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RESEARCH PAPER

Isolation and molecular characterization of *ERF1*, an ethylene response factor gene from durum wheat (*Triticum turgidum* L. subsp. *durum*), potentially involved in salt-stress responses

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Abstract

As food crop, wheat is of prime importance for human society. Nevertheless, our understanding of the genetic and molecular mechanisms controlling wheat productivity conditions has been, so far, hampered by the lack of sufficient genomic resources. The present work describes the isolation and characterization of *TdERF1*, an *ERF* gene from durum wheat (*Triticum turgidum* L. subsp. *durum*). The structural features of *TdERF1* supported the hypothesis that it is a novel member of the *ERF* family in durum wheat and, considering its close similarity to *TaERF1* of *Triticum aestivum*, it probably plays a similar role in mediating responses to environmental stresses. *TdERF1* displayed an expression pattern that discriminated between two durum wheat genotypes contrasted with regard to salt-stress tolerance. The high number of *cis*-regulatory elements related to stress responses present in the *TdERF1* promoter and the ability of TdERF1 to regulate the transcription of ethylene and drought-responsive promoters clearly indicated its potential role in mediating plant responses to a wide variety of environmental constrains. *TdERF1* was also regulated by abscisic acid, ethylene, auxin, and salicylic acid, suggesting that it may be at the crossroads of multiple hormone signalling pathways. Four *TdERF1* allelic variants have been identified in durum wheat genome, all shown to be transcriptionally active. Interestingly, the expression of one allelic form is specific to the tolerant genotype, further supporting the hypothesis that this gene is probably associated with the susceptibility/tolerance mechanism to salt stress. In this regard, the *TdERF1* gene may provide a discriminating marker between tolerant and sensitive wheat varieties.

Key words: BAC sequencing, *cis*-regulatory elements, ethylene, ethylene response factor, salt stress, transactivation assay, *Triticum durum*, wheat.

Abbreviations: ABA, abscisic acid; ABRE, ABA-responsive element; BAC, bacterial artificial chromosome; CRT, C-repeat element; DRE, dehydration-responsive element; ERF, ethylene response factor; EST, expressed sequence tag; GFP, green fluorescent protein; GR, Grecale; LTRE, low-temperature-responsive element; NLS, nuclear localization signal; OR, Om Rabiaa; SA, salicylic acid; SD, standard deviation; TF, transcription factor; UTR, untranslated region.

Introduction

The importance of wheat in economical and nutritional terms has made this crop a major target of different strategies aimed at increasing yield. Drought and salinity represent two major environmental factors that currently reduce wheat productivity. It is particularly critical in low-rainfall areas such as Mediterranean countries including southern Europe and northern Africa where drought and salinity are major constraints affecting cereal crops. Durum wheat (*Triticum turgidum* L. subsp. *durum*, or *Triticum durum*), also known as macaroni wheat, is the hardest of all wheat varieties and is characterized by its high protein content. To date, durum wheat is the only tetraploid wheat species of commercial importance that is widely cultivated. However, facing the rapidly growing food demand of the expanding world population, the harsh environmental conditions associated with global climate change represent a real obstacle to the increase in wheat production. In particular, salinity and drought have enormous impact on wheat and other crop yields. They exert osmotic stress and cause water deficit in plants and consequently affect plant growth and development (Jin *et al.*, 2013). These impacts have driven plants to evolve various survival strategies (Dong *et al.*, 2012). Thus, it is critical to decipher the mechanisms underlying the responses of wheat to environmental stresses in order to design efficient strategies dedicated to improving stress tolerance and crop productivity in these species. A common feature associated with plant adaptation to adverse environmental conditions is the regulation of genes involved in stress tolerance. Transcription factors (TFs) are known to mediate stress signal transduction pathways regulating downstream target gene expression and lead to stress tolerance (Shinozaki and Dennis, 2003; Chen and Zhu, 2004; Yamaguchi-Shinozaki and Shinozaki, 2005). Among these, the AP2/ERF TFs are specific to plants and comprise a large number of family members, reaching 163 in the *Arabidopsis thaliana* model plant. Ethylene response factor (ERF) and dehydration-responsive element (DRE)-binding protein (DREB)/CBF subfamilies belonging to the AP2/ERF family (Nakano *et al.*, 2006) are known to play crucial roles in plant adaptation to several environmental stresses. DREB/CBF members are important in abiotic stress tolerance such as osmotic and cold stress (Morran *et al.*, 2011). They have been reported to recognize the DRE/C-repeat (CRT) element present in the promoter of target genes (Stockinger *et al.*, 1997). On the other hand, ERFs bind to the GCC-box found in the promoter of ethylene-inducible and pathogen-related genes (Ohme-Takagi and Shinshi, 1995), thus mediating biotic stress responses. ERFs have also been shown to be involved in abiotic stress and, for instance, TaERF3, known as a pathogen-inducible TF (Zhang *et al.*, 2007), promotes tolerance to salt and drought stresses in hexaploid wheat (*T. aestivum*) (Rong *et al.*, 2014). So far, studies on AP2/ERF TFs in wheat remain scarce. In bread wheat, 117 AP2/ERF sequences have been identified, including 57 DREBs and 47 ERFs. Among the latter, only four *ERF* genes (*TaERF1*, *TaERF3*, *TaPIEP1*, and *TaERF4*) have been characterized and shown to be involved in abiotic stress responses (Xu *et al.*, 2007; Zhang *et al.*, 2007; Dong *et al.*, 2012; Rong *et al.*, 2014). Likewise, two *DREB* genes, TaDREB2 and TdDREB3, were shown to be involved in drought and frost stresses (Morran *et al.*, 2011). Yet, sequencing of the durum wheat genome remains a difficult task, and lacking such an essential resource hampers the identification of the genetic factors and mechanisms underlying responses to environmental stresses. In durum wheat, very few AP2/ERF sequences have been identified to date, and only three genes belonging to the DREB subfamily shown to be involved in drought stress have been partially characterized: TdDRF1 (Latini *et al.*, 2007. 2008. 2013., TdDREB2, and TaDREB3 (Morran *et al.*, 2011). By contrast, the literature is lacking any report describing the characterization of an *ERF* gene from tetraploid wheat species.

In the present study, we identified, isolated, and performed a partial functional characterization of the first *ERF* gene in durum wheat, named *TdERF1*. *TdERF1* displayed a strong homology with TaERF1 from *T. aestivum* (Xu *et al.*, 2007). It was induced by high-salt treatment in two durum wheat varieties, Grecale (GR) and Om Rabiaa (OR), shown to be salt-tolerant and -sensitive, respectively, suggesting that *TdERF1* may be involved in salt-stress responses. *TdERF1* is also regulated by various phytohormones including abscisic acid (ABA) and salicylic acid (SA), ethylene, and auxin, suggesting that this *ERF* gene is at the cross-roads of multiple hormone signalling pathways. More interestingly, the TdERF1 protein was present in four allelic forms that behaved differently when challenged with GCC-box- and DRE-containing promoters. These data provide new leads towards improving durum wheat tolerance to abiotic stresses.

Materials and methods

Three genotypes of tetraploid *T. turgidum* L. subsp. *durum* ($2n=4\times=28$) were used in this study; Langdon LDN#65 for PCR bacterial artificial chromosome (BAC) screening, and OR and GR for functional analyses. The latter two were a local Tunisian variety and an Italian variety introduced in Tunisia, respectively.

BAC library and screening strategy

The BAC library of tetraploid wheat LDN#65 contains 516 096 clones individually maintained on 384-well plates. The average insert size of BAC clones was estimated to be 131 kb resulting in a coverage of five genome equivalents (Cenci *et al.*, 2003). The library is organized in a two-dimensional pool and BAC library screening was performed as described by Cenci *et al.* (2003 and 2004). (Supplementary Fig. S1 at *JXB* online).

