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Isolation, promoter analysis and expression profile of *Dreb2* in response to drought stress in wheat ancestors

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ABSTRACT

Drought is one of the most important abiotic stresses, constraining crop production seriously. The dehydration responsive element binding proteins (DREBs) are important plant-specific transcription factors that respond to various abiotic stresses and consequently induce abiotic stress-related genes that impart stress endurance in plants. Wild species are naturally exposed to various abiotic stresses and potentially harbor suitable alleles through natural selection. In this study we isolated and characterized *Dreb2* from *Triticum urartu* (GenBank: KF731664), *Aegilops speltoides* (GenBank: KF731665) and *Aegilops tauschii* (GenBank: KF731663), the A, B and D genome ancestors of bread wheat, respectively. Analysis of over 1.3 kb upstream region of the gene revealed the presence of several conserved *cis*-acting regulatory elements including ABA-responsive elements, low temperature responsive elements, and several light and environmental signaling related motifs potentially vindicate *Dreb2* responses to environmental signals. Moreover, the gene exhibited an alternative splicing, conserved among orthologous genes in grasses, and produced a non-functional isoform due to splicing in an exon resulted frame-shift creating an early stop codon before the functional domain. The expression analysis of *Dreb2* under normal and different levels of dehydration stress conditions indicated that the two active spliced isoforms are up-regulated when the plant exposed to drought stress whereas the non-functional isoform is downregulated in severe drought.

1. Introduction

As sessile organisms, plants are constantly challenged by a wide range of environmental stresses. Abiotic stresses such as drought, high salinity, and cold are common adverse environmental conditions that significantly influence plant growth and productivity worldwide (Lata and Prasad, 2011).

Progress in developing abiotic stress tolerance in crops is a challenge for plant breeders due to the multigenic nature of abiotic stress responses, low heritability, and high genotype × environment interactions (Fleury et al., 2010). Therefore, the mechanisms underlying stress tolerance and adaptation have long been the focus of intensive researches (Mir et al., 2012; Umezawa et al., 2006). Large-scale transcriptome analysis has revealed activation of many genes by abiotic stresses that can broadly be classified into two groups. One group constitutes genes that encode proteins to protect the cells from the effects of water stress (reviewed by dos Reis et al., 2012) and a second group of genes comprises regulatory proteins that further regulate stress signal transduction and modulate gene expression (Agarwal P.K. et al., 2006; Reguera et al., 2012; Shinozaki and Yamaguchi-Shinozaki, 2007). The second group controls the expression of the first group. Transcription factors (TFs) interact with *cis*-elements in the promoter regions of various stress-responsive genes to regulate their expression and are thought to be optimal targets for engineering broader stress response (Cattivelli et al., 2008; Golldack et al., 2011; Hussain et al., 2011; Lindemose et al., 2013).

The dehydration-responsive element-binding (DREB)/C-repeat binding factor (CBF) proteins, initially identified in *Arabidopsis*, are a group of TFs containing a single APETALA2 (AP2) DNA binding (Liu et al., 1998). DREBs play a key role in imparting tolerance to multiple stresses in plants, generally in an ABA-independent manner (Akhtar et al., 2012; Xu M. et al., 2011). DREBs interact with dehydration/C-repeat responsive element (DRE/CRT) *cis*-elements identified in the

Abbreviations: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to RNA; mRNA, messenger RNA; nt, nucleotide(s); UTR, untranslated region(s); TFs, transcription factors; DREB, dehydration-responsive element-binding; AP2, APETALA2; CBF, c-repeat binding factor; SNPs, single nucleotide polymorphisms; ERF, ethylene responsive factor; LT, low temperature; LTR, low temperature regulatory; RWC, relative water content; ABRE, ABA-responsive elements; ABRE-CE, ABRE coupling element(s); LEA, late embryogenesis abundant; COR, cold-responsive.

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promoter regions of various target genes such as rd29a, genes encoding late embryogenesis abundant (LEA) and cold-responsive (COR) proteins (Kobayashi et al., 2008) known to act in stress responses to activate a cascade of endogenous genes, providing tolerance to stresses (reviewed by Mizoi et al., 2012; Lindemose et al., 2013). DREBs in *Arabidopsis* are divided into six subgroups (A-1 to A-6) in which DREB2/CBF2 are known to respond to dehydration and high salinity, but not cold in an ABA-independent manner (Liu et al., 1998; Mizoi et al., 2012). However, a cross-talk with various abiotic signals and also ABA-dependent pathways has been reported in various species (Egawa et al., 2006; Roychoudhury et al., 2013; Shinozaki and Yamaguchi-Shinozaki, 2000, 2007; Zhao et al., 2010).

Genetic engineering of DREBs can improve tolerance to drought, salinity, heat, and cold (Todaka et al., 2012). However, the use of different promoters resulted in contradictory effects on stress tolerance and plant growth. Transgenic plants constitutively over-expressing DREBs exhibit hindered plant growth and delayed flowering (Agarwal P.K. et al., 2006; Kovalchuk et al., 2013; Morran et al., 2011; Saint Pierre et al., 2012; Shen et al., 2003) while expression of DREBs under the control of drought-inducible promoters significantly improved drought tolerance without stunting plant development (Bihani et al., 2011; Kovalchuk et al., 2013; Reis et al., 2014). *Cis*-acting regulatory elements of promoter have great impact on gene expression and consequently gene function, in particular in stress responsive genes; thus understanding regulatory gene network in stress responsive cascades depends on successful functional analyses of *cis*-acting elements (Yamaguchi-Shinozaki and Shinozaki, 2005). Studying the natural regulatory elements present in *Drebs* promoter is expected to advance our understanding of their complex regulatory networks of abiotic stress responses and the cross-talks between different signaling pathways during the adaptation of plants to various abiotic stresses (Lata and Prasad, 2011).

