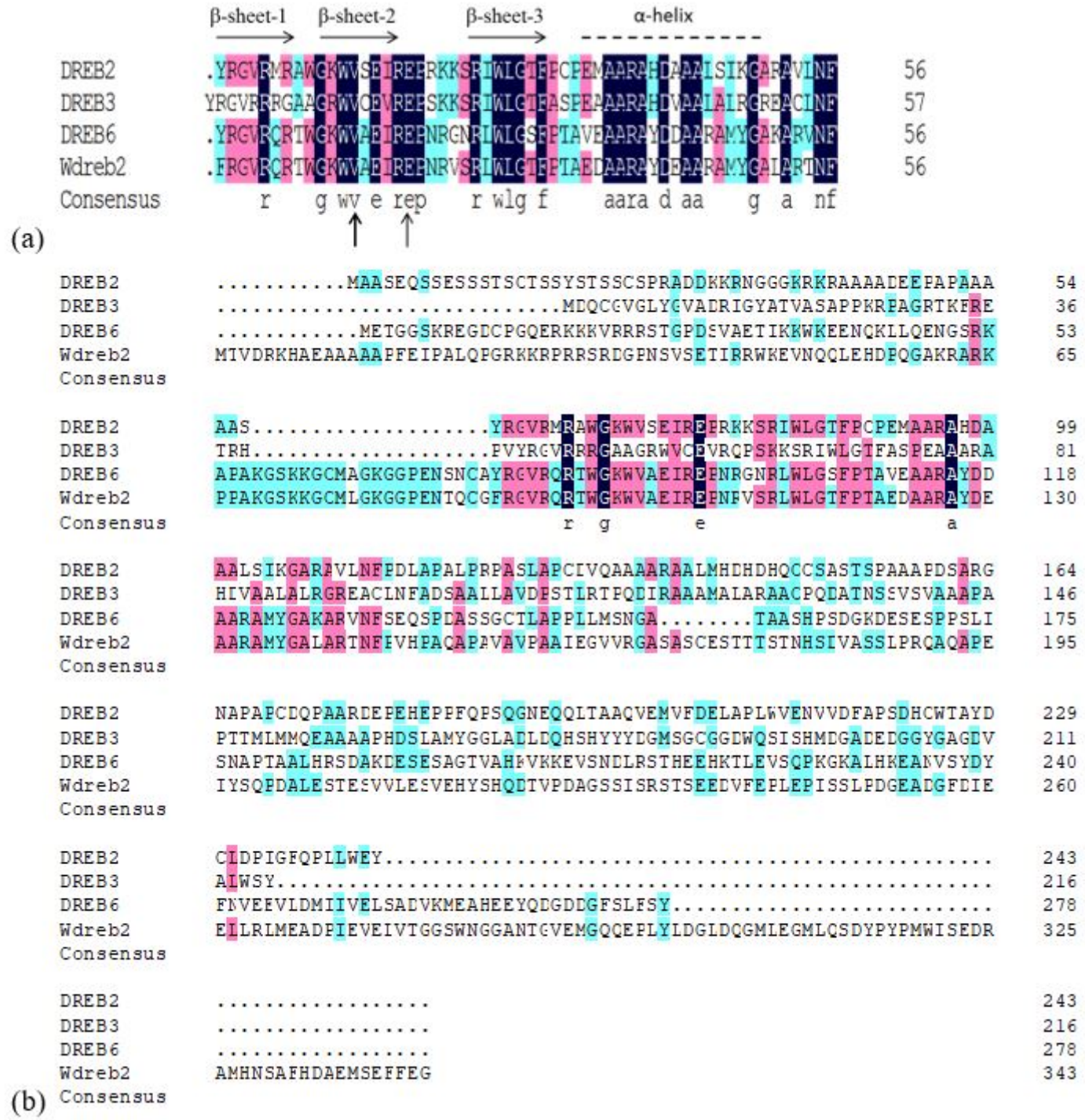

Figure 1

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.

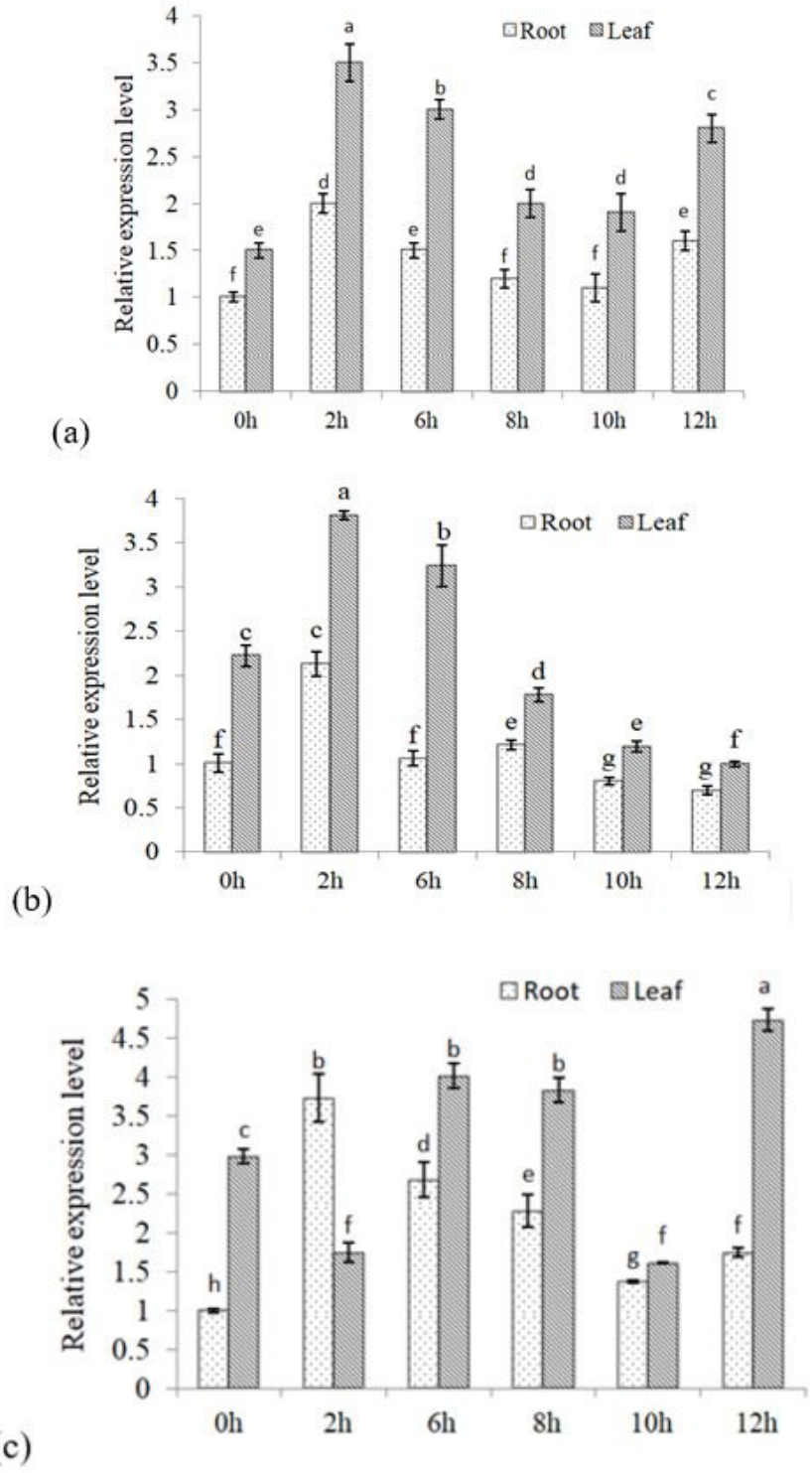

Figure 2

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 ($P < 0.05$).

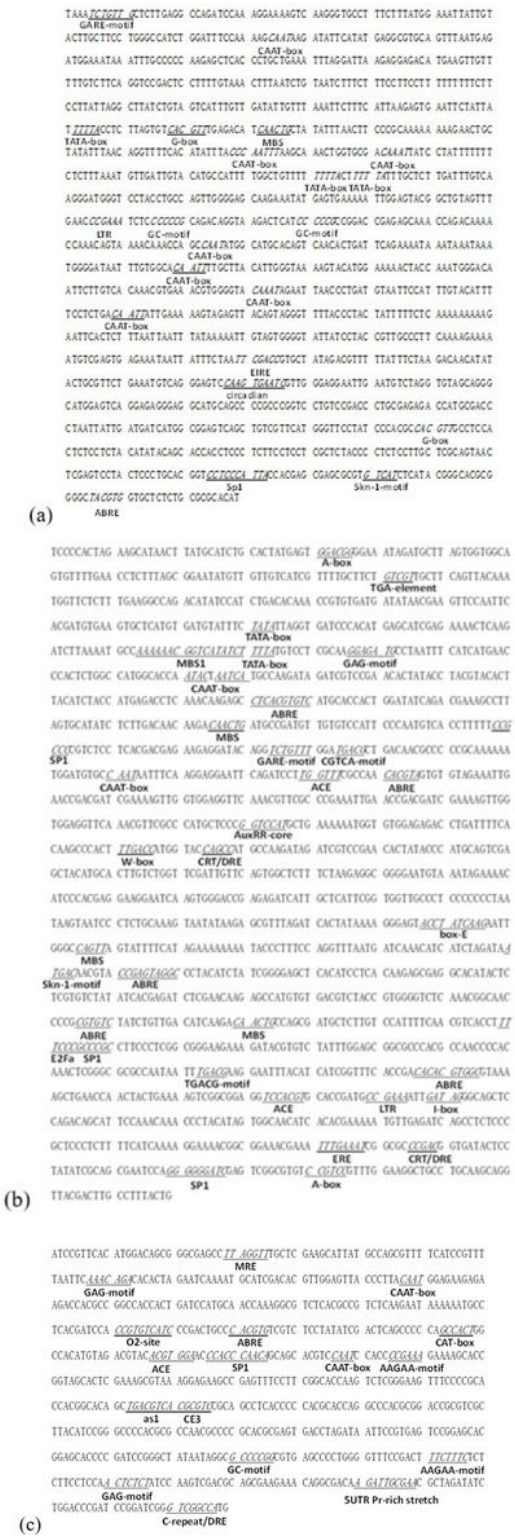

Figure 3

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.

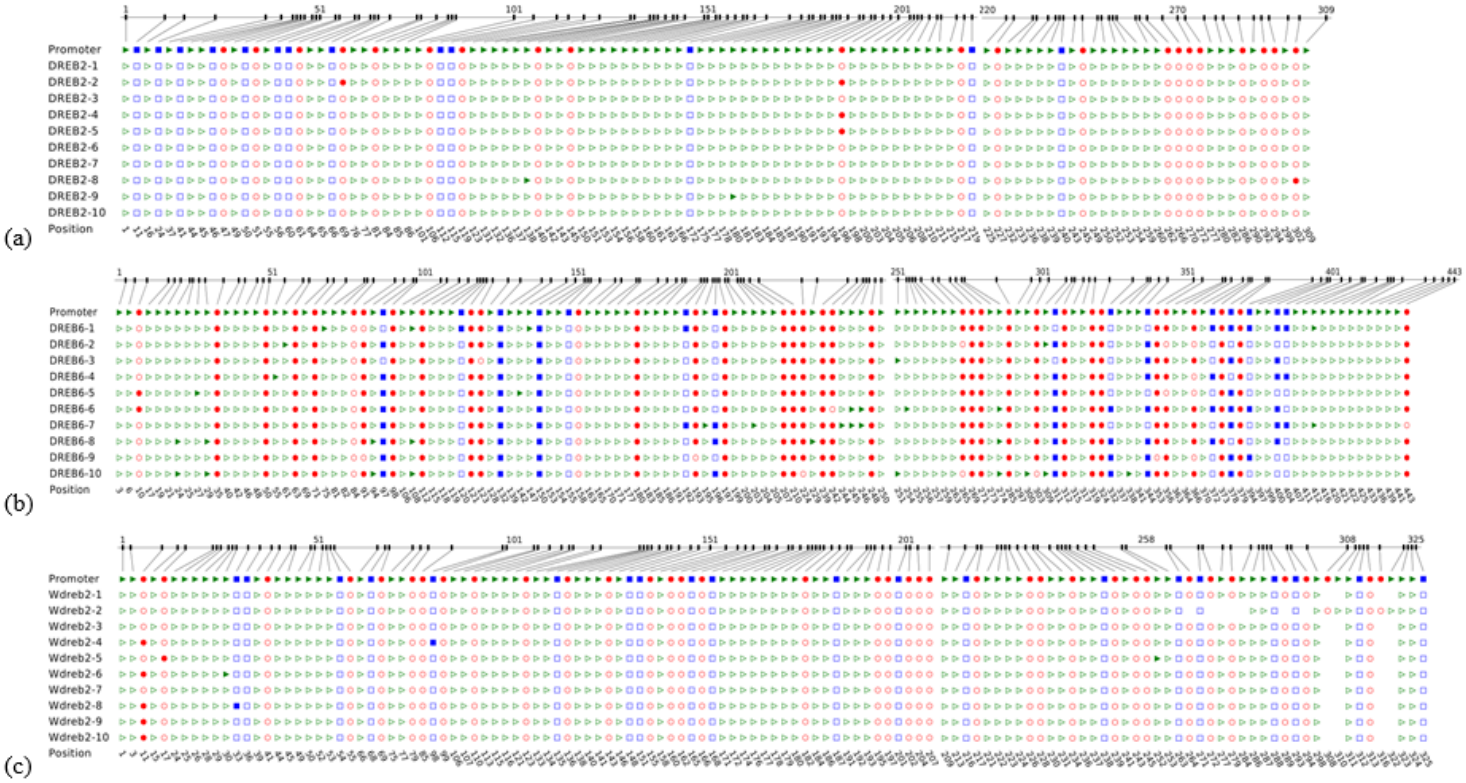

Figure 4

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.

