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# Isolation and molecular characterization of wheat (*Triticum aestivum*) Dehydration Responsive Element Binding Factor (DREB) isoforms

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**Abstract**

Due to adverse effects of abiotic stresses on plant growth and development, we focused on an effective abiotic stress responsive element known as Dehydration responsive element binding factor (DREB) in *Triticum aestivum*. The objective of the research was to isolate and molecularly characterize two wheat DREB2 (WDREB2) gene isoforms. In order to isolate WDREB2 isoforms, plants were exposed to cold stress. Total RNA was extracted from cold-treated plant leaves and first-strand cDNA was synthesized. Two isoforms were isolated by specific primers and submitted in NCBI database (Accession No. JQ004969 and HQ171443). Searching for similar sequences to isolated isoforms by NCBI BLASTn resulted in 10 sequences in *Triticum aestivum* with ≥ 95% identity and ≥ 90% coverage. Multiple alignments on homologous sequences were preformed. The results showed that all these homologous sequences are related to one definite gene, WDREB2, which has 3 different alternate spliced forms or isoforms. β Isoform that lacks transcription activation domain is inactive while α is an active isoform. In order to verify α isoform activity, WDREB2 α isoform was expressed in tomato using *Agrobacterium* mediated transformation and α isoform-expressed plants were tested. Phonotypical comparison between transgenic and normal plants under cold stress confirmed the activity of WDREB2α isoform. In addition to laboratory research, 2 available sequences, submitted as wheat DREB gene promoter (Accession No GU785009 and GU785008) were selected and promoter analysis was carried out. Several motif sites such as ABRE, C-repeat/DRE and MBS, which are important Cis acting elements in abiotic stress response were identified.

**Keywords:** Abiotic stress; Alternative splicing; α isoform; β isoform; Transcription fator; *Triticum aestivum.*
**Abbreviations:** DREB- Dehydration responsive element binding factor; WDREB- wheat dehydration responsive element binding fator; ABA- Abscisic acid; ABRE- Abscisic acid responsive element; C-repeat/DRE- Dehydration responsive element binding factor; bZIP- Basic zipper; MBS- myeloblastosis gene binding site.

## Introduction

Plants are exposed to a wide range of environmental stresses during their life. Plant growth and development are seriously affected by abiotic stresses. Abiotic stresses such as cold and drought lead to physiological and developmental changes in plants, which are due to occurring changes in plant genes expression profile (Kobayashi et al., 2004). Genes and proteins involved in compatibility and tolerance to abiotic stresses are categorized in two distinctive functional and regulatory genes groups (Liu et al., 1998) . Transcription factors which are involved in gene expression regulatory networks are categorized in the second group. It has been shown that transcription factors lead to regulation of inducible genes through interactions with Cis acting elements in the promoter region of many stress inducible genes (Sakuma et al., 2006). Molecular study of regulatory networks suggests the existence of ABA independent and ABA-dependent signal transduction pathways that convert the initial stress signal into cellular responses (Seki et al., 2007). Some transcription factors are expressed through abscisic acid pathways while others have independent expression to abscisic acid hormone (Nakashima et al., 2006). Most ABA-inducible genes such as rd22 contain a conserved cis acting element named ABRE in their promoter regions and are regulated by transcription factors such as bZIP, MYB and MYC (Shinozaki, 1997). However, ABA-independent stress inducible genes such as RD29A are regulated by other transcription factors like DREB (Narusaka et al., 2003). Regulatory networks study shows that many of these signal pathways overlapped each other (Shinozaki et al., 2000 & Knight et al., 2001). Dehydration responsive element binding factors, DREB transcription factors, are members of the AP2/ERF family which consist of many important regulatory and stress responding genes. DREB transcription factors have a sharp and transient response to abiotic stress condition and induce the expression of downstream functional genes which are involved in abiotic stresses. In fact, they specifically bind to a distinct region in the promoter of target genes known as DRE/CRT sequence, which activate the transcription of genes (Shinozaki et al., 2000). DRE region consists of 5bp conserved sequence CCGAC known as C-repeat sequence. DREB proteins bind to this region and regulate genes expression. In *Arabidopsis*, DREB/CBF contains two subclasses, DREB1/CBF and DREB2, which are induced by cold and dehydration, respectively (Shinozaki et al., 2000). The *Arabidopsis* genome contains six DREB1genes and eight DREB2 genes (Nakashima et al., 2009). DREB1/CBF and DREB2 homologue genes were identified in various plants such as