Bread wheat (*Triticum aestivum* L.) is an allohexaploid species formed through interspecies hybridization of *Triticum urartu* (AA genome) with probably *Aegilops speltoides* (BB genome) (approximately 0.36 ± 0.18 MYA) followed by one or more rare hybridization of the resulted wild tetraploid wheat (AABB) with *Aegilops tauschii* (DD genome) 7000 to 10,000 years ago (Dvorak and Akhunov, 2005; McFadden and Sears, 1944). Gene flow from *Ae. tauschii*, as the last genome joint to bread wheat, was an important source of wheat genetic diversity (Wang J. et al., 2013). *Ae. tauschii* distributed over a wide spectrum of climatic conditions envisage harboring potentially useful alleles of different stress tolerant genes that can provide new genetic variability for adaptation to stress-prone environments in wheat breeding programs (Chen et al., 2008; Dubcovsky and Dvorak, 2007; Trethowan and Mujeeb-Kazi, 2008).

Because of their critical role in abiotic stresses response, DREBs have been extensively studied in different plant species (Mizoi et al., 2012). However, the potential regulatory elements of their promoter are less studied and also our knowledge about the ancestors of current DREBs in crops is limited. To explore *Dreb2* gene in wheat ancestors, we isolated and characterized the orthologous genes of wheat *WDreb2*, previously demonstrated to respond to various abiotic stresses and to improve drought tolerance (Egawa et al., 2006; Kobayashi et al., 2008), in *T. urartu*, *Ae. speltoides* and *Ae. tauschii*. The potential regulatory elements present in their promoter were investigated and the quantitative differential expressions of the alternatively spliced isoforms of the gene were evaluated in response to various levels of dehydration conditions.

2. Material and methods

2.1. Isolation and characterization of AetDREB2

Total RNA was extracted from dehydrated seedlings of *Ae. tauschii* ssp. *tauschii* (accession no. GS2505). First-strand complementary DNA (cDNA) was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) and *AetDreb2* was isolated via designed primers on the conserved regions of common wheat *WDreb2* (GenBank: AB193608), barley *HvDRF1* (GenBank: AY223807) and available ESTs in database (Supplementary Table 1). The PCR amplicons were separated on 1.5% gel and three different resolved fragments were isolated separately from the gel, ligated in pGEM easy vector (Promega) and upon cloning in *Escherichia coli*, sequenced using Sanger sequencing (ABI 3130xl DNA Sequencer). Total genomic DNA was isolated from *Ae. tauschii* seedling plants using modified CTAB method (Murray and Thompson, 1980). The full-length genomic sequence of the *AetDreb2* gene was isolated using different primer pairs designed on the available cDNA sequences (Supplementary Table 1) and applied on genomic DNA. Exon/intron structure of the gene was obtained by alignment of three different cDNAs and genomic sequence in NCBI BLASTn. The complete *AetDreb2* orthologs were isolated from *T. urartu* (accession no. GS2214) and *Ae. speltoides* (accession no. GS2150) using the primers reported in Supplementary Table 1. The seeds were provided by Scuola Superiore Sant' Anna.

2.2. Isolation and characterization of the promoter

The 5′ upstream region of *AetDreb2* was isolated using a GenomeWalker™ Universal kit (Clontech, Canada) following the manufacturer's protocol. The putative *cis*-acting regulatory elements were predicted using PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/PLACE/) databases. The corresponding promoter was isolated from *T. urartu*, *Ae. speltoides* and wheat (*T. aestivum* cv. Chinese spring) using primers reported in Supplementary Table 1.

2.3. Protein structure and phylogenetic analysis

The molecular weight and isoelectric point of the protein were calculated in http://isoelectric.ovh.org/files/calculate.php. Secondary structure of putative proteins, position of alpha-helix and beta sheets in the AP2 domain and 3D structure of the protein were predicted using the PredictProtein server (http://www.predictprotein.org) and ExPASy website (http://www.expasy.ch). The schematic helical wheel diagram of AP2 domain was constructed in http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html. A homology modeling analysis of *AetDREB2* was carried out using the SWISS-MODEL program (http://swissmodel.expasy.org/).

The phylogenetic analysis of DREB2 related genes, including six *Arabidopsis* DREBs which belong to 1A to 6A sub-groups (Mizoi et al., 2012), was carried out on amino acid sequences of AP2 DNA-binding domain and the unambiguous positions aligned in CLUSTALW 2.0 (Larkin et al., 2007), using neighbor-joining method in MEGA6 (Tamura et al., 2013).

2.4. Dehydration stress conditions and sample preparation

To study specifically the expression profile of the three spliced forms of *Dreb2* in response to dehydration stress, seeds were germinated in petri-dish and seedlings were transferred in PERLIGRAN (Deutsche Perlite, Dortmund, Germany) in a growth chamber under 16/8 h light and darkness regime at 25 °C. Seedlings at two-leaf stage with similar leaf size were selected for control and dehydration treatments. Plants were placed on a dry filter paper at room temperature and leaf samples were collected every 30 min to have a different range of RWC; the first leaf of each individual sample was collected for RNA extraction and was immediately frozen in liquid nitrogen and stored at −80 °C while the second fully expanded leaf was collected to measure the leaf RWC. To measure RWC, the leaf fresh weight (FW) was immediately recorded; leaves were soaked overnight in distilled water at room temperature in darkness and were dried for 48 h at 80 °C to measure turgid weight (TW) and dry weight (DW), respectively. RWC was calculated according to the Barrs and Weatherley (1962) formula: RWC (%) =