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Identification of a novel gene (*Hsdr4*) involved in water-stress tolerance in wild barley

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Abstract Drought is one of the most severe stresses limiting plant growth and yield. Genes involved in water stress tolerance of wild barley (*Hordeum spontaneoum*), the progenitor of cultivated barley, were investigated using genotypes contrasting in their response to water stress. Gene expression profiles of water-stress tolerant vs. water-stress sensitive wild barley genotypes, under severe dehydration stress applied at the seedling stage, were compared using cDNA-AFLP analysis. Of the 1100 transcript-derived fragments (TDFs) amplified about 70 displayed differential expression between control and stress conditions. Eleven of them showed clear difference (up- or down-regulation) between tolerant and susceptible genotypes. These TDFs were isolated, sequenced and tested by RT-PCR. The differential expression of seven TDFs was confirmed by RT-PCR, and TDF-4 was selected as a promising candidate gene for water-stress tolerance. The corresponding gene, designated *Hsdr4* (*Hordeum spontaneum* dehydration-responsive), was sequenced and the transcribed and flanking regions were determined. The deduced amino acid sequence has similarity to the rice Rho-GTPase-activating protein-like with a Sec14 p-like lipid-binding domain. Analysis of *Hsdr4* promoter region that was isolated by screening a barley BAC library, revealed a new putative miniature inverted-repeat transposable element (MITE), and several potential stress-related binding sites for transcription factors (MYC, MYB, LTRE, and GT-1), suggesting a role of the *Hsdr4* gene in plant tolerance to dehydration stress. Furthermore, the *Hsdr4* gene was mapped using wild barley mapping population to the long arm of chromosome 3H between markers *EBmac541* and *EBmag705*, within a region that previously was shown to affect osmotic adaptation in barley.

Keywords cDNA-AFLP · Dehydration stress · Gene expression · Drought tolerance · Wild barley

Abbreviations

cDNA-AFLP	cDNA-amplified fragment length polymorphism
TDF	transcript-derived fragment
RT-PCR	reverse transcription polymerase chain reaction
Q-PCR	quantitative expression analysis by real-time PCR
UTR	untranslated region
SNP	single nucleotide polymorphism
QTL	quantitative trait locus
MITE	miniature inverted-repeat transposable element

The sequences reported in this paper have been deposited in the NCBI and dbEST databases. The accession numbers are incorporated in the text. The accession number of *Hsdr4* is DQ464370. The accession numbers of the ESTs are: TDF-2, EB174194; TDF-3, EB174195; TDF-4, EB174196; TDF-5, EB174197; TDF-7, EB174198; TDF-9, EB174199; TDF-10, EB174200; TDF-11, EB174201; TDF-12, EB174202; TDF-13, EB174203; TDF-14, EB174204.

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Introduction

Water deficit is one of the prevalent causes of crop yield loss. Increased plant tolerance to water deficit is considered among the most important abiotic parameters that can contribute to increased grain production, in particular in Mediterranean regions (Araus et al. 2003). Plant genetic adaptation to environmental stress is displayed in physiological and biochemical responses, controlled by changes in gene expression. Several major classes of genes have been noted that are altered in response to water-deficit stress; genes involved in signaling and gene regulation and gene products that are proposed to support cellular adaptation to water-deficit stress are among the most frequently altered in gene expression (Hazen et al. 2003; Hazen et al. 2005; Ito et al. 2006; Yamaguchi-Shinozaki and Shinozaki 2005). Although a large number of genes and proteins responding to stresses have been studied, most molecular components of the signaling transduction pathway involved in gene regulation under stress are still unidentified, and their precise functions in either tolerance or sensitivity remain unclear. Comprehensive profiling of stress-associated metabolites, combined with stress metabolomics of major crop plants may be a key factor in molecular breeding for tolerance (Vinocur and Altman 2006).

Numerous studies have shown that wild progenitors of cultivars comprise one of the major genetic recourses of plant tolerance to stressful environments (Ellis et al. 2000; Nevo et al. 2002). Habitats of wild barley (*Hordeum spontaneum*), the progenitor of cultivated barley, differ in water availability, temperature, soil type, altitude, and vegetation, generating thereby a high potential of adaptive diversity in wild barley to abiotic stresses. Adaptive genetic diversity in natural populations was revealed by protein and DNA markers (Nevo et al. 1979; Owuor et al. 2003; Turpeinen et al. 2003; Suprunova et al. 2004; Ozkan et al. 2005), indicate the potential of wild barley as a source for drought-resistance alleles for breeding purposes.

Functional genomic tools can be applied for identifying and isolating the genes involved in plant abiotic stress tolerance (Langridge et al. 2006). These tools include variety of molecular techniques available to identify and clone differentially expressed genes. Among the techniques based on assaying nucleic acid hybridization, microarray technology plays an ever-increasing role in unraveling the molecular genetic basis of plant reaction to stress (Chao et al. 2005; Gulick et al. 2005; Kim and von Arnim 2006). However, microarray technology has relatively high start-up costs and its utility is limited by low sensitivity for detection of rarely expressed transcripts and difficulties in distinguishing transcripts from homologous genes. Among the genome-wide expression analysis techniques based on PCR and gel separation and visualization procedures, cDNA amplified fragment length polymorphism (cDNA-AFLP) (Bachem et al. 1996, 1998) has proved the most popular technique. cDNA-AFLP overcomes some of the limitations in hybridization-based techniques and is considered a valid alternative/complementation to microarrays (Volkmuth et al. 2003). This technique does not require prior sequence information and has good reproducibility and sensitivity compared to microarray technologies (Reijans et al. 2003). cDNA-AFLP results correlate well with Northern, quantitative expression analysis by real-time PCR (Q-PCR), and microarray analysis (Donson et al. 2002; Avrova et al. 2003; Breyne et al. 2003).

In the present study, we have used the cDNA-AFLP technique to screen for candidate transcripts, which are differentially expressed between sensitive and tolerant wild barley genotypes under dehydration stress. Several transcript-derived fragments (TDFs) that were differentially expressed in the tolerant genotype, as compared to the sensitive genotype, were isolated. TDF4 was analyzed as a novel candidate gene for drought tolerance in wild barley. The novel water stress inducible gene; designated *Hsdr4* (*Hordeum spontaneum* dehydration-responsive 4) was sequenced and analyzed and its involvement in drought stress tolerance in barley is discussed.

Materials and methods

Plant material

Two water stress sensitive genotypes (JS1 and JS2) and two water stress tolerant genotypes (JR1 and JR2) of wild barley (*Hordeum spontaneum*) were used in this study. These genotypes were selected from a collection of 400 genotypes originating from diverse eco-geographic regions in Israel and Jordan and surroundings. Selection was done based on measurements of water loss rate (WLR) and relative water content (RWC) after severe dehydration stress, and displayed different patterns in the dynamics of drought-induced expression of dehydrin genes in response to dehydration stress (described in details by Suprunova et al. 2004).

Stress treatments

Seedlings were grown in a greenhouse at 22°C, with a photoperiod of 12 h light/12 h dark, in Murashige and

Skoog basal salt mixture (MS) (Sigma) solution, circulated by air pumps. Ten-day-old seedlings were subjected to water stress by complete draining of the MS solution from the container. Leaf tissues were harvested from control plants (time 0), and after 3 h and 12 h of stress. Leaves were frozen in liquid nitrogen and stored at –80°C for RNA extraction. Two to three seedlings from each genotype were tested independently in each step of the gene expression analysis.

cDNA-AFLP analysis

Total RNA was extracted from barley leaf tissue using EZ-RNA Total RNA Isolation Kit (Biological Industries, Beit Haemek LTD, Israel). Poly(A)$^+$ RNA was prepared from 30 μg of total RNA using oligo (dT) coupled to paramagnetic beads (PolyA Tract mRNA Isolation System IV, Promega). Double-stranded cDNA synthesis was carried out with the Universal RiboClone cDNA Synthesis System (Promega) according to the manufacturer's instruction.

cDNA-AFLP analysis was conducted according to Bachem et al. (1998) with a slight modification. Double-stranded cDNA (100 ng) was digested by *Mse*I/*Eco*RI enzyme combination and than ligated to appropriated adaptors. For pre-amplification reactions non-selective *Mse*I + 0 and *Eco*RI + 0 primers were used. Following the pre-amplification step, the products were diluted (10×) with TE buffer and 5 μl were used for final selective amplifications using the following primer combinations: *Eco*RI-C/*Mse*I-TC; *Eco*RI-C/*Mse*I-CG; *Eco*RI-AC/*Mse*I-GG; *Eco*RI-AC/*Mse*I-CC; *Eco*RI-CA/*Mse*I-TC; *Eco*RI-CA/*Mse*I-CC; *Eco*RI-CA/*Mse*I-CG; *Eco*RI-CA/*Mse*I-CCA; *Eco*RI-CA/*Mse*I-CCC; *Eco*RI-CA/*Mse*I-CCG; *Eco*RI-CA/*Mse*I-CGA; *Eco*RI-CA/*Mse*I-CGC; *Eco*RI-CA/*Mse*I-CGG; *Eco*RI-CC/*Mse*I-GG; *Eco*RI-CC/*Mse*I-TC. The *Eco*RI primers were radioactively labeled by [γ-^{33}P]ATP. Selective amplification products were separated on 5% polyacrylamide sequencing gels using Sequi-Gen GT System (Bio-Rad). The polyacrylamide gels were dried onto 3 MM Whatmann paper and positionally marked before being exposed to Kodak Biomax MR Films for 3 days.

Isolation and sequencing of cDNA-AFLP fragments

X-ray films and gels were aligned according to the markers on the gels, and transcript-derived fragments (TDFs) that showed clear differential amplification in sensitive vs. tolerant barley genotypes under dehydration were excised from the gels and incubated in 50 μl H_2O overnight at room temperature. The eluted TDFs were re-amplified using the method developed by Brugmans et al. (2003) for converting AFLP markers into single-locus markers. The method includes three steps: (1) Each of the TDFs was excised from the cDNA-AFLP gel and re-amplified with its corresponding selective cDNA-AFLP primers under the same PCR conditions that were used for the active PCR step. Recovered TDFs were separated on 2% agarose gel and purified (QIAquick Gel Extraction Kit, Qiagen); (2) The re-amplified fragments were used as templates for second PCR using a set of 16 degenerated *Mse*I primers and 16 degenerated *Eco*RI primers. The amplification products were separated on 2% agarose gel. Based on the results of the quality and quantity of the different amplification products we were able to determine the extra selective nucleotides adjacent to the corresponding ends of *Mse*I and *Eco*RI selective primers; (3) Based on this analysis, new specific primers were designed for each TDF, that were used for final PCR amplification using the first re-amplified TDF as a template. The final PCR products were separated on 2% agarose gel, purified, and directly sequenced. Each TDF was sequenced using the corresponding new selective primers as sequencing primers.

The homologues for all TDF sequences were determined using BLAST algorithm (Altshul et al. 1997) by comparison with database at National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) and the *Triticeae* EST database (''HarvEST'') (http://harvest.ucr.edu/).

Reverse Transcription (RT-PCR) analysis

Equal amounts of total RNA from leaves were treated with RNase-free *DNase* I (DNA Free kit, Ambion, USA), following the manufacturer's instruction. First-strand cDNA was synthesized from 3 μg of total RNA, using universal oligo(dT)$_{15}$ primer and 200 units of SuperScript II reverse transcriptase (GIBCO-BRL), at 42°C for 1 h in a 20 μl reaction volume. The resulting single-strand cDNA was amplified using specific primers for each TDF that were designed, based on the obtained TDFs sequences. To preserve the highest RT-PCR stringency, only primers with annealing temperatures higher than 55°C were designed. PCR products were sampled after 20, 25, 30, and 35 cycles to determine the linearity of the PCRs. For each TDF specific PCR conditions were determined, including annealing temperature and cycle's number that provided the sensitivity needed to detect the differences in expression (Table 1). Barley *α-tubulin* gene amplified with

Table 1 Primer sequences and PCR conditions used in RT-PCR analysis of seven TDFs

TDFs name	Oligonucleotide sequence (5′ to 3′)	Amplicon size (bp)	PCR conditions
TDF-2	TGACACTTGCTACTAATCACCG CAATTCTTTTGACAGGGCTTTA	128	94°C, 30 s; 55°C, 30 s; 72°C, 30 s; (for 35 cycles)
TDF-3	GTC GA AGAC AGGAC AGGC AT ATGT ATC GGACCC ACGGCT A	173	94°C, 30 s; 56°C, 30 s; 72°C, 30 s; (for 25 cycles)
TDF-4	TGAGATTCCAGATTTTGTTACT ACC ATTTGTCTTC AT ACCTAC C	143	94°C, 30 s; 55°C, 30 s; 72°C, 30 s; (for 25 cycles)
TDF-5	GACTGGGTTCTACACTCCT ATAGCATTCTCTTCCTGATAG	210	94°C, 30 s; 55°C, 30 s; 72°C, 30 s; (for 30 cycles)
TDF-11	ATCGCAGAGATAGCTCATTCTCA ATCACAATATCTGCTCCAAGTTC	94	94°C, 30 s; 55°C, 30 s; 72°C, 30 s; (for 30 cycles)
TDF-13	GGCGTGAACCCGATGAAGATG ACCCCTCGCCGTCTCCTAAACT	220	94°C, 30 s; 60°C, 30 s; 72°C, 30 s; (for 30 cycles)
TDF-14	CAGCGGGTTGAGGAGGACAG CTCGATCAGAGATTCTATGCG	174	94°C, 30 s; 56°C, 30 s; 72°C, 30 s; (for 25 cycles)

the specific primers 5′-AGTGTCCTGTCCACCC-ACTC-3′ and 5′-CCAAGGATCCACTTGATGCT-3′ (acc. no. U40042) was used as a constitutive control in all experiments. The RT-PCR products were visualized by electrophoresis on 2% agarose gel.

Generation of the 3′- and 5′-ends of cDNA

The FirstChoice RLM-RACE kit (Ambion, USA) was used for rapid amplification of cDNA ends according to the manufacturer's instruction. Two gene-specific primers (5′-TCGGCTGGAATATCTATGGG-3′ and 5′- ACCATTTGTCTTCATACCTACC-3′) and two gene-specific inner primers (5′-TGAGATTCCAGA TTTTGTTACT-3′ and 5′-TGGTCGGTGCTCAAG-TATCT-3′) were designed based on the 223-bp TDF-4 sequence. Total RNA of wild barley genotype (JR1) dehydrated for 12 h was used as a template for the RACE procedure. RACE-PCR products were cloned in a pGEM-Teasy vector (Promega, Madison, USA), and six independent clones were sequenced. Based on the sequences of the 5′- and 3′-untranslated regions the specific primers (5′-CAGCAGGAGGGGCGGC CGGC-3′ and 5′-GCATCGCCTTTTGCTATGACAT-3′) were designed and used for PCR amplification of the whole *Hsdr4* gene from genomic DNA of wild barley.

BAC library screening

The Bacterial Artificial Chromosome (BAC) library of *H. vulgare L.* cv. Morex (Yu et al. 2000) was used for isolation of the *Hsdr4* promoter region. A set of 17 high-density filters printed with the BAC library clones in a 4×4 double-spotted array was screened with a 143 bp fragment from *Hsdr4* cDNA as a ^{32}P-labeled probe. Hybridizations of the BAC library high-density filters and genomic Southern blots were performed as described by Dubcovsky and Dvorak (1994). Five positive BAC clones (61L6, 76G22, 76G24, 275F6, and 422I22) carried the *Hsdr4* gene were confirmed by PCR using *Hsdr4* specific cDNA primers. The DNA from these BAC clones was isolated using PCIψClone BACDNA Kit (Princeton Separation, USA). *Hin*dIII digested fragments of BAC clone's DNA were separated on 1% agarose gel using pulse-field electrophoresis system (Bio-Rad), blotted onto a Hybond N^+ membrane and hybridized with [^{32}P]-labeled 143 bp fragment from cDNA of *Hsdr4* according to standard procedures (Sambrook et al. 1989). BAC clone 76G24 was chosen for isolation of the *Hsdr4* promoter region.

Quantitative expression analysis of *Hsdr4* by real-time PCR (Q-PCR)

Gene quantification of *Hsdr4* gene was performed using ABI PRISM 7000 Sequence Detection System and SYBR Green PCR Master Mix (Applied Biosystems, USA). Total RNA was extracted from seedling shoots of two sensitive (JS1 and JS2) and two tolerant (JR1 and JR2) genotypes under control and after 3 h and 12 h of dehydration. A specific primer pair was designed, based on sequence *Hsdr4* gene considering the exon–intron structure (forward 5′-CCGGGCTTTA TTCCTGGCT-3′ and reverse 5′-TTTCCAGTA-CAACCCTCCGCT-3′). The standard curve was generated for the *Hsdr4* gene using serial dilutions of an experimental cDNA sample that showed the maximal amount of target gene in preliminary RT-PCR analysis (cDNA sample of JR1 genotype after 12 h dehydration). In order to account for differences in target RNA presented in each sample, *Hsdr4* gene quantities were normalized to the barley *α-tubulin* as an internal

housekeeping gene, which was amplified with forward (5′-TCCATGATGGCCAAGTGTGA-3′) and reverse (5′-CTCATGTACCGTGGGGATGTC-3′) primers (acc. no. U40042). Five independent plant samples for each genotype were examined in triplicates. Student's *t*-test and StatSoft package (Version 6.0) were used to evaluate the expression data.

Genetic mapping of the *Hsdr4* gene

A wild barley mapping population derived from a cross of *H. spontaneum* accessions MA10-30 × WQ23-38 (Chen 2005) was used to determine the chromosomal location of the *Hsdr4* gene. Using pair of primers (5′-CGAGGAGTGGCACGACTGCGT-3′ and 5′-CCTCTGGCAACTCGGTGCGGAG-3′), one of the introns of *Hsdr4* gene was amplified and sequenced for both parental lines. The genomic DNA of 135 plants was used for PCR amplification followed by *Taq1* restriction of PCR products. Using the presence/absence restriction site polymorphism of F_2 plants and aligning our data with existing mapping data for the MA10-30 × WQ23-38 mapping population, the map location of the barley *Hsdr4* gene was determined. The MultiPoint program for efficient multipoint mapping (http://www.multiqtl.com), based on Evolutionary Strategy algorithm (Mester et al. 2004), was used for mapping.

Analysis of the *Hsdr4* promoter and gene sequence

The obtained nucleotide and deduced amino acid sequences of *Hsdr4* were analyzed with DS Gene program (Accelrys, Software Inc., England). Analysis of the promoter sequence was performed using the Eukaryotic Promoter Database, EPD, (http://www.epd.isb-sib.ch). To search for known promoter motifs and transcription factor binding sites, the software Signal Scan of the Plant *Cis*-acting Regulatory DNA elements database was used (Higo et al. 1999), (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html).

Results

Differential expression of wild barley under water stress by cDNA-AFLP analysis

cDNA-AFLP analysis was performed using seedlings of two contrasting genotypes (JS1 and JR1) subjected to dehydration stress in order to identify genes that are involved in water-stress tolerance of wild barley. Differentially expressed fragments were detected by selective amplifications using fifteen primer combinations (PCs). An example of a typical cDNA-AