

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

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## Research article

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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 plant response 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 in this study, their CDS sequence was composed of 732bp, 837bp or 1035bp, respectively, one 712bp 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 mildly methylated. Compared with the control, the level of promoter methylation decreased in *DREB2* and *DREB6* as stressed for 2h, then increased along with the increase of stress time, which was opposite in *Wdreb2* promoter, the status of promoter methylation also had significant change under drought stress. Further analysis showed that promoter methylation of *DREB6* or *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 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 character of plant is 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 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 plant resistance 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 for expression regulation of DREB transcription factor is less studied, especially the epigenetic regulation has not yet been reported.

DNA methylation is a major epigenetic modification, and plays crucial role in growth and development of plant [11], but DNA methylation of plant is easily affected by physiological status, developmental stage and environmental factors [12]. Under drought stress, the state of DNA methylation would change in plant, 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 plant response to adverse stress, could regulate the expression of stress-resistant genes through changes of DNA methylation, and then would improve plant resistance to stress [16]. For example, the physiological processes of *Rice* response to drought stress was related to DNA methylation [13], the change of DNA methylation status was closely related to drought resistance of trees [17], furthermore, methylation or demethylation of gene in plant would lead to 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 sustainable increase of wheat yield [20]. At present, studies on wheat response to drought stress are basically limited 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 plant response 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 was 732bp, 837bp and 1035bp respectively, *DREB2* and *Wdreb2* had no intron, but one 712bp 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  $\beta$  folds and one  $\alpha$  helix, simultaneously, V (valine) and E (glutamate) were very conserved at 14th or 19th of AP2/EREBP domain (Fig. 1, a). The nucleotide sequences or amino acid sequences of *DREB2*, *DREB6* and *Wdreb2* were further compared by DNAMAN, it was found that the similarity was low with only 33.24% identity among their amino acid sequences (Fig. 1, b), their AP2/EREBP domains had 73.25% identity, even the identity reached to 83.93% between AP2/EREBP domains of *DREB6* and *Wdreb2* (Fig. 1, a).

Homologous sequences of *DREB2*, *DREB6* and *Wdreb2* were analyzed and compared (Table 1, Fig. S2), the similarity of wheat *DREB2* was 95% with *Aegilops tauschii* ERF, was about 60% with TINY of *Oryza sativa*, *Sorghum bicolor* or *Zea mays*, and AP2/EREBP domain of *DREB2* was the same to that from *Aegilops tauschii* and *Zea mays* (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, *Triticum aestivum* DREBW73, *Aegilops biuncialis* DREB2 or *Leymus multicaulis* DREB2, and AP2/EREBP domain of *DREB6* was the same to that from *Thinopyrum elongatum*, *Aegilops biuncialis* and *Agropyron mongolocum* (Fig. S2, b). In addition, the similarity of wheat *Wdreb2* with *Aegilops tauschii* DREB2B reached up to 99%, was also higher with *Aegilops speltoides* DREB, *Triticum turgidum* DRF or *Triticum dicoccoides* DREB, and was about 95% (Table 1). Furthermore, AP2/EREBP domain of *Wdreb2* was the same to that from *Aegilops tauschii* and *Aegilops speltoides* (Fig. S2, c).

### 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.

Compared with the control, the expression level of *DREB2* increased under drought stress, and reached to the highest level as stressed for 2h, which was significantly higher than the control ( $P < 0.05$ ). Along with the increase of stress time, the expression level of *DREB2* decreased, was the lowest as stressed for 10h, 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 2h, 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 10-12h, and was the lowest under drought stress for 12h ( $P < 0.05$ ) (Fig. 2, b). As shown in Fig. 3 (b), compared with the control, the expression level of *Wdreb2* in root significantly increased under drought stress, was obviously higher the control as stressed for 2h, and the expression level of *Wdreb2* in leaf also significantly increased when stressed for 6-8h, especially stressed for 12h ( $P < 0.05$ ).

## Promoter analysis of wheat *DREB*

In this study, the promoter of *DREB2*, *DREB6* and *Wdreb2* was cloned, their length was respectively 1735bp, 1792bp or 649bp, and was analyzed by Plant CARE and PLACE. As shown in Fig. 3 and Table S1-S3, the promoter of *DREB2*, *DREB6* and *Wdreb2* contained basic regulatory element, such as TATA-box and CAAT-box, and there were 26, 18 and 5 TATA-boxes in the promoter of *DREB2*, *DREB6* and *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 as, 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).