PCR primer design and PCR amplification for BAC screening

In order to design primers that amplified the exon parts of the AP2/ERF gene family in durum wheat, we aligned all the sequences of AP2/ERF retrieved from several databases using the *T. aestivum* AP2/ERF conserved domain as the query (NCBI: http://www.ncbi.nlm.nih.gov/; GrainGenes: http://wheat.pw.usda.gov/GG2/index.shtml; Gramene: http://www.gramene.org/; TIGR: http://www.jcvi.org/; HarvEST: http://harvest.ucr.edu/; Phytozome: http://www.phytozome.net/; PTDB: http://planttfdb.cbi.edu.cn/; and plant GDB: http://www.plantgdb.org/). Key words were also used to expand investigations

trying to find genes without or with truncated conserved domain. Multiple alignments of the DNA sequences were performed by Clustal W software (Larkin *et al.*, 2007). In order to avoid an amplification of the exon–intron junction, prediction of the exon boundaries within Triticeae expressed sequence tags (ESTs) were performed based on rice genomic sequences, and PCR primers were then designed to cover exons of the entire selected sequence using Perl primer tool v.1.1.2.1 (Marshall, 2004) (Supplementary Table S1 at *JXB* online). Primers were tested on genomic DNA of LDN#65 before BAC library screening. Total DNA was extracted from wheat Langdon 65 variety using the Plant DNAzol® reagent. PCR conditions used were the following: initial denaturation at 95 °C for 5 min, followed by 45 cycles of 20 s at 95 °C, 16 s at 60 °C, and 20 s at 72 °C, performing a melting curve with an increment of 0.5 °C per cycle. PCR products for the selected BACs were separated by electrophoresis (2% agarose).

BAC sequencing, assembly, and annotation via the TriAnnot Pipeline

BAC DNA was extracted using a NucleoSpin® 96 Flash kit (Macherey-Nagel) and the insert size was estimated with *Not*I digestion (FastDigest NotI; fermentas). The BAC DNA was sequenced with 454 technology on a GS Junior Roche system (Kit 454 Titanium; Roche). The sequence data assembly was performed with Newbler software sold by 454 Life Sciences/Roche for 454 data (Veras *et al.*, 2013). Sequenced BAC DNA was analysed using TriAnnot Pipeline v.3.8 improved for wheat species (http://wheat-urgi.versailles.inra.fr/Tools/TriAnnot-Pipeline) allowing annotation, masking of transposable elements, and gene structure organization (Leroy *et al.*, 2012).

Isolation of TdERF1 *cDNA sequences from OR and GR cultivars*

Total RNA from salt-treated and untreated leaves and roots from OR and GR genotypes were extracted using a Pure Link Plant RNA Reagent kit (Invitrogen). Total RNA was DNase treated (Promega), and first-strand cDNA was reverse transcribed from 2 µg of total RNA using an M-MLV Reverse Transcriptase kit (Promega) according to the manufacturer's instructions The first-strand cDNA from different samples was used as a template for PCR amplification of the *TdERF1* gene with the primers pairs listed in Supplementary Table S1. Samples were denatured for 30 s at 98 °C and then run for 35 cycles of 10 s at 98 °C, 30 s at 60 °C, and 40 s at 72 °C, with a final extension of 5 min at 72 °C. A wheat actin gene fragment used as an internal standard was synthesized by two primers (Supplementary Table S1). The PCR program was 5 min of denaturation at 95 °C and then 25 cycles of 30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C, with a final extension of 5 min at 72 °C. The PCR products were purified, cloned into a pGEM®-T Easy vector (Promega) according to the protocol instructions, and then sequenced with both forward and reverse vector primers (Supplementary Table S1) to identify new ERF sequences.

Alignment, phylogenetic tree, and sequence analysis

Sequence identities were determined using BLAST tools such as BLASTN, BLASTX, or BLASTP available from the NCBI web server. Conserved-domain positioning was identified using the NCBI conserved domain search tool. Motif detection was performed with the MEME program (v.3.5.7, http://meme.sdsc.edu/meme/meme.html) (Bailey *et al.*, 2009). Sequences alignments were performed using DNAMAN software (http://www.lynnon.com/). A phylogenetic tree of *TdERF1* and various selected heterologous of AP2/ERF members was constructed via FigTree (v.1.4.0.exe).

Abiotic stress and hormonal treatments

Using sterile conditions, seeds of two independent genotypes from *T. turgidum* subsp. *durum*, OR and GR, were first sterilized for 48 h at 4 °C for initialization of germination. Seedlings were sown in recipient Magenta™ vessels containing 50 ml of 50% MS-based medium (Murashige and Skoog, 1962) and were left for 10 d in an *in vitro* growth chamber maintained at a controlled photoperiod of 14 h during the day at 25 °C with 80% humidity and an intense luminosity of 250 µmol m^{-2} s^{-1}, and for 10 h during the night at 20 °C. They were then subjected to abiotic and hormonal stress treatments. For salinity treatment, seedlings were transferred into 50% MS medium containing 200 mM NaCl for 6 or 24 h. Hormone treatments were made by soaking seedlings in solutions containing 200 µM ABA, 200 µM auxin, or 200 µM SA for 4 h. For ethylene treatment, the hormone was delivered as a gas at 200 µl l^{-1} concentration in a sealed Plexiglas® chamber and incubated for 4 h. Leaves and roots were then harvested separately, dropped immediately into liquid nitrogen, and stored at –80 °C for RNA extraction.

Gene expression analysis

First-strand cDNA generated from total RNA including salt-treated and untreated samples from either the OR or GR genotype was subjected to quantitative expression analysis. This latter was performed in a fluometric thermal cycler (DNA Engine Opticon 2; MJ Research, Walthan, MA, USA) using SYBR Green fluorescent dye following the manufacturer's instructions. Results were shown using SDS2.2 software on an Applied Biosystem 7900 HT Fast Real-Time PCR System. Comparisons of repeated samples were assessed using C_T values among the three replications. Linear data were normalized to the mean C_T of 26S rRNA as an internal reference gene and the relative expression ratio was calculated using the formula $2^{-\Delta\Delta CT}$. *T. aestivum TaMYB73* transcription factor (GenBank accession no. JN969051) (He *et al.*, 2012) and *T. aestivum* salt tolerance-related gene *TaSTRG* (GenBank accession no. EF599631) (Zhou *et al.*, 2009) are both salt-stress-involved genes and they were shown to improve salt-stress tolerance in *A. thaliana* and rice. These two genes were used to better see the expression pattern of *TdERF1*. The gene-specific primers used for PCR are listed in Supplementary Table S2 at *JXB* online.

Sublocalization assay

The coding region of TdERF1 lacking the termination codon was cloned into a pGreen-GFP vector, which allowed the transient expression of TdERF1 protein tagged with green fluorescent protein (GFP) under the control of the cauliflower mosaic virus 35S (CaMV 35S) and the nopaline synthase (NOS) terminator. Protoplasts used for transfection were obtained from a suspension of cultured tobacco BY-2 cells (Leclercq *et al.*, 2005). They were then transfected using a modified polyethylene glycol method (Abel and Theologis, 1994): 0.2 ml of protoplast suspension (0.5×10^6) was transfected with 50 µg of shared salmon sperm carrier DNA and 10 µg of either 35S::TdERF1-GFP or 35S::GFP (as a control) plasmid DNA. Transfected protoplasts were incubated at 25 °C for 16 h and analysed for GFP fluorescence by confocal microscopy. All transient expression assays were conducted at least three times.

Transient expression of TdERF1 protein

The *TdERF1* full-length clone was PCR amplified and then introduced in the sense orientation within *Sma*I site of the pGreen binary vector using T4 ligase. The constructs were under the transcriptional control of the CaMV 35S promoter and NOS terminator. Protoplasts for transfection were obtained according to the method described above. For co-transfection assays, aliquots of protoplasts (0.5×10^6) were transformed either with 10 µg of the reporter vector alone containing the GCC-box or DRE-box synthetic promoters (Pirrello *et al.*, 2012) separately fused to the GFP reporter gene or in combination with 10 µg of TdERF1 construct as the effector plasmid. Transcription assays were performed in three replicates. After 16 h, GFP expression was analysed and quantified by flow cytometry (FACS Calibre II instrument; BD Biosciences, San Jose, CA, USA).

Results

Isolation of an AP2/ERF *gene through PCR screening of the durum wheat BAC library*

With the aim of better understanding the genetic and molecular basis of plant responses to environmental constraints and considering the reported role of *AP2/ERF* genes in stress responses, the present study attempted to isolate members of this gene family in durum wheat (*T. durum*). BLAST search mining of the available sequence information at the *T. durum* EST databases allowed, in a first approach, the identification of 10 partial cDNA clones potentially encoding AP2/ERF transcriptional regulators. One particular clone (GenBank accession no. AJ610963) displayed high similarity to the *TaERF1* gene (GenBank accession no. AY781352), which was shown to be involved in biotic and abiotic stress responses (Xu *et al.*, 2007). To test whether a gene sequence corresponding to *TaERF1* was present in the *T. durum* genome, forward (TdEST-F) and reverse (TdEST-R) primers were designed (Supplementary Table S1) and used in a PCR on genomic DNA extracted from *T. durum* LDN#65 variety. The PCR amplification yielded a fragment with the expected size (486 bp). Subsequent screening of the BAC library resulted in the isolation of one positive BAC clone putatively harbouring the wanted *ERF* gene (Fig. 1A).

BAC sequencing and annotation

Full sequencing of the isolated BAC by 454 technology revealed that it contained a wheat genomic DNA insert of 158 kb, slightly larger than the size estimated on the gel (130 kb) after restriction enzyme digestion. A total number of 9359 reads with an average size of about 400 bp per read allowed us to reach 25× coverage of the BAC sequence. The reads were assembled in five contigs ranging from 500 to 78 875 bp. Analysis of the contig sequences indicated that one contig contained a well-conserved AP2/ERF domain. Investigation via TriAnnot Pipeline annotation revealed that the selected contig harboured a complete *AP2/ERF* gene, here named *TdERF1*, made of two exons and a single intron, a gene structure commonly shared among other monocot plants such as *T. aestivum*, *Oryza sativza*, *Hordeum vulgare* and *Zea mays*. The endpoints of the unique intron were also verified with standard GT/AG boundary analysis.

Gene structure of TdERF1 *and phylogenetic relationship with members of the* AP2/ERF *family*

The genomic sequence of the new *AP2/ERF* gene was 2451 bp with a total size for the predicted cDNA of 1577 bp and a complete open reading frame of 1065 bp. The length of the predicted 5'-untranslated region (UTR), exon 1, intron, exon 2, and 3'UTR were 111, 259, 875, 806, and

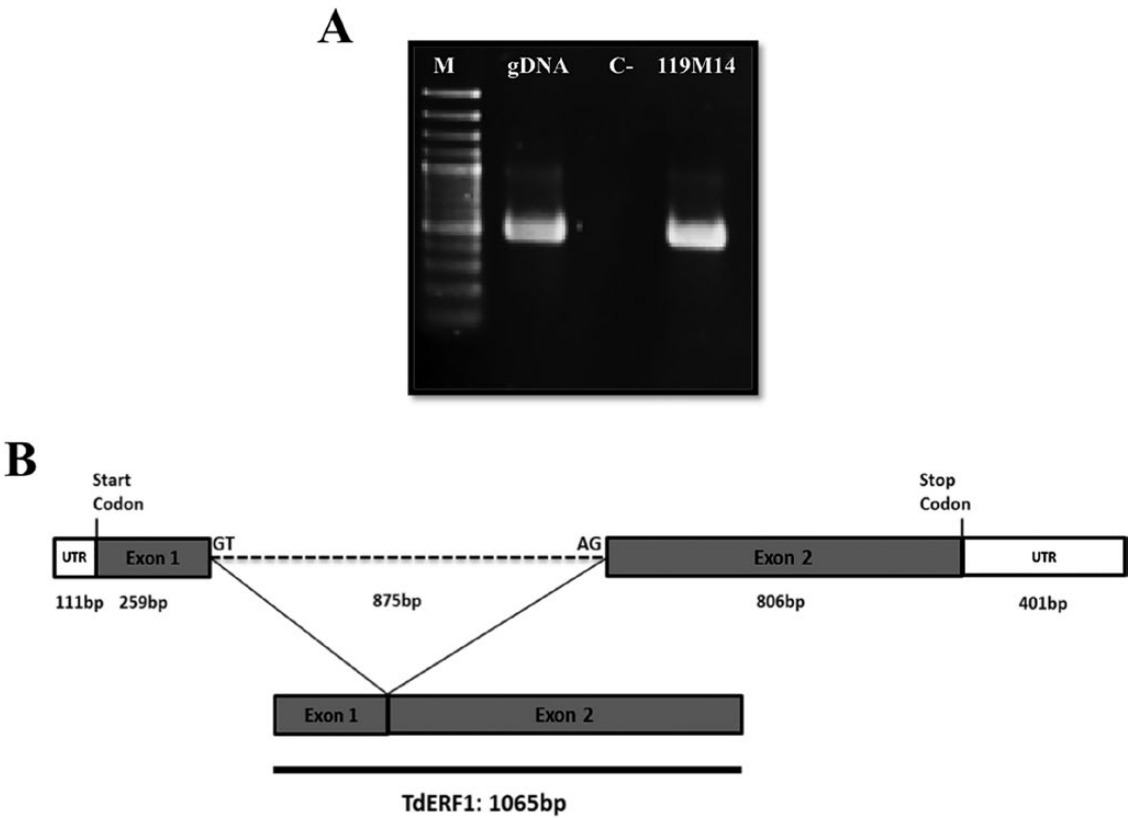

Fig. 1. Isolation of *TdERF1* by screening of a durum wheat BAC library and its genomic structure. (A) Amplification of a TdERF1 partial sequence on a positive BAC clone. M, 1 kb marker; gDNA (genomic DNA), positive amplification of a TdERF1 fragment using durum wheat genomic DNA as template; 119M14, positive amplification of a TdERF1 fragment using the 119M14C BAC clone as template; C-, no amplification in the absence of genomic DNA used as negative control. (B) Genomic structure of the durum wheat *TdERF1* gene; white and dark grey portions are untranslated regions and exons, respectively. The dashed line represents the intron; GT and AG are the intron borders. The dark line is the complete open reading frame.

401 bp, respectively (Fig. 1B). The derived TdERF1 protein contained 355 aa. Deeper analysis of the deduced protein sequence showed a conserved AP2/ERF DNA-binding domain of 59 aa. This domain shared high amino acid identity, ranging from 66.7 to 97%, with previously described ERF proteins from other species. TaERF1 from bread wheat was the closest homologue and displayed extremely high (97%) sequence identity in the AP2 domain. According to the conventional classification (Mazarei *et al.*, 2002), the AP2/ERF domain was divided into two conserved segments, YRG and RAYD, and the protein formed three β-sheets and a single α-helix (Fig. 2B). *TdERF1* was predicted to belong to the ERF subfamily of TFs based on the presence in the conserved domain of an alanine (A) at residue 14 and aspartic acid (D) at residue 19 (Sakuma *et al.*, 2002). The predicted full-length TdERF1 protein contained several motifs (Fig. 2A) including the highly conserved N-end motif (MCGGAIL) typical of group VII of *Arabidopsis* ERF subfamily members (Nakano *et al.*, 2006). This motif was shown recently to be involved in an oxygen-sensing mechanism in *Arabidopsis* by controlling the release of the Rap2.12 ERF protein from the plasma membrane and its import to the nucleus where it activates the expression of hypoxia-responsive genes (Licausi *et al.*, 2011). Three basic amino acid regions (K_{27}KKR/K_{106}RKRK/R_{117}RRP), potentially acting as nuclear localization signals (NLSs), were located in the N-terminal moiety. In the N-terminal part, the acidic amino acid region (E_{45}EEFQEEEGDAEFEADFEGFVEAEEESDGE) was predicted to be the putative core sequence of a transcriptional activation domain. Four potential *N*-linked glycosylation sites with an NXS/T core sequence (N_{224}MS, N_{240}FS, N_{280}ES, and N_{291}ST) were present in the C-terminal moiety of the TdERF1 protein. The C-terminal region also contained a putative phosphorylation site, T_{263}PDITS, that qualified TdERF1 as a potential phosphorylation substrate for one or more protein kinases, especially the homologues of the TaMAPK1 protein kinase (Xu *et al.*, 2007). Alignment of the conserved AP2/ERF domain of TdERF1 with those of *Arabidopsis* ERF proteins (Fig. 3) showed that the durum wheat ERF protein belonged to group VII (Nakano *et al.*, 2006). ERF proteins from other species most closely related to TdERF1 were *T. aestivum* TaERF1, tomato Sl-ERF.E2 and Sl-ERF.E1, *O. sativa* OsEREBP1, soybean GmERF4, and hot pepper CaERFLP1 (Supplementary Fig. S2 at *JXB* online). Taken together, these structural features indicated that *TdERF1* is a novel member of the *ERF* family in durum wheat and, given its high sequence homology with *TaERF1* and *OsEREBP1*, it can be postulated that it plays similar roles in mediating responses to biotic and abiotic stresses.

Targeting of TdERF1 protein to the nucleus

The presence of three putative NLSs in the TdERF1 sequence suggested that the encoded protein is likely to be targeted to the nucleus. Indeed, fluorescence microscopy analysis associated with image overlay techniques demonstrated that TdERF1–GFP fusion protein was exclusively localized to the nucleus (Fig. 4A), while control cells transformed with GFP alone displayed fluorescence distributed throughout the cytoplasm (Fig. 4B). These data confirmed the nuclear localization of TdERF1.

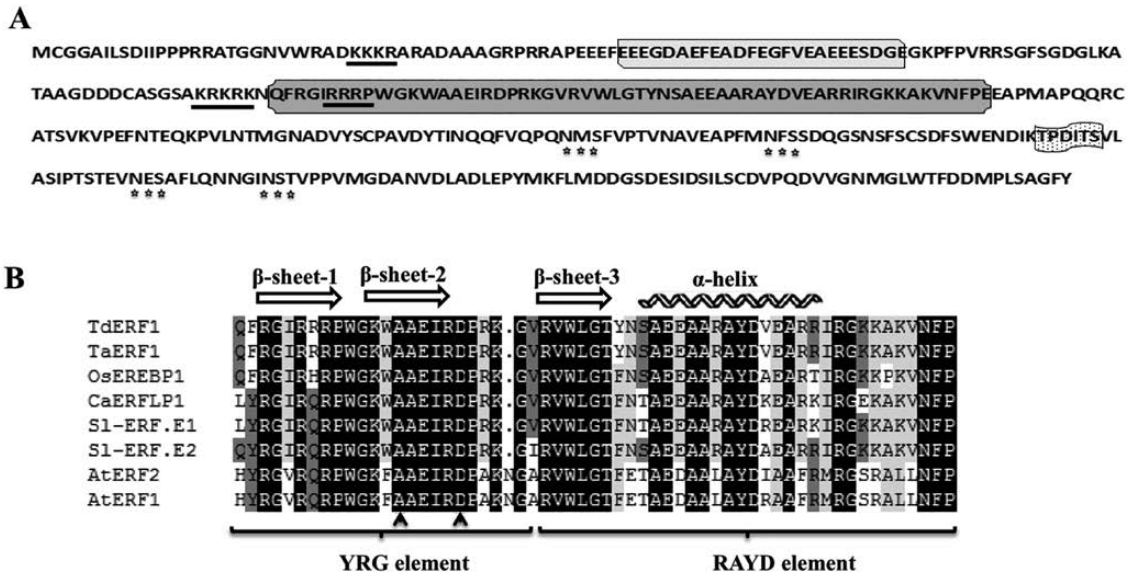

Fig. 2. TdERF1 protein sequence analysis. (A) Amino acid sequence of TdERF1. The ERF domain is shown in dark grey. Three basic amino acid regions are underlined and the acidic amino acid region is framed and shaded light grey. A putative phosphorylation site is shown in dashed box and four potential *N*-linked glycosylation sites are designated by three stars. (B) Multiple alignment of the AP2/ERF conserved domain of TdERF1 with those of other closely related ERF proteins using DNAMAN software (http://en.bio-soft.net/format/DNAMAN.html), including *T. aestivum* TaERF1 (GenBank accession no. AY271984), rice OsEREBP1 (AF193803), tomato Sl-ERF.E1 and Sl-ERF.E2 (AY192368 and AY044235, respectively), hot pepper CaERFLP1 (AY529642), and *Arabidopsis* AtERF1/2 (AB008103 and AB008104, respectively). Black, light grey, and dark grey shading represent 100, 75, and 50% similarity, respectively. The conserved YRG and RAYD elements are indicated by brackets. Three β-sheet and an amphipathic α-helix are indicated over the corresponding sequences. Dark arrowheads indicate amino acids discriminating ERF from DREB-type TFs (Sakuma *et al.*, 2002).

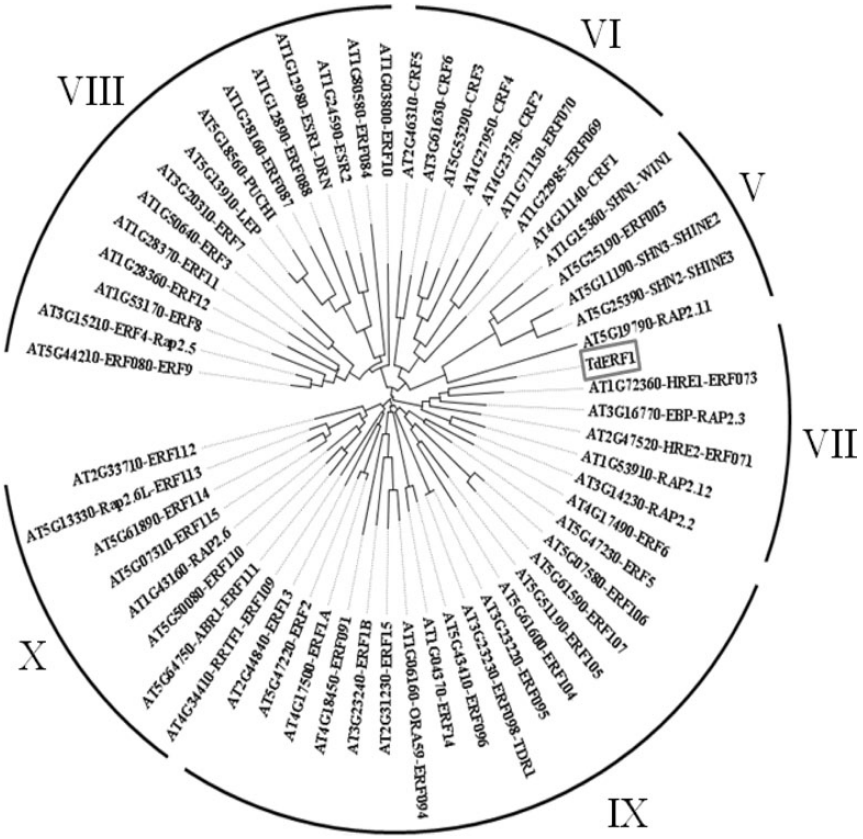

Fig. 3. Phylogenetic analysis of the conserved AP2/ERF domain of TdERF1 with those of *Arabidopsis* ERF proteins. The *Arabidopsis* ERF sequences were downloaded from GenBank. The TdERF1 is boxed. Alignment of protein sequences was performed with Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the radial tree was visualized with the FigTree (v.1.4.4.exe) program. The branch length represents the calculated distance between the sequences.

OR and GR genotypes of durum wheat display contrasting tolerance to salt stress

Two genotypes of *T. durum*, named GR and OR, were assessed for their salt-stress tolerance using germination capacity as a test. Under standard growth conditions, the overall germination rates of the GR and OR varieties were 100% (Fig. 5A). When challenged with an increasing gradient of NaCl for 14 d, the germination rate of the sensitive genotype OR was severely reduced and did not exceed 23, 6, and 3% of the total seeds under 100, 200, and 300 mM NaCl, respectively. By contrast, germination rate was significantly higher in the salt-tolerant GR with 100, 93, and 66% of germinating seeds in 100, 200, and 300 mM NaCl, respectively (Fig. 5A). The measurement of stomatal conductance showed that both GR and OR were affected by salinity at 14 d upon application of salt stress compared with normal conditions. Nevertheless, OR was more severely affected than GR at both concentrations (100 and 200 mM NaCl) of salt tested (Fig. 5B), suggesting that the stomatal aperture is more sustained in the tolerant GR variety after 14 d under salt-stress conditions.

Isolation of TdERF1 *cDNA and its allelic forms from the OR and GR genotypes*

Appropriate primers (Supplementary Table S1) encompassing the transcribed sequence of TdERF1 from LDN#65 variety were used to PCR amplify the corresponding cDNAs in the OR and GR durum wheat varieties. The amplified fragments were cloned into a pGreen vector and sequenced, revealing the presence of four allelic forms of TdERF1 in the OR genotype and a single form in the GR variety (Fig. 6). The open reading frame of the longest version of the *TdERF1* alleles was 1068 bp with a derived protein of 356 aa, hereafter referred to as TdERF1-E for the extended form. The short version was 1065 bp (355 aa) and is referred to as TdERF1-S. The extended allelic form TdERF1-E had a 3 nt insertion (CAG) at position 144, which added a glutamine residue (G) to the derived protein similar to that described in *T. aestivum* TaERF1 (Xu *et al.*, 2007). These two forms were identified in the OR variety, whereas only the extended form was isolated in the GR genotype. The *TdERF1* gene identified in the LDN#65 variety was 1065 bp, corresponding to the TdERF1-S variant. Interestingly, two variants of TdERF1-S proteins were identified within the OR genotype differing by the nature of the amino acid at position 332 where a leucine (L) residue was substituted by a proline (P). These two allele variants were named TdERF1-S-L and TdERF1-S-P, with respect to the nature of the amino acid at position 332. The TdERF1-S protein sequence from LDN#65 genotype had a proline and shared 100% identity with TdERF1-S-P from the OR variety at both the nucleotide and amino acid sequence levels. Two allelic variants of the extended form,

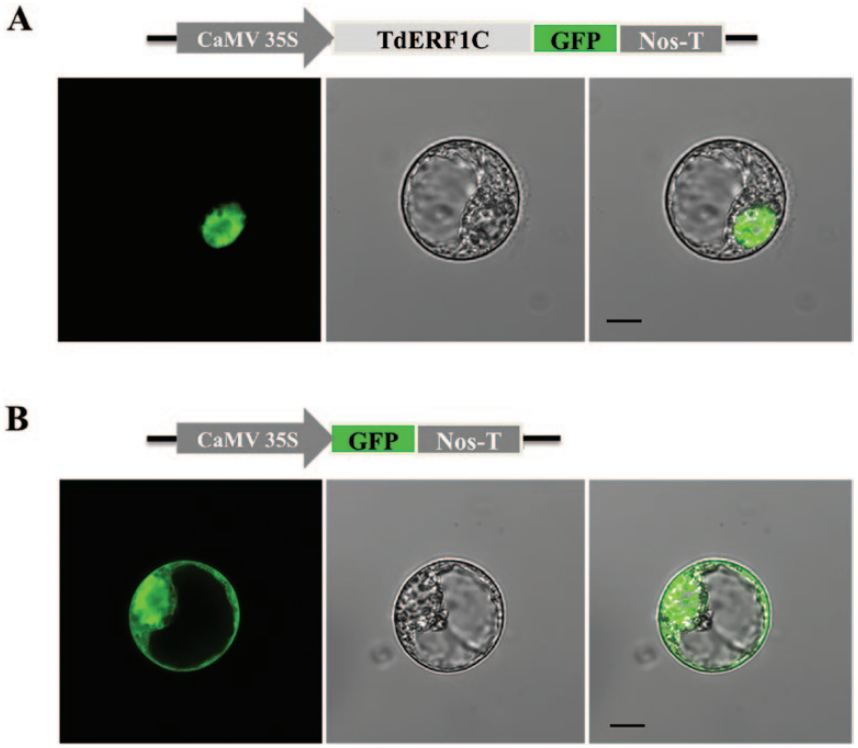

Fig. 4. Nuclear localization of TdERF1 protein fused to a GFP tag. Constructs consisting of either 35S::TdERF1-GFP (A) or 35S::GFP (B) were used to transiently transform tobacco protoplasts. The subcellular localization of the TdERF1–GFP fusion protein or GFP alone was analysed by confocal laser-scanning microscopy (left panel). Light micrograph (central panel) and fluorescence (left panel) images are merged (right panel) to illustrate the different location of the two proteins. Bars, 10 µm. (This figure is available in colour at *JXB* online.)

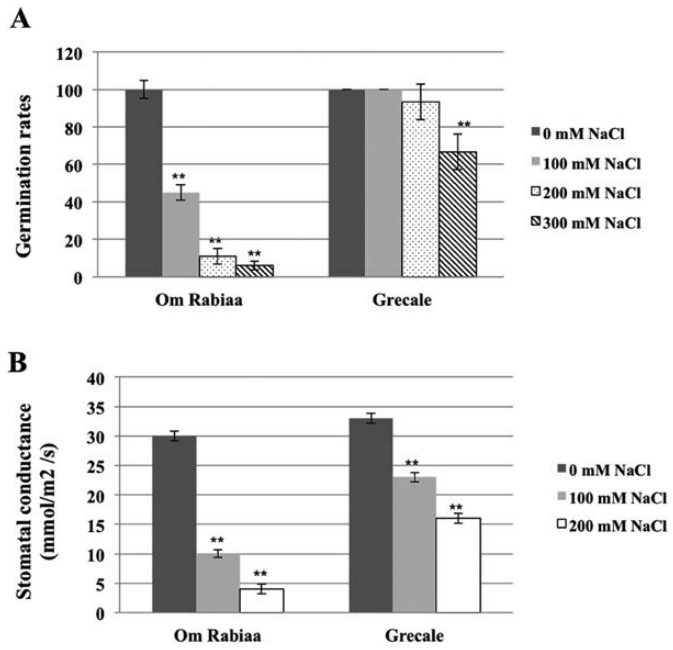

Fig. 5. Relative tolerance to salt stress between OR and GR, two durum wheat genotypes that contrast with regard to salt-stress tolerance. Comparative analysis of germination rate (A) and stomatal conductance (B) is shown between OR and GR without and after salt treatment (0, 100, 200, and 300 mM NaCl). The experiment lasted 14 d and was carried out in triplicate for each NaCl concentration and for each analysis. Values are means±standard deviation (SD) ($n \geq 30$) of three replicates. *$0.01 < P < 0.05$; ***$P < 0.001$ (Student's *t*-test).

TdERF1-E-L and TdERF1-E-P, were present in the OR variety, while only the TdERF1-E-P variant was detected in the GR genotype. Since durum wheat has two genomes, AA and BB, these allelic forms might arise from genome A or B and could share the same functions as their closest homologue in *T. aestivum*, TaERF1 (Xu *et al.*, 2007).

The expression pattern of TdERF1 under salt-stress conditions in two contrasting genotypes

The expression pattern of *TdERF1* in response to short-term salt stress, in both leaves and roots, was analysed in the OR and GR genotypes of durum wheat shown to be sensitive and tolerant to high salt, respectively. Specific primers (Supplementary Table S2 at *JXB* online) non-discriminating between allelic forms of TdERF1 were designed and used in a quantitative real-time PCR. Upon high-salt treatment (200 mM NaCl), the expression levels of *TdERF1* was altered in the leaves and roots of both genotypes, but with a greater effect on the expression in leaves (Fig. 7). After 6 h of salt treatment, *TdERF1* transcript accumulation in leaves (Fig. 7A) showed a fast increase in the GR genotype while it was decreased in the OR variety. Thereafter, the expression of the *ERF* gene in leaf tissues displayed a dramatic increase at 24 h in both genotypes, even though the upregulation was substantially higher in GR (28-fold) than in OR (13-fold). The induced expression of *TdERF1* at 24 h is in line with the upregulated expression of *TaMYB73* and *TaSTRG*, two salt-stress-induced genes. In treated OR roots, application of salt stress induced a decrease in transcript levels of *TdERF1* after 6 h treatment and then the expression level went back to normal after 24 h of stress treatment (Fig. 7B). The transcript abundance of *TdERF1* in treated GR roots dropped to seven times less than in control plants after 24 h (Fig. 7B).

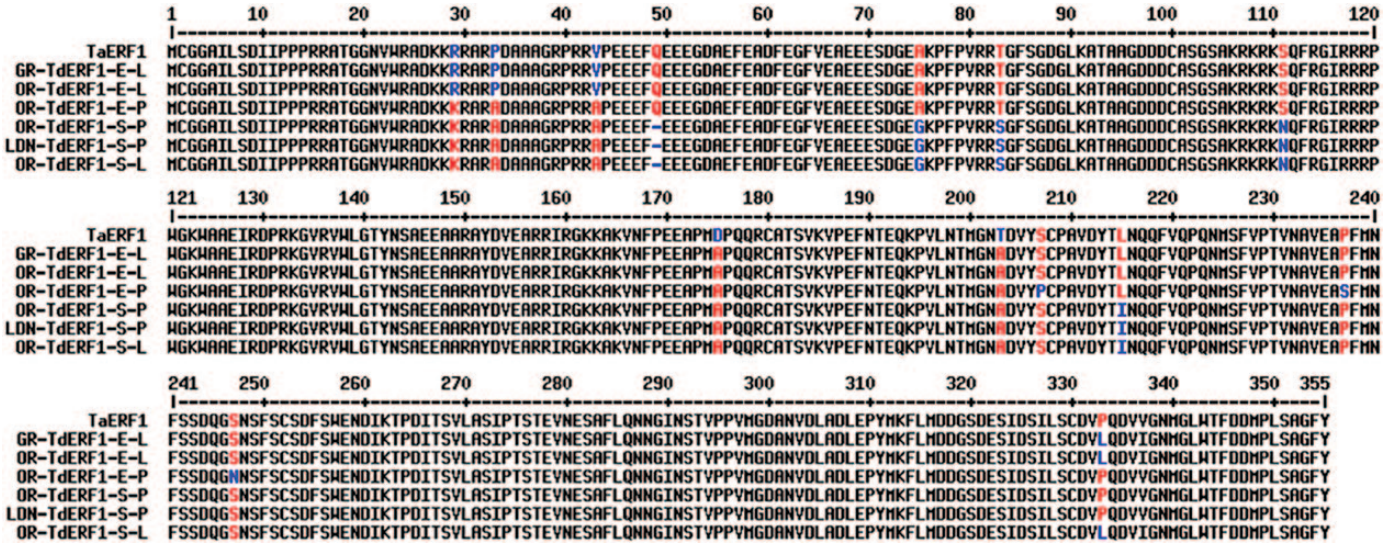

Fig. 6. Alignment of different allelic forms of TdERF1 with that of *T. aestivum* TaERF1. E, extended; S, short; L, leucine; P, proline. Red and blue represent high and low consensus colours, respectively. (This figure is available in colour at *JXB* online.)

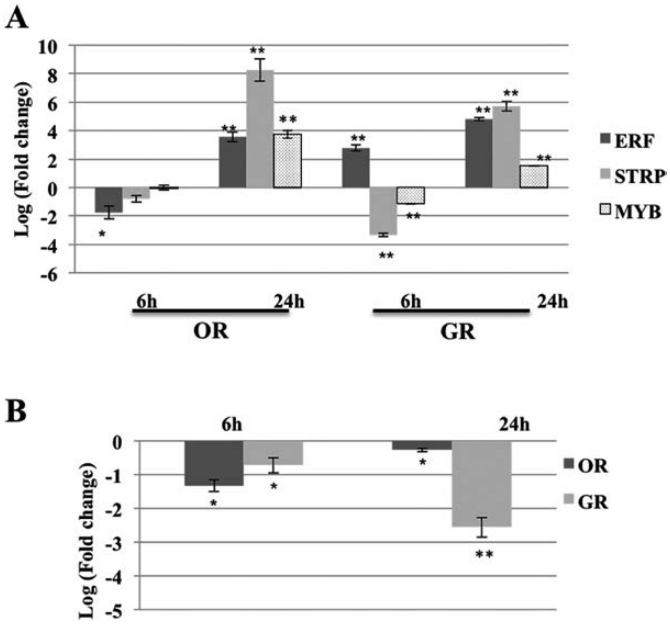

Fig. 7. Expression pattern of *TdERF1* in response to salt stress. Expression profile of *TdERF1* in leaves (A) and roots (B) from GR and OR durum wheat genotypes following 200 mM NaCl treatment. The levels of *TdERF1* transcripts were assessed by real-time quantitative PCR. mRNA accumulation was monitored in 10-d-old roots and leaves, after 6 and 24 h of NaCl treatment (200 mM NaCl). For each sample, relative fold changes were determined by normalizing the C_T value of the *TdERF1* gene in different tissues in both sensitive and tolerant varieties to the C_T value of Td26S (internal control) and by calculating relative to a calibrator using the formula $2^{-\Delta\Delta CT}$. $\Delta\Delta$ C_T refers to fold differences in TdERF1 expression relative to untreated tissues. *TaMYB73* (GenBank accession no. JN969051), a Myb transcription factor gene (He *et al.*, 2012) and the salt tolerance-related gene *TaSTRG* (GenBank accession no. EF599631) from *T. aestivum* (Zhou *et al.*, 2009) were used as salt-stress-induced genes. The experiment was carried in triplicate. Values are means±SD ($n \geq 30$) of three replicates. *$0.01<P<0.05$; ***$P<0.001$ (Student's *t*-test).

Expression pattern of TdERF1 *in response to hormone treatments*

Given that *ERF* genes are considered an important component of the ethylene signaling mechanism, the ethylene responsiveness of *TdERF1* was tested in the sensitive OR and tolerant GR genotypes. The data (Fig. 8A) indicated that ethylene negatively regulated *TdERF1* expression in the tolerant GR genotype, while it had no significant effect on the expression of this gene in the sensitive OR variety. To assess whether the expression of *TdERF1* was regulated by other hormones in the tolerant GR genotype, the effects of auxin, SA, and ABA were investigated. Transcript levels of *TdERF1* were upregulated after 4 h of exogenous treatment with both ABA and SA,whereas they were negatively regulated by auxin (Fig. 8B).

Putative cis*-acting elements identified in the* TdERF1 *promoter region*

Building on the BAC sequence data generated above, a 1 kb 5' regulatory region upstream of the transcription start of the *TdERF1* gene was subjected to *in silico* analysis using a plant *cis*-acting regulatory DNA elements (PLACE) signal scan to search for putative *cis*-regulatory elements potentially involved in the control of *TdERF1* gene expression. The data indicated the presence of a large number of conserved *cis*-regulatory elements that are putative targets for TFs reported to mediate responses to environmental stresses or to stress-related hormones (Table 1). Among these, the ABA-responsive elements (ABREs) are likely to be responsible for the upregulation of *TdERF1* expression upon ABA treatment (Fig. 8B). ABRE-like motifs (ACGTG) and ABRE-related motifs (ACGTGKC and TACGTGTC) were also found in the promoter region of *TdERF1*. The MYB-core element (TAACTG) and a number of MYB-related motifs (YAACKG, CNGTTR, and GGATG), as well as a MYC (CANNTG) motif and the MYC-related motifs (CATGTG and CACATG), were present in the promoter. DRE (TACCGACAT), CRT (RCCGAC), and low-temperature-responsive elements (LTREs) (CCGAC), all containing the CCGAC motif that forms the core of the DRE sequence, were well represented in the promoter of *TdERF1*. DRE-like elements such as CBFHv (RYCGAC) and DRE/CRT (RCCGAC) were also identified. Two GCC-box motifs (AGCCGCC) that serve as target sequences for ERF proteins were found in the *TdERF1* promoter. Four RAV1-A motifs (CAACA), to which RAV1

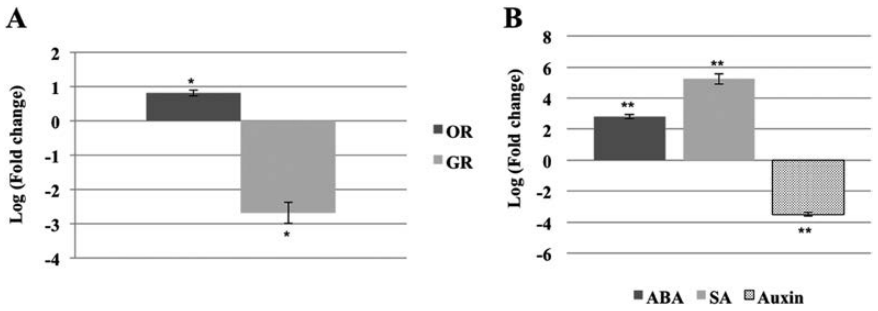

Fig. 8. Hormonal regulation of *TdERF1* expression. (A) *TdERF1* transcript accumulation upon exogenous ethylene treatment in both GR and OR durum wheat genotypes. (B) *TdERF1* expression in the GR stress-tolerant variety upon ABA, SA, and auxin treatments assessed by quantitative real-time PCR in 10-d-old seedling. Relative fold changes were determined by normalizing the C_T value of *TdERF1* to the C_T value of Td26S (internal control) and by calculating relative to a calibrator using the formula $2^{-\Delta\Delta CT}$. $\Delta\Delta C_T$ refers to fold differences in *TdERF1* expression in treated relative to untreated seedling. Values are means±SD ($n \geq 30$) of three replicates. *$0.01<P<0.05$,; ***$P<0.001$ (Student's *t*-test).

Table 1. *Cis-regulatory elements present in the TdERF1 promoter region*

A 1 kb DNA fragment corresponding to the 5'-regulatory region upstream of the transcription start of the *TdERF1* gene was subjected to *in silico* analysis using a PLACE signal scan to search for putative *cis*-regulatory motifs potentially involved in the control of *TdERF1* gene expression. R, A/G; Y, C/T; K, G/T; N, any nucleotide.

Cis-element	Sequence core	Number	Conditions	References
W-box	TGAC	13	Biotic stress	(Eulgem *et al.*, 1999. 2000.
RAV	CAACA	1	Ethylene	(Kagaya *et al.*, 1999)
GCC-box	GCCGCC	2	Ethylene, dehydration	(Wu *et al.*, 2007)
LTRE	CCGAC	2	Low temperature	(Baker *et al.*, 1994)
DRE/CRT	R(A/G)CCGAC	2	Dehydration, cold	(Dubouzet *et al.*, 2003; Skinner *et al.*, 2005)
CBH-Hv	RYCGAC	2	Dehydration, cold	(Xue, 2002; Svensson *et al.*, 2006)
ARF	TGTCTC	1	Auxin	(Ulmasov *et al.*, 1999; Hagen and Guilfoyle, 2002)
MYB	YAACKG	8	ABA, drought, high salt	(Abe *et al.*, 1997) (see text)
MYC	CANNTG	10	ABA, drought, high salt	(Abe *et al.*, 1997) (see text)
ABRE	YACGTGKC	8	ABA, drought, high salt	(Yamaguchi-Shinozaki and Shinozaki, 2005)

proteins can bind through their AP2 and B3-like domains, were also present in the *TdERF1* promoter. Moreover, seven cores of TGAC-containing W-box elements that are the target of WRKY proteins were identified in the *TdERF1* promoter. Six W-box-like motifs were also found in the *TdERF1* promoter. In line with the observed auxin regulation of *TdERF1*, the promoter region also contained one auxin response element with the typical TGTCTC motif shown to be the target of auxin response factor proteins. As summarized in Table 1, the promoter region of *TdERF1* was highly rich in *cis*-acting elements, and most of these were related to stress-induced gene expression, suggesting the putative role of this *ERF* gene in wheat responses to a variety of environmental stresses.

TdERF1 modulates the transcriptional activity of GCC-box- and DRE-containing promoters

The capacity of the TdERF1 protein to drive transcription from synthetic promoters either containing ethylene-responsive or drought-response elements was tested using a transactivation assay in a single-cell system. The system was based on co-transfection of tobacco protoplasts with an effector and a reporter construct. The reporter construct allowed the expression of the GFP reporter under the control of either an ethylene-inducible promoter containing the GCC-box motif or a drought-responsive promoter containing the DRE/CRT *cis*-acting element. In addition, to assess whether the allelic variation of TdERF1 impacted on the ability to modulate the transcriptional activity of target promoters, four effector constructs were made, allowing the expression of TdERF1-E-L, TdERF1-E-P, TdERF1-S-L, and TdERF1-S-P proteins. The transactivation assay data revealed that all allelic forms of TdERF1 acted as inducers of both ethylene- and drought-responsive promoters (Fig. 9A, B). However, the TdERF1-E-P extended variant was the most active on the GCC-box synthetic promoter (Fig. 9A) whereas the TdERF1-S-P short variant displayed higher induction on the DRE/CRT synthetic promoter (Fig. 9B). These data suggest that the allelic forms of TdERF1 have differential affinity to target promoters and may therefore be involved in activating the transcription of different genes.

Discussion

Even though wheat is one of the world's most important crops, the main genetic factors that limit the productivity of these agricultural species under constrained environmental conditions remain poorly understood. The high complexity and the size

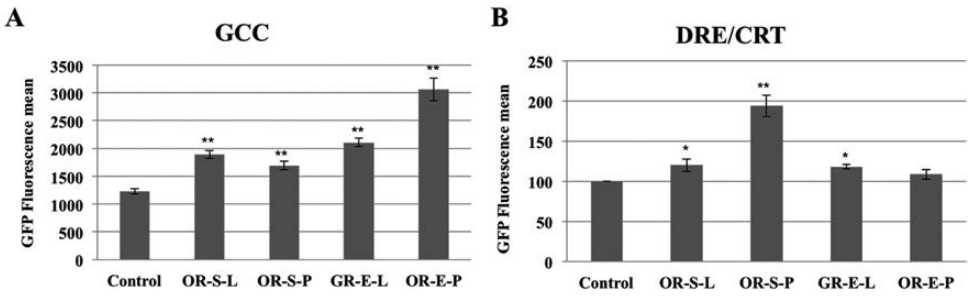

Fig. 9. Ability of the TdERF1 protein to regulate the transcriptional activity of ethylene- and drought-responsive promoters. TdERF1 differentially regulates the expression of a GFP reporter gene driven by ethylene (A) or drought-responsive synthetic promoters (B) in a transactivation assay. TdERF1 protein was challenged with a GCC-box- or DRE/CRT-containing promoter in a single-cell system to allow assessment of the reporter gene activity. The fluorescence was assessed by flow cytometry. Four different allelic forms of TdERF1 were tested for GCC-box-and DRE/CRT-containing promoters. The basal fluorescence obtained by co-transformation with the promoter fused to the reporter gene and with the empty vector was taken as reference. Values are means±sSD ($n \geq 30$) of three independent biological replicates. *$0.01 < P < 0.05$; ***$P < 0.001$ (Student's *t*-test).

of the wheat genome have largely hampered the development of genomic resources, and the lack of a full-genome sequence represents a major limitation for designing efficient breeding strategies towards improving wheat yield. Such a situation is even more critical for durum wheat, which is cultivated mainly in Mediterranean countries known to suffer from chronic droughts. The present study describes the isolation and partial functional characterization of TdERF1 from durum wheat. It was shown that TdERF1 belongs to the ERF family of TFs whose members have been reported to mediate environmental stress responses in various plant species. Since the genome sequence information is not available for durum wheat, the strategy used to isolate the *TdERF1* gene built on the screening of the BAC library constructed at the University of California (Cenci *et al.*, 2003) and stored at the French Plant Genomic Resource Center (http://cnrgv.toulouse.inra.fr/). One positive BAC clone was identified as potentially bearing an *AP2/ERF* gene. The BAC screening and sequencing approach was very powerful to obtain the complete genomic sequence of the putative *AP2/ERF* gene including the promoter region and *cis*-acting elements, providing much information about eventual relationships with other regulators leading to final responses.

The *AP2/ERF* gene family is regarded as the second largest family of plant TFs, with the highest number of members being found in maize (185, of which 151 compose the *ERF* subfamily), while the model plant *Arabidopsis* has 147 members (of which 122 comprise the *ERF* subfamily) (Yan *et al.*, 2013). The *ERF* family remains less investigated in monocotyledonous plants such as wheat than in dicotyledonous such as *Arabidopsis* and tomato (Nakano *et al.*, 2006; Pirrello *et al.*, 2012). So far, in the absence of the durum wheat genome sequence, the only sequence information available in public databases corresponds to ESTs (listed above) and genomic BAC clones (http://cnrgv.toulouse.inra.fr/). The lack of sufficient genomic resources strongly restricts the discovery of genes and gene families, and in this context, the *TdERF1* gene isolated in this study represents the first characterized ERF-type TF from durum wheat. This *ERF* gene shared a strong identity (97%) and displayed similar structural features to its putative orthologue, *TaERF1*, from bread wheat (Xu *et al.*, 2007). *TaERF1* and its homologues were assigned to chromosomes 7A and 7B (Hossain *et al.*, 2004; Xu *et al.*, 2007) in bread wheat, suggesting that *TdERF1* might be also accommodated within chromosome 7A/B. Phylogenetic analysis assigned *TdERF1* to group VII within the *ERF* subfamily (Nakano *et al.*, 2006). The derived protein contained the distinctive and highly conserved N-terminal MCGGAII/L pattern, typical of ERF group VII members, first described in tomato (Tournier *et al.*, 2003; Pirrello *et al.*, 2012) and shown recently to control the lifetime pathway of *Arabidopsis* RAP2.12 for proteasomal protein degradation in hypoxia conditions (Licausi *et al.*, 2011). *TdERF1* contains a single intron located in the N0-flanking conserved site of the *ERF* domain similar to *TaERF1* and to *Arabidopsis* group VII, suggesting that divergence of monocots and dicots occurred later than the divergence from the ancestor of this group evolving an intron (Xu *et al.*, 2007). While most ERF proteins contain one or two putative NLS domains, the TdERF1 sequence and its closest homologue, TaERF1, revealed three putative NLS motifs (Fig. 2A), suggesting that the nuclear targeting of the encoded proteins may operate through the synergistic control of these NLSs. The nuclear localization of TdERF1 fused to the GFP reporter protein was confirmed by transient expression assays in protoplast cells. Of particular note, NLS3 is located in the AP2/ERF domain and might be involved in both DNA-binding affinity and nuclear localization (Xu *et al.*, 2007). TaERF1 has been shown to be implicated in phosphorylation cascades involving TaMAPK1 (Xu *et al.*, 2007) and mediating the ethylene signal transduction pathway (Broekaert *et al.*, 2006), as well as responses to pathogen signals (Gu *et al.*, 2000; Yap *et al.*, 2005). Similarly, the C-terminal region of TdERF1 contains a putative phosphorylation site; however, whether these structural similarities between TdERF1 and its TaERF1 homologue are indicative of functional conservation remains to be tested.

A number of ERF genes isolated from various plant species have been shown to be involved in biotic and abiotic stress responses, while others are involved in the regulation of growth and development (Nakano *et al.*, 2006; Licausi *et al.*, 2011; Mizoi *et al.*, 2012). However, reports on the involvement of wheat ERF proteins in stress responses remain poorly documented. In bread wheat, only four ERF genes (TaERF1, TaERF3, TaPIEP1, and TaERF4) have so far been reported to be implicated in abiotic stresses (Xu *et al.*, 2007;

Zhang *et al.*, 2007; Dong *et al.*, 2010 and 2012). In a context where no functional characterization has yet been carried out for ERFs in durum wheat, our data showed that the *TdERF1* gene was inducible by high-salt treatment. Moreover, *TdERF1* expression in response to salt stress displayed distinctive patterns in two durum wheat genotypes with contrasting behaviour regarding tolerance to abiotic stresses. In the tolerant GR variety, *TdERF1* was strongly induced by salt stress within few hours (6 h), while it was downregulated in the sensitive OR variety. Therefore, the *TdERF1* pattern of expression may provide a useful marker for discriminating among wheat varieties with regard to their response to abiotic stress and can potentially be used as a tool in breeding programmes aiming at creating wheat varieties with improved tolerance to salt stress. In the line with the stress-induced expression of *TdERF1* is the ability of the encoded protein to bind and modulate the activity of a drought-responsive promoter containing the DRE/CRT *cis*-acting element and the ethylene-inducible promoter containing the GCC-box motif. The transactivation assay data support the hypothesis that this ERF member might be part of the mechanisms underlying the observed higher tolerance to salt stress. Of particular note, the *TdERF1* promoter harbours a number of *cis*-regulatory elements reported to mediate responses to environmental stresses or to stress-related hormones at the transcriptional level. ABA is known to play a key role in response to abiotic constrains such as drought, low temperature, and osmotic stress (Fujita *et al.*, 2005). The presence of several conserved ABREs (YACGTGKC) (Yamaguchi-Shinozaki and Shinozaki, 2005) is probably responsible for the upregulation of *TdERF1* expression upon ABA treatment. It is known that MYB and MYC factors act in an ABA-dependent manner at a later stage of stress responses to high-salt and water stresses (Urao *et al.*, 1993; Abe *et al.*, 1997). Thus, the upregulation of TdERF1 by exogenous ABA application and the presence of MYB/MYC elements in the *TdERF1* promoter suggest that MYB and MYC TFs might mediate the ABA-dependent expression of *TdERF1* during abiotic stress responses, notably drought and salt stress. ERF proteins regulate a variety of environmental stress responses as well as plant developmental processes via interaction with the GCC-box and the DRE to activate the expression of stress-related genes (Zhang *et al.*, 2004; Wu *et al.*, 2007). In our study, two canonical GCC-boxes were present in the promoter of *TdERF1*, which make it a putative target for ERF TFs known for their binding affinity to this motif. On the other hand, DRE elements have been shown to be essential *cis*-acting elements for regulating *RD29* induction in response to dehydration and cold. CRTs and LTREs, both containing DRE sequences, have been reported to regulate cold-inducible promoters (Baker *et al.*, 1994). Interestingly, these elements as well as DRE-like motifs such as CBFHv (Xue, 2002; Svensson *et al.*, 2006) are present in the *TdERF1* promoter, indicating eventual involvement of TdERF1 in drought and cold signalling pathways. Auxin is often regarded as a 'master' hormone in plant growth and is especially important in cell division and differentiation processes, which were shown to be associated with the expression of auxin-regulated genes (Guilfoyle and Hagen, 2007). Our data showing that TdERF1 transcript levels are outstandingly decreased after ethylene and auxin treatment, and the presence of both auxin and ethylene response elements in the *TdERF1* promoter, indicate that these two hormones contribute to the regulation of *TdERF1*, and position this wheat ERF member at the crossroads of auxin and ethylene signalling pathways. A further argument for the putative involvement of TdERF1 in stress responses is the presence in the *TdERF1* promoter of four RAV1-A (CAACA) motifs acting as putative targets for RAV TFs (Kagaya *et al.*, 1999). These data suggest that the *TdERF1* promoter can be a common target for different members of AP2/ERF TFs, including ERF and RAV. *Cis*-acting elements related to biotic stress were strongly present in the *TdERF1* promoter, such as W-box known to be the target element of the WRKY DNA-binding protein, which contributes to plant defence responses (Eulgem *et al.*, 1999. 2000). Six W-box-like motifs were identified in the *TdERF1* promoter that have been shown to be involved in elicitor-responsive defence genes in tobacco (Yamamoto *et al.*, 2004) and in wounding-induced activation of the *ERF3* gene (Nishiuchi *et al.*, 2004). After SA treatment, *TdERF1* transcript levels were highly upregulated, suggesting that the *TdERF1* gene may contribute to SA responses in response to biotic stress. All these results suggest possible cross-talk between ethylene, auxin, ABA, and SA in regulating *TdERF1* gene expression. Moreover, the different *cis*-regulatory elements related to stress responses present in the *TdERF1* promoter indicate the potential role of this gene in mediating plant responses to a wide variety of environmental constraints.

Despite the crucial importance of wheat as a food and feed crop, the genetic factors controlling the productivity of this agricultural species in harsh environmental conditions are not well known. This study reports on the isolation of *TdERF1*, an *ERF* gene whose expression pattern discriminates between two contrasting durum wheat varieties in terms of salt-stress tolerance. In addition, the promoter structure and the ability of the encoded protein to regulate the transcription of ethylene and a drought-responsive promoter clearly indicate its putative involvement in mediating salt-stress responses. Interestingly, four allelic variants of the *TdERF1* gene have been identified and shown to be expressed at the transcriptional level. However, under stress condition, the expression of one allelic variant was specific to the tolerant variety, further supporting the hypothesis that this gene is likely to be part of the susceptibility/tolerance mechanism to salt stress. In this regard, the *TdERF1* gene may provide a good marker for discriminating tolerant and sensitive wheat varieties. Furthermore, the DNA polymorphism detected in the *TdERF1* coding sequence can give rise to genetic markers that may potentially be used in marker-assisted selection strategies.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Primers design for durum wheat BAC screening and isolation of *TdERF1* cDNA sequence.

Supplementary Table S2. Primers design for quantitative real-time PCR carried out to assess the expression of the *TdERF1* gene at the transcript level.

Supplementary Fig. S1. Two-dimensional pooling strategy of the *Triticum turgidum* LDN#65 BAC library.

Supplementary Fig. S2. Phylogenetic analysis of the TdERF1 protein and related full-length AP2/ERF family protein sequences.

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