rice, wheat, diploid wheat (*Triticum monococcum*), barley, wild barley (*Hordeum spontaneum*), maize, sorghum (*Sorghum bicolor*), rye (*Secale cereale*) and oat (*Avena sativa*) (Zhao et al., 2009). HvDRF1 is identified in barley as a dehydration responsive element binding factor induced in drought and has different isofroms. These isoforms are produced through alternate splicing mechanism and are post transcriptional regulated ( Xue et al., 2004 & Terashima et al., 2009 ). ZmDREB2A in corn is responsive to cold and high temperature (Shen et al., 2003). Wheat DREB2 transcription factor (WDREB2) is homologue of DREB2A in *Arabidosis*. WDREB2 has also high homology with HvDRF1. WDREB2 transcription factor codes a DRE/CRT binding protein and have an important role in abiotic stress responses (Nakashima et al., 2009). The WDREB2 expression is activated by cold, drought, salt and exogenous ABA treatment. It activates Cold responsive/ late embryogenesis abundant (Cor/Lea) genes (Egawa et al., 2006). In this research 2 isoforms of WDREB2 were isolated from *Triticum aestivum* cDNA source and molecularly characterized. We also verified the role of WDREB2 molecularly active isoform in cold stress. Besides, according to the importance of DREB transcription factor, promoter analysis of two wheat DREB gene promoters (Accession No GU785009 and GU785008) was carried out.

## Results and Discussion

### *β Isoform isolation and molecular characterization of different isoforms*

After cold stress treatment for 4 hours, RNA extraction and cDNA synthesis, WDREB fragment, which was 1286 bp in length from the Sardari cultivar was isolated with specific primer (Table 1) and confirmed by sequencing (Fig. 1). BLASTn search was done in order to find homologous sequences in *Triticum aestivum*. Search for homologous sequences based on nucleotide similarity by NCBI BLASTn showed that there are 10 sequences in *Triticum aestivum* with ≥ 95% identity and ≥ 90% coverage to our isolated fragment (Fig. 2). These 10 sequences were selected for further sequence analysis. Multiple alignments were carried out between the isolated fragment and 10 sequences by VectorNTI9 alignment tool. Sequences comparison by alignments showed that all sequences including isolated fragment and 10 homologous sequences are identical except for two internal insertion 53 and 91 bp (Fig. 3). Isolated fragment and 4 of homologous sequences including (Accession No AY781357, AY781356, AY781351 and AY781349) had only 53 bp insertion. In this case, the existence of just 53 bp insertion results in frame shift and causes a stop codon in the exon4 position. A coded protein from this sequence lacks essential activation domain and cannot activate transcription of downstream genes. So this isoform is considered as an inactive isoform. Accession No AY781354 and AY781358 have both 53bp and 91 bp internal insertions and codes for complete protein with essential activation domain. They are considered as active isoforms. In sequences including (Accession No AY781359, AB193608, AY781350 and AY781355) neither 53bp nor 91 bp were found (Fig. 4). However, no frame-shift happens in this situation and they code for active protein with ability for transcription activation of downstream genes. We focused on inactive and one of the active isoforms. Since all of these sequences, isolated isoform and 10 sequences, were isolated from cDNA source without any intron, we examined the sequences from the point of different arrangements of exons.

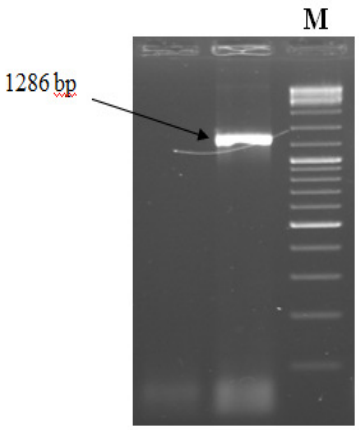


**Fig 1.** Gel electrophoresis of PCR product of WDREB2 β isoform. M = GeneRuler 100bp DNA ladder. Complete CDS of β isoform in 1286bp amplified by RT-PCR is shown.

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| JQ004969.1 | Triticum aestivum DREB AP2 binding factor beta isoform mRNA, comp | 2372 | 2372 | 100% | 0.0 | 100% |
| AY781357.1 | Triticum aestivum DREB transcription factor 5C (DREB5) mRNA, comp | 2255 | 2255 | 100% | 0.0 | 98% |
| AK332563.1 | Triticum aestivum cDNA, clone: WT004_E18, cultivar: Chinese Spring | 2060 | 2060 | 100% | 0.0 | 96% |
| AY781356.1 | Triticum aestivum DREB transcription factor 4C (DREB4) mRNA, comp | 2060 | 2060 | 100% | 0.0 | 96% |
| AB193608.1 | Triticum aestivum Wdreb2 mRNA for EREBP/AP2 type transcription fa | 1958 | 2212 | 96% | 0.0 | 100% |
| AY781359.1 | Triticum aestivum DREB transcription factor 5B (DREB5) mRNA, comp | 1956 | 2162 | 95% | 0.0 | 99% |
| AY781358.1 | Triticum aestivum DREB transcription factor 5A (DREB5) mRNA, comp | 1956 | 2258 | 100% | 0.0 | 99% |
| AY781351.1 | Triticum aestivum DREB transcription factor 3C (DREB3) mRNA, comp | 1912 | 1912 | 95% | 0.0 | 95% |
| AY781355.1 | Triticum aestivum DREB transcription factor 4B (DREB4) mRNA, comp | 1757 | 1967 | 96% | 0.0 | 96% |
| AY781354.1 | Triticum aestivum DREB transcription factor 4A (DREB4) mRNA, comp | 1755 | 2063 | 100% | 0.0 | 96% |
| HQ171443.1 | Triticum aestivum DREB2 transcription factor alpha isoform mRNA, cc | 1718 | 2024 | 91% | 0.0 | 98% |
| AY781350.1 | Triticum aestivum DREB transcription factor 3B (DREB3) mRNA, comp | 1648 | 1819 | 91% | 0.0 | 95% |
| AY781349.1 | Triticum aestivum DREB transcription factor 3A (DREB3) mRNA, comp | 1646 | 1915 | 95% | 0.0 | 95% |

**Fig 2.** Result of NCBI BLASn search for isolated isoform. 10 sequences in *Triticum aestivum* submitted as different DREBs showed ≥ 95% identity and ≥ 90% coverage to the isolated fragment (Accession No JQ004969).

By studying multiple alignment and comparing similar/dissimilar parts we supposed that sequences have different exons. 53 bp and 91 bp internal insertion were considered as exon 2 and 3 (Fig. 5). Exon 1 and exon 4 were supposed to be upstream of exon2 and downstream of exon 3 respectively. All the sequences have exon1 and 4. Differences in all sequences originated from exon2 (53 bp insertion) and exon3 (91 bp insertion) (Fig. 5). Exon 2 and 3 have fundamental role in determination of isoforms type. In order to specify these Sequences, we used α, β, γ symbols like Egawa's et al symbol for alternative spliced forms. Those sequences with 53bp internal insertion were categorized as inactive ß isoform, those with 53bp and 91 bp internal insertions categorized as α isoform and those without any insertion categorized as δ isoform (Fig. 5). Similar evidence about WDREB2 has been reported. DREB gene was studied in wheat and it was suggested that different isoforms are produced by alternative splicing mechanism (Egawa et al., 2006). Egawa et al report was based on genomic library studies and comparing genomic libraries with Expression Sequence Tags (ESTs). Our study confirmed Egawa's report. However our study was based on complete CDS isolated isofrms and we studied all relative submitted sequences for *Triticum aestivum*. In addition, Our study revealed that there are not different DREB genes in *Triticum aestivum*.

**Table 1.** Primer sequences and general information.

| Primer name | Primer sequence (5′ to 3′) | Reference |
|---|---|---|
| TaDREBF | GACAAGATTGCGAACGCTAGA | (Kobayashi *et al*., 2008) |
| TaDREBR | CCGACCAAACACCATAGACA | (Kobayashi *et al*., 2008) |
| Partial DREBF | GCCATGACGGTAGATCGG | |
| Partial DREBR | GCTGCCTCGTCATAAGCACG | |
| SDREBF | CGGGAGCCAAATCGGGTGAG | |
| SDREBR | GGTCCAAGCCATCCAGGTAGAGAGG | |
| EDREBF | AGCAGTAATCTTCCCTGTAATG | |
| EDREBR | GTTGTTGGTTCACTTCTTTCC | |

**Table 2.** Different Type of WDREB2 isoforms and exon numbers.

| Accssesion number | Description | Isoform type & exon number |
|---|---|---|
| AB193608.1 | *Triticum aestivum* Wdreb2 mRNA for EREBP/AP2 type transcription factor, complete cds | γ. Exon1,4 |
| AY781359.1 | *Triticum aestivum* DREB transcription factor 5B (DREB5) mRNA, complete cds, alternatively spliced | γ. Exon1,4 |
| AY781355.1 | *Triticum aestivum* DREB transcription factor 4B (DREB4) mRNA, complete cds, alternatively spliced | γ. Exon1,4 |
| AY781357.1 | *Triticum aestivum* DREB transcription factor 5C (DREB5) mRNA, complete cds, alternatively spliced | β. Exon 1,2,4 |
| AY781358.1 | *Triticum aestivum* DREB transcription factor 5A (DREB5) mRNA, complete cds, alternatively spliced | α. Exon 1,2,3,4 |
| AY781356.1 | *Triticum aestivum* DREB transcription factor 4C (DREB4) | β. Exon 1,2,4 |
| AY781354.1 | *Triticum aestivum* DREB transcription factor 4A (DREB4) mRNA, complete cds, alternatively spliced | α. Exon 1,2,3,4 |
| AY781350.1 | *Triticum aestivum* DREB transcription factor 3B (DREB3) mRNA, complete cds, alternatively spliced | γ. Exon 1,4 |
| AY781351.1 | *Triticum aestivum* DREB transcription factor 3C (DREB3) mRNA, complete cds, alternatively spliced | β. Exon 1,2,4 |
| AY781349.1 | *Triticum aestivum* DREB transcription factor 3A (DREB3) mRNA, complete cds, alternatively spliced | β. Exon 1,2,4 |