## Distribution of CpG island in *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 507bp-644bp, 826bp-960bp, 1149bp-1584bp or 1631bp-1735bp of *DREB6* promoter, and one CpG island with 559bp existed in the promoter of *Wdreb2* (Fig. S3, c). Furthermore, there were also functional elements in the CpG island, such as abscisic acid response elements, light response elements, low temperature response element, and so on (Fig. 3, Table S1-S3).

CpG island in the promoter of *DREB2*, *DREB6* and *Wdreb2* was further examined from leaf by bisulfite sequencing PCR (BSP), and found that there were more CHH sites and less CHG sites in the promoter of *DREB2*, *DREB6* and *Wdreb2*, but methylation rate of CG was the highest (Fig. S4). In the promoter 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. S4, a). As shown in Fig. S3 (b), in the promoter 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 of *Wdreb2*, methylation rate of CG, CHG or CHH was 1.89%, 1.0% and 0.29%, respectively, which were all mildly methylated (Fig. S4, c).

## Methylation level of *DREB* promoter under drought stress

Under drought stress, cytosine methylation altered in the promoter of *DREB2*, *DREB6* and *Wdreb2* from leaf (Fig. 4). Compared with the control, methylation rate of CG in the promoter of *DREB2* decreased obviously ( $P<0.01$ ), was 0.5% or 1.42% as stressed for 2h and 10h, but methylation rate of CHG and CHH increased significantly as stressed for 10h ( $P<0.01$ ), and drought stress for 2h had no effect on methylation of CHG and CHH. Further analysis showed that methylation level of *DREB2* promoter was obviously lower or higher than the control when stressed for 2h or 10h, this difference was very significant ( $P<0.01$ ) (Fig. 5, a).

As shown in Fig. 5 (b), methylation level of *DREB6* promoter changed under drought stress, compared with the control, methylation rate of CG and CHG was obviously lower or higher as stressed for 2h and 12h ( $P<0.01$ ), although methylation rate of CG and CHG was significantly lower as stressed for 2h, the promoter of *DREB6* was still heavily CG cytosine methylated (>60 %) and moderately CHG cytosine methylated (>20 %). As stressed for 2h or 12h, methylation rate of CHH was obviously higher or lower than the control, but this change was less than that of CG and CHG ( $P<0.01$ ), methylation level of *DREB6* promoter was significantly higher when stressed for 12h (Fig. 5, b).

Furthermore, methylation level of *Wdreb2* promoter also changed under drought stress, was significantly higher or lower than the control when stressed for 2h and 12h ( $P<0.01$ ) (Fig. 5, c). Methylation rate of CG, CHG and CHH was respectively 2.16%, 1.5% or 1.02% as stressed for 2h, and was obviously higher than the control ( $P<0.01$ ), however was significantly lower than the control as stressed for 12h ( $P<0.01$ ).

### **Methylation status in *DREB* promoter under drought stress**

As listed in Table 2, methylation status in the promoter 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 2h, but 2 CG site, 3 CHH sites and 1 CHG sites were in hypermethylation status as stressed for 10h, furthermore, there were 3 CG sites and 1 CHH site in demethylation status under drought stress.

Under drought stress, the number of hypermethylation and demethylation sites also changed in *DREB6* promoter, as stressed for 2h, 8 CHH sites and 1 CHG site were hypermethylated, 7 CHH sites and 1 CG site were in demethylation status, 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 12h (Table 2). Along with the increase of stress time, the number of hypermethylation 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 2h, 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 12h.

### **Correlation analysis between promoter methylation and expression of *DREB***

The correlation between promoter methylation and expression of *DREB2*, *DREB6* and *Wdreb2* in leaf was analyzed by SPSS software. As shown 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 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 12h (Fig. 2, a; Fig. 5, a).

## Discussion

DREB transcription factor plays an important role in plant response 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 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, one 712bp intron was found in *DREB6*, AP2/EREBP domain of *DREB2*, *DREB6* and *Wdreb2* had 73.25% identity, the amino acid at 14th or 19th were respectively V and E in their AP2/EREBP domain. However, the similarity was lower among nucleotide sequences or amino acid sequences of *DREB2*, *DREB6* and *Wdreb2*, BLAST 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 and generally reached to the highest level under drought stress for 2h, 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 2h, 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, cis-acting regulatory elements in the promoter provide 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 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 found, some studies found that DNA methylation could regulate the expression of stress-responsive genes, and play an important role in plant response 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 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 change of methylation state in the promoter of gene [34]. Under drought stress, methylation level altered in the promoter of *DREB2*, *DREB6* and *Wdreb2*, compared with the control, methylation level in *DREB2* and *DREB6* promoter decreased as stressed for 2h, then increased along with the increase of stress time, which was opposite in *Wdreb2* promoter. Furthermore, methylation status in the promoter of *DREB2*, *DREB6* and *Wdreb2* had significant change under drought stress, such as demethylation and hypermethylation, Zilberman also found that the expression of gene could be respectively promoted or inhibited by demethylation and hypermethylation of promoter [35].

Further analysis showed that promoter methylation of *DREB6* or *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 the promoter of *DREB2* maybe belong to low CpG-contain promoter. Similarly, promoter of *z1B4* and *z1B6* was almost not methylated in *Zea mays* [37], DNA methylation was not found in the promoter of some genes and only occurred in its coding region in *Arabidopsis* or tomato [38, 39]. In addition, one CpG island was also found in coding region of *DREB2*, *DREB6* and *Wdreb2*, and the CpG island almost covered the whole coding region of *DREB2*. However, it is unclear on the relation between DNA methylation in the coding region and gene expression of wheat *DREB*, the mechanism of DNA methylation regulating gene expression of wheat *DREB* needs to be further studied.

## 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 drought stress of wheat seedlings

Cultivation of wheat seedlings was performed according to methods and conditions used by Duan et al. [40], wheat seeds were firstly surface-sterilized for 10 min by 0.1%  $\text{HgCl}_2$ , 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  $24 \pm 1^\circ\text{C}$  with 45% relative humidity and 14h photoperiod of 4500 lux illumination intensity, and were irrigated with 5 ml distilled water every two days.

At the three-leaf stage, wheat seedlings were removed from nutrition soil and put in 15% PEG<sub>6000</sub> solution for 2h, 6h, 8h, 10h or 12h. After subjected to drought stress, roots and leaves of wheat seedlings were frozen with liquid nitrogen and store at -80°C. 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 [41], 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 seedling 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<sub>2</sub>O and was stored at -80°C. Furthermore, the integrity of total RNA was verified by 1.0% agarose gel electrophoresis, the yield of total RNA was determined by UV spectrophotometer at 260 nm, and the purity of total RNA was checked by determining A<sub>260</sub>/A<sub>280</sub> ratio.

Furthermore, cDNA was synthesized by reverse transcription using the extracted total RNA as template, 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 obtain *DREB* in wheat, specific primers of *DREB* were designed according to sequences of *DREB2* (GU785008), *DREB6* (AY781361) and *Wdreb2* (AB193608), these primers for PCR amplification were listed in Table S5. In this study, genomic DNA and cDNA were respectively used as template to obtain DNA sequence or cDNA sequence of *DREB* from wheat seedlings.

PCR reaction system of *DREB* cloning was composed of 2.0µl template, 1.0µl each primer (10µM), 10.0µl 2xTaqMix and 6.0µl ddH<sub>2</sub>O. PCR procedure was at 95°C for 5min, followed by 35 cycles of 94°C for 30s, 55°C for 30s and 72°C for 1min, finally extension at 72°C for 5min. PCR amplification products were detected by 1% agarose gel electrophoresis, the target fragment was obtained by gel extraction and recycling, and then was sequenced in Vazyme (Nanjing, China).

In addition, the analysis of target sequences was performed in the following, the exon, intron and ORF of *DREB* in wheat was analyzed with ProtParam, the conserved domain and amino acid sequence encoded by wheat *DREB* were analyzed by CD-search in NCBI, BLASTP was used to search similar sequences of amino acid sequence encoded by wheat *DREB*, and domain or homologous sequences of wheat *DREB* were 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 was prepared on ice, consisted of 1.0μl AceQ qPCR SYBR Green Master Mix, 0.5μl each primer (10μM), 2.0μL cDNA, and 16.0μl dH<sub>2</sub>O. The reaction procedure of qRT-PCR was pre-denaturation at 95°C for 5min followed by 40 cycles of 95°C for 10s and 60°C for 30s.

In addition, the relative expression level of *DREB* gene under drought stress was normalized and analyzed by the comparative Ct ( $2^{-\Delta\Delta Ct}$ ) method [42]. The calculation formula was as follows: Relative expression level =  $2^{-\Delta\Delta Ct}$ ,  $\Delta\Delta Ct$  (target gene) =  $\Delta Ct$  (treatment group) -  $\Delta Ct$  (control group),  $\Delta Ct$  (target gene) =  $Ct$  (target gene) -  $Ct$  (reference gene). Furthermore, three biological replicates were set up and each qRT-PCR experiment was repeated three times in this study.

## Isolation and analysis of promoter sequences

The promoter was cloned to further analyze expression pattern of *DREB* in wheat, 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 amplification system of promoter was 20μl, consisted of 2.0μl DNA, 10.0μl 2xTaqMix, 1.0μl each primer (10μM) and 6.0μl ddH<sub>2</sub>O. The reaction conditions of PCR procedure was at 95°C for 5min, followed by 40 cycles of 94°C for 30s, 55°C for 30s and 72°C for 1.5min, finally extension at 72°C for 5min. PCR amplification products were separated with 1% agarose gel electrophoresis, and the target fragment was obtained by gel extraction and recycling, then was sent to Vazyme (Nanjing, China) for sequencing. Furthermore, PlantCARE and PLACE were used to analyze cis-acting elements in the amplified promoter sequences.

## 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 EMBOSSCpGPlot. According to the analysis of CpG island, amplification primers of bisulfite sequencing PCR (BSP) were designed by MethPrimer, MethylPrimerExpressv1.0 and PrimerPremier5.0 (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 of wheat leaf was firstly treated with EZDNAMethylation-Lightning™Kit (ZymoResearch, America), then was used as template in BSP amplification of *DREB* promoter. BSP reaction system was 30.0μl and composed of 2.0μl bisulfite-treated DNA, 1.0μl each primer (10μM), 3.0μl

10×buffer (Mg<sup>2+</sup>), 1.0μl dNTP, 1.0μl Relia™ hot-start Taq polymerized aes, 21.0μl dH<sub>2</sub>O. PCR amplification procedure was pre-denaturation at 95°C for 4min followed by 40 cycles ( 94°C for 30s, 55°C for 30s, 72°C for 40s), and final extension at 72°C for 5min. PCR amplification products were detected with 1% agarose gel electrophoresis, only target fragment was amplified, then the target fragment was cut, purified and sequenced (GENEray, 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 ratio were performed with CyMATE and Kismeth.

### **Statistical analysis**

Statistical analysis of data was performed in this study, expression 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; qRT-PCR: quantitative real-time PCR.

## **Declarations**

### **Ethics approval and consent to participate**

Not applicable.

### **Consent for publication**

Not applicable.

### **Availability of data and material**

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

### **Competing interests**

The authors declare that they have no competing interests.

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### Authors' contributions

HD and YZ conceived this experiments. YZ obtained and analyzed experiment data. HW and WJ analyzed experiment data and wrote this paper. ZD, XW, and QQ participated in text editing. LJ, YZ and HD revised this manuscript. All authors read and approved the final manuscript.

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## Supplementary Information

**Fig. S1 The nucleotide sequence and amino acid sequence of wheat *DREB* gene.** (a), (b) and (c) represented the sequence of *DREB2*, *DREB6* and *Wdreb2*, respectively. The box and underline respectively represented nuclear localization signal and AP2/EREBP domain.

**Fig. S2 Multiple alignment of wheat DREB in and its homologous sequences.** (a), (b) and (c) was the alignment between homologous sequences with *DREB2*, *DREB6* or *Wdreb2*, respectively.

**Fig. S3 The distribution of CpG island in the promoter of wheat *DREB* gene.** (a), (b) and (c) represented the prediction result of CpG island in the promoter of *DREB2*, *DREB6* or *Wdreb2*, respectively, CpG island was shown by the shaded part.

**Fig. S4 Bisulfite sequencing result for the promoter of wheat *DREB* gene.** 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.

**Table S1 The cis-acting elements in the promoter of *DREB2***

**Table S2 The cis-acting elements in the promoter of *DREB6***

Table S3 The cis-acting elements in the promoter of *Wdreb2*

Table S4 Correlation between promoter methylation and expression of *DREB* gene

Table S5 Primers used in this study

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