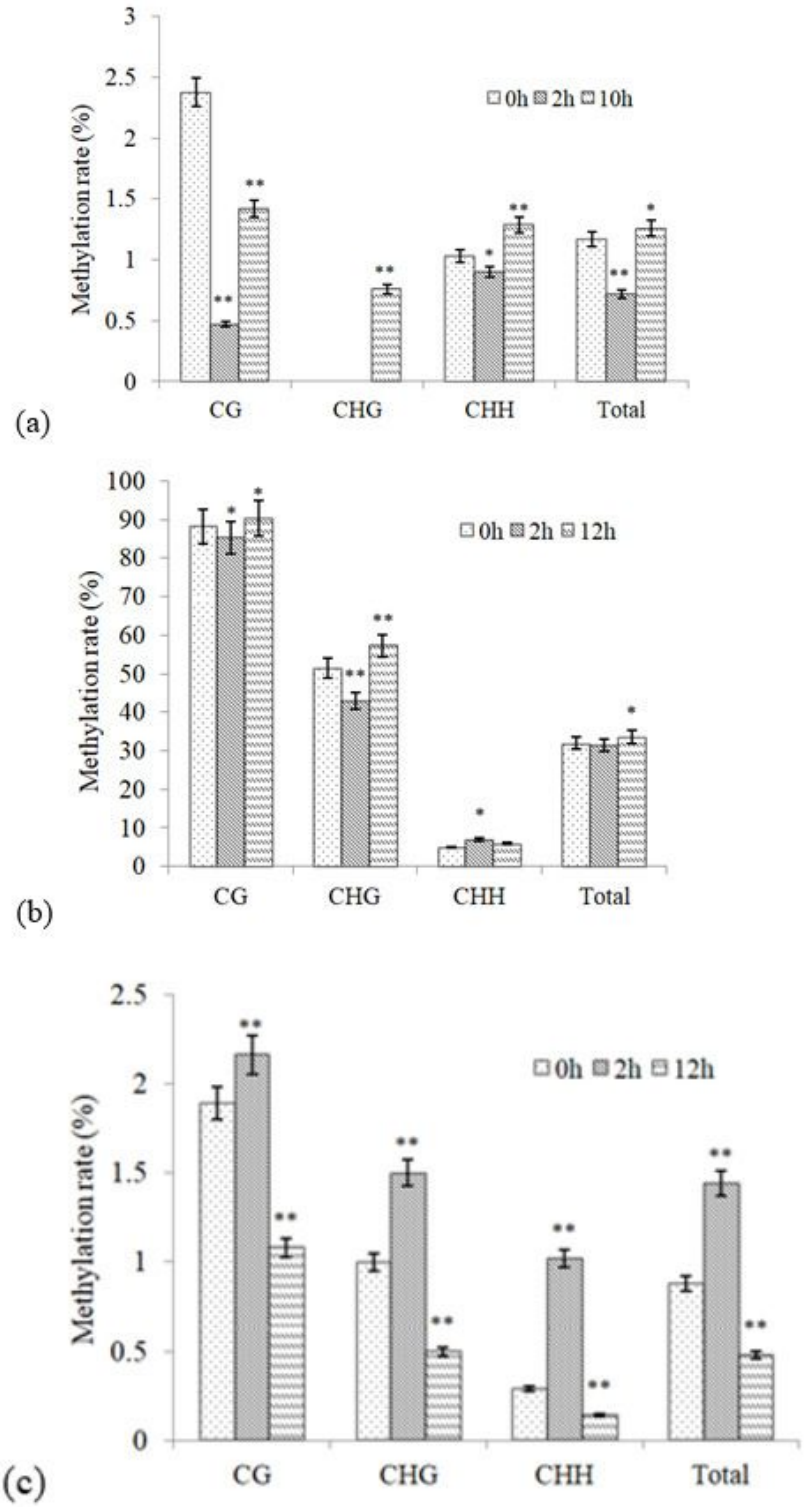

Figure 6

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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