According to alignments of isolated and homologous sequences, the results of our studies led to the conclusion that all of these CDS are resulted from definite gene, WDREB2. Since the types of these isoforms are not determined in NCBI, the types of sequences in Genebank were identified according to alignment between isolated isoform and other homologous sequences (Table 2). Finally, isolated isoform was confirmed and registered in NCBI as ß isoform mRNA complete CDS of wheat DREB gene with (Accession No. JQ004969) (Fig. 6).

***α Isoform isolation***

In order to isolate the active isoform of the DREB gene, partial DREB primer was used for identification of the recombinant clone (Table 1). Partial DREB primer set was designed based upon multiple sequence alignments between β isolated fragment and other homologous sequences .This amplifies the end part of exon1- the first part of 4 and internal exons 2 and 3 in case they exist- and produced 550,450,384 bp fragment on α, ß and δ isoforms respectively. So the clones with a 550 bp amplified fragment were identified as α isoform (Fig. 7). After sequencing, the isoform was confirmed and registered in NCBI as α isoform mRNA complete CDS of wheat DREB gene (Accession No. HQ171443) (Fig. 8). This isoform has high homology with (Accession No AY781354 and AY781358) and has all four exons. The amino acids sequence coded by this isoform's open reading frame was determined using vectorNTI9. This protein has 393 amino acids (Fig. 9). Deduced amino acid sequence was analyzed by Pfam. Amino acids sequence 136-178 was defined as AP2 domain. AP2 region has particular motifs that are involved in binding to special sequences in the promoter region of downstream genes. Having Ap2 domain, α isoform is considered as active isoform. However β isoform lacks this domain and it is inactive. This domain is a distinctive characteristic in DREB subfamily and has an important role in transcription factor binding to the target genes promoter.

***Verification of WDREB2 α isoform activity***

In order to confirm α isoform activity, it was cloned in pBI121 vector under control of 35S promoter and *Nos* terminator. The construct was designated as *35S::Wdreb2* and used for tomato transformation. Since tomato is one of the most sensitive plants to abiotic stresses particularly cold,

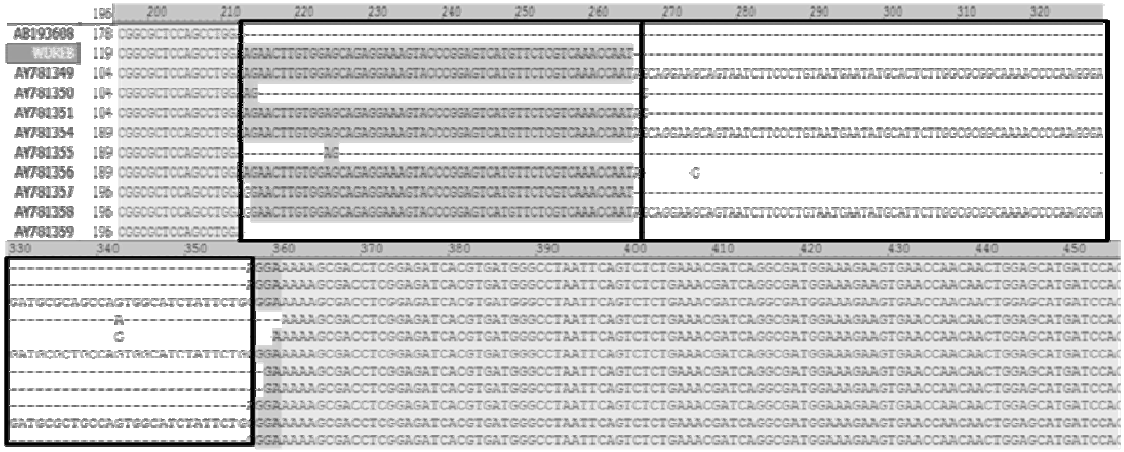

**Fig 3.** Multiple alignment between the WDREB2 and 10 homologous sequences by VectorNTI9 alignment tool. All sequences including isolated fragment and 10 homologous sequences are identical except for two internal 53 and 91 bp insertions. The box indicates two internal insertion 53 and 91 bp.


**Fig 4.** 2 Internal insertions in WDREB2. The arrows indicate two internal insertions 53 and 91bp which may not exist in different isoforms. α isoform has both 53bp and 91bp internal insertions, ß isoform has only 53bp insertion and δ isoform has none of these insertions.

this plant was used for α isoform activity test. *35S: Wdreb2* construct was transferred into tomato genome by *Agrobacterium tumefaciens*-mediated transformation. Expression of α isoform WDREB2 in tomato was just used in order to verify if this isoform, which is structurally active with essential activation domain, is really functional and can cause cold stress tolerance. After kanamycin selection, the regenerated tomatoes were analyzed to identify transgenic plants. In order to verify the existence of WDREB2 α isoform gene, PCR and RT-PCR with specific primer were carried out (Fig. 10and11). To confirm α isoform gene expression and activity, bioassay test under cold stress treatment was performed. Phonotypical characteristics between normal and transgenic plants were compared under cold stress. The bioassay test was just used as a kind of quantal assay, testing the response of α isoform-expressed and non-expressed plants to cold stress. The results were either tolerance or intolerance with adverse phenotypical effects. To examine the survival ability of non-transgenic and transgenic plants, the plants were cultured in the same composition soil and the same pots. Under cold treatment at 4° C for different time intervals (4, 10, 24 hours), significant differences between α isoform-expressed tomatoes and the control plant, were observed. After being exposed to cold stress, it was observed that leaves of non-transgenic plant became wilted and curled, whereas the transgenic plants did not (Fig. 12). After 24 hours exposing to cold, leaves and shoots of non transgenic plants were dried irreversibly.

***Promoter analysis of DREB gene***

Although high homology between selected wheat DREB genes (Accession No. GU785009 and GU785008) with our isoforms was not observed using Blast search and alignment tools, we chose these gene promoters for analysis since they belong to DREB subfamily. All of these subfamily genes have the same and distinct characteristics. Besides, these were the only wheat DREB promoter sequences submitted in the genebank. Promoter analysis of (Accession No GU785008 and GU785009) submitted as DREB2 and DREB3 promoters respectively are illustrated in (Table 3and4). As it can be observed, there is one C-repeat/DRE motif (TGGCCGAC) in the promoter region of DREB3, which is a critical region for DREB activity and has an important role in cold and dehydration-responsiveness. The DRE/CRT motif is recognized by DREB/C-repeat-binding factor (CBF) proteins. It was an interesting point to find a DRE/CRT motif in a DREB promoter gene since it binds to the same motif in the promoter region of downstream stress inducible genes. So, it may predict that the expression of DREB gene is regulated by some other transcription factors such as DREB which interact with this unique motif. In addition, 2 and 5 ABRE cis-acting elements were identified in different positions of DREB2 and DREB3 promoters, respectively. ABRE cis-acting elements are involved in the abscisic acid responsiveness. Although DREB genes are categorized as the transcription factors involved in ABA independent pathways in previous studies (Nakashima et al., 2009; Shinozaki and Yamaguchi- Shinozaki 2000; Chen et al, 2008), these results reject this idea due to the existence of ABRE elements in the promoter region. ABRE motifs are recognized by AREB / ABF proteins. These proteins bind specifically to ABRE motifs in the promoter region of genes that are expressed in ABA dependent pathways (Nakashima et al., 2000). As Shinozaki et al reported in (2000), ABA dependent and independent pathways overlap in many cases.

Moreover, the result from the promoter analysis of DREB promoter showed that 3 and 2 MBS motifs are located in the wheat DREB3 and DREB2 promoters. In fact, MBS are appropriative binding sites for MYB transcription factors which have a major role in abiotic stresses responses (Tuteja, 2007). In addition, 1 LTR motif site was found in the DREB2

**Table 3.** Promoter analysis of wheat DREB2 gene (Accession No GU785008). Different Cis acting elements with their sequences and functions in the promoter are shown**.**

| Site Name | Motif number | Sequence | function |
|---|---|---|---|
| ABRE | 5 | CGTACGTGCA | cis-acting element involved in the abscisic acid responsiveness |
| ACE | 3 | ACGTGGA | cis-acting element involved in light responsiveness |
| Box II | 2 | CCACGTGGC | part of a light responsive element |
| C-repeat/DRE | 1 | TGGCCGAC | regulatory element involved in cold- and dehydration-responsiveness |
| CAT-box | 2 | GCCACT | cis-acting regulatory element related to meristem expression |
| CCGTCC-box | 2 | CCGTCC | cis-acting regulatory element related to meristem specific activation |
| CGTCA-motif | 4 | CGTCA | cis-acting regulatory element involved in the MeJA-responsiveness |
| G-Box | 11 | CACGTG | cis-acting regulatory element involved in light responsiveness |
| GA-motif | 1 | AAAGATGA | part of a light responsive element |
| GAG-motif | 2 | AGAGATG | part of a light responsive element |
| GARE-motif | 1 | TCTGTTG | gibberellin-responsive element |
| GC-motif | 2 | CCACGGGG | enhancer-like element involved in anoxic specific inducibility |
| GCN4_motif | 2 | TGTGTCA | cis-regulatory element involved in endosperm expression |
| GT1-motif | 2 | GGTTAAT | light responsive element |
| MBS | 3 | CGGTCA | MYB Binding Site |
| OCT | 1 | CGCGGATC | cis-acting regulatory element related to meristem specific activation |
| Skn-1_motif | 6 | GTCAT | cis-acting regulatory element required for endosperm expression |
| TCA-element | 1 | CCATCTTTTT | cis-acting element involved in salicylic acid responsiveness |
| TCCC-motif | 1 | TCTCCCT | part of a light responsive element |
| TGA-element | 1 | AACGAC | auxin-responsive element |
| TGACG-motif | 4 | TGACG | cis-acting regulatory element involved in the MeJA-responsiveness |
| as-2-box | 1 | GATAatGATG | involved in shoot-specific expression and light responsiveness |
| box II | 1 | TCCACGTGGC | part of a light responsive element |
| chs-CMA2a | 1 | GCAATTCC | part of a light responsive element |
| circadian | 1 | CAANNNNATC | cis-acting regulatory element involved in circadian control |
| rbcS-CMA7a | 1 | GGCGATAAGG | part of a light responsive element |

**Table 4.** Promoter analysis of wheat DREB3 gene (Accession No GU785009). Different Cis acting elements with their sequences and functions in the promoter are shown.

| Site Name | Motif number | Sequence | function |
|---|---|---|---|
| ABRE | 2 | ACGTGGC | cis-acting element involved in the abscisic acid responsiveness |
| ACE | 1 | AAAACGTTTA | cis-acting element involved in light responsiveness |
| Box | 4 | ATTAAT | part of a conserved DNA module involved in light responsiveness |
| G-Box | 10 | CACGTT | cis-acting regulatory element involved in light responsiveness |
| GA-motif | 1 | AAGGAAGA | part of a light responsive element |
| GARE-motif | 1 | TCTGTTG | gibberellin-responsive element |
| MBS | 2 | CAACTG | MYB binding site involved in drought-inducibility |
| P-box | 1 | CCTTTTG | gibberellin-responsive element |
| TCA-element | 2 | GAGAAGAATA | cis-acting element involved in salicylic acid responsiveness |
| TGA-element | 1 | AACGAC | auxin-responsive element |

promoter gene that is responsible for low temperature responsiveness. Multiple putative *cis*-acting elements, which are involved in light responses such as G-Box, Box II and ACE were detected within the promoter. The exact number of *cis*-acting regulatory elements and their position within the promoter region are listed in (Table 3and4). Some other regulatory elements related to hormone responses like GARE-motif and TGA-element were found in these 2 promoter regions. Such regulatory elements are responsible for gibberellins and auxin responses.

## Materials and methods

### *Plant Materials and Cold Stress Treatment*

Seedlings of native Iranian cultivar, Sardari, of bread wheat (*Triticum aestivum* L.), which is one of the most abiotic stress tolerant of native cultivars were grown at 25ºC for10 days. For cold stress treatment, plants were placed in a 4ºC chamber and sampling was carried out after 4 hours from plant leaves. Samples were fixed in liquid nitrogen and preserved at -70 ºC for RNA extraction. For transgenic plants bioassay, regenerated tomatoes and non-transgenic types were placed in a 4ºC chamber for cold stress treatment. These plants were cultured in similar pots containing the same composition of peat, vermiculite and sand to avoid environment interaction.

### *Primer screening and PCR amplification*

Specific primers for WDREB2 gene were used. In order to distinguish 2 isoforms of WDREB2 genes partial primer set were designed with VectorNTI9 software based upon multiple sequence alignments between β isolated fragment and other homologous sequences. To verify WDREB2 gene integration into transgenic plants genome and verification of WDREB2 gene expression in transgenic plants, SDREB and EDREB primer set were used and designed with allel-ID5 software (Table1). The PCR were carried out as follows: 3 min at 94 ºC for initial denaturation, 35 cycles at 94°C (1min), 55 °C (1min), 72°C (2min) and a final extension at 72°C for 10 min. PCR products were separated on 1% agarose gels. $\alpha$ and β isoform were cloned into pTZ vector by PCR InsTA clone $^{TM}$ Kit (Fermentas) for sequencing.

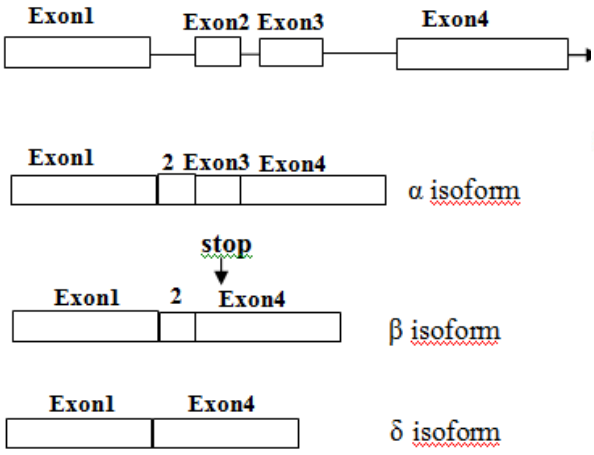


**Fig5.** Different Types of isoforms by different exons. 53bp and 91bp internal insertion were considered as exon2 and 3. Exon1 and 4 were supposed to be upstream of exon2 and downstream of exon3 respectively. All the sequences have exon1 and 4. Differences in all sequences originated from exon2 (53 bp insertion) and exon3 (91 bp insertion).

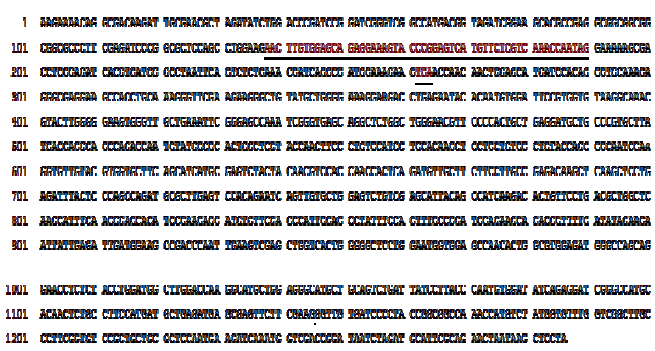

**Fig 6.** WDREB2 β isoform sequence (Accession No. JQ004969). Exon2 (53 Bp insertion) and the stop codon are underlined. The existence of just 53 bp insertion results in frame shift and causes a stop codon in the exon4 position.

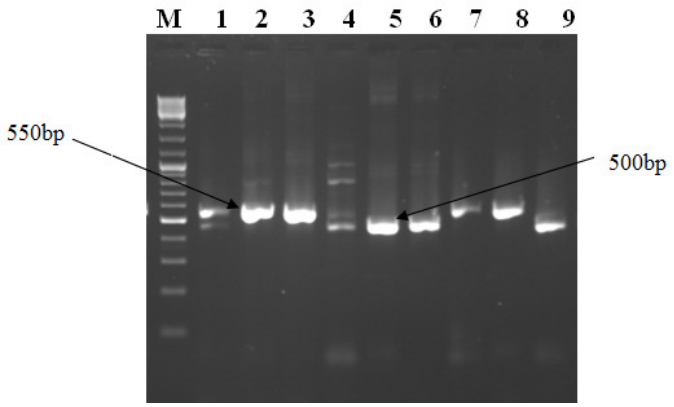


**Fig 7.** Gel electrophoresis of PCR product of recombinant bacterial clones under primer Partial DREB in order to isolate α isoform. 1 = GeneRuler 100bp DNA ladder. 3,4 indicates 550 bp fragment related to clones containing α isoform. 6,7 indicates 500 bp fragment related to clones containing B isoform.

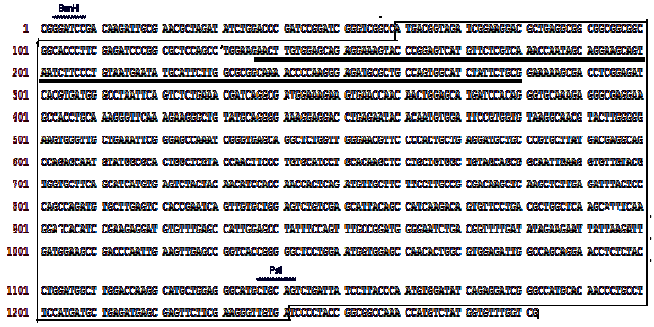

**Fig 8.** WDREB2 α isoform sequence (Accession No. HQ171443). The box indicates the 1182-bpopen reading frame. The underlines indicate exon 2&3.

***DNA, RNA Extraction and cDNA synthesis***

Total RNA was extracted from leaves using (-Plus) RNX$^{TM}$ kit (cinagene, Tehran, Iran). First cDNA strand was synthesized by cDNA synthetase fermentas kit using MuLV and oligo dT primer. In order to verify WDREB gene integration in transgenic tomatoes, genomic DNA was extracted using CTAB method (Gawel et al., 1991).

***Sequence Analysis***

BLAST search was done to align our isolates with those already existing in the NCBI genebank. In addition, multiple alignments between homologous sequences and isolated ones were done using Mega4 software. Deduced amino acid sequence analyses were also carried out by Pfam site. http://pfam.sanger.ac.uk/

***DNA construction and Agrobacterium tumefaciens Mediated Transformation***

As described, β isoform lacks essential domain for transcription activation and is an inactive isoform while α isoform seems to be active. So α isoform which was isolated by specific primer set, cloned in pBI121 binary vector under the control of 35s promoter. This construct was used for tomato transformation in order to verify α isoform activity.

***Promoter analysis of wheat DREB gene***

In addition to laboratory research, 2 available sequences in NCBI, submitted as wheat DREB gene promoter (Accession No. GU785009 and GU785008) were selected and promoter analysis was carried out. These two genes were determined as drought responsive element binding factor with promoter region. NBlast search and alignments using NCBI database

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  1 MTVDRKDAEA AAAAAPFEIP ALQPGRTCGA EESTRSHVLV KPIAGSSNLP
 51 CNEYAPLARQ NPKGDALPVA SILRKKRPRR SRDGPNSVSE TIRRWKEVNQ
101 QLEHDPQGAK RARKPPAKGS KKGCMQCKGG PENTQCGFRG VRQRTWGKWV
151 AEIREPNRVS RLWLGTFPTA EDAARAYDEA ARAMYGALAR TNFPVHPAQA
201 PAVAVAAAIE GVVRGASASC ESTTTSTNHS DVASSLPRQA QALEIYSQPD
251 VLESTESVVL ESVEHYSHQD SVPDAGSSIS RSTSEEDVFE PLEPISSLPD
301 GESDGFDIEE LLRLMEADPI EVEPVTGGSN NGGANTGVEI GQQEPLYLDG
351 LDQGMLEGML QSDYFYPMNI SEDRAMHNPA FHDAEMSEFF EGL*
```

**Fig 9.** Deduced amino acid sequence from WDREB2 α isoform. The underline indicates amino acids 136-178 defined as AP2 domain.

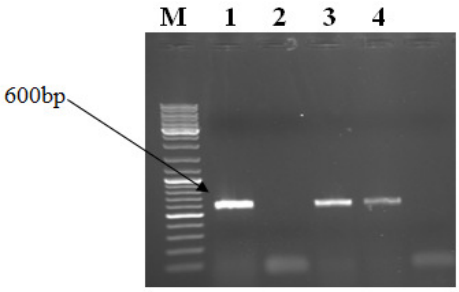


**Fig 10.** Gel electrophoresis of PCR product of WDREB2 gene under SDREB primer. M = GeneRuler 100bp DNA ladder. 1 showes α isoform fragment in wheat as a positive control. 2 indicates normal tomato in which targeted band is absent. 3&4 indicates α isoform-expressed tomato. Wheat and α isoform-expressed tomatoes has a WDREB2 gene and relative 600bp fragment was amplified by specific primer while normal tomatoes do not have this gene.

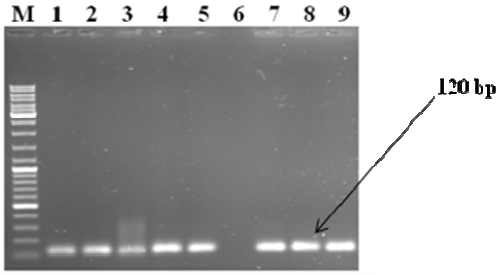


**Fig 11.** Gel electrophoresis of RT-PCR product of WDREB2 gene under EDREB primer. M = GeneRuler 100bp DNA ladder. 1-5,8,9 lanes shows 120bp α isoform fragment in α isoform-expressed tomatoes. 7 showes α isoform fragment in wheat as a positive control.

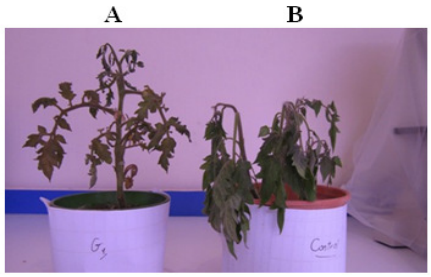


**Fig 12.** Phonotypical comparison between normal and α isoform-expressed tomatoes. A α isoform-expressed plant shows more tolerance to 4°C cold stress after 10 hours. B normal tomatoes, significant stress effects such as wilting in leaves and stem can be observed.

and Mega4 were carried out to verify the homology of isolated isforms with this gene. Promoter analysis was carried out via the Plant CARE site (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

## Conclusion

Two isoforms of WDREB2 were isolated (Accession No. JQ004969 and HQ171443) and molecularly characterized. Multiple alignments on homologous sequences to isolated CDS showed that all 10 homologous sequences are related to one definite gene, WDREB2, which has 3 different alternate spliced forms or isoforms. β Isoform that lacks transcription activation domain is inactive while α is an active isoform. The isoform type of all submitted CDS as *Triticum aestivum* DREB transcription factor in NCBI was determined. The activity of α isoform was confirmed through its expression in tomato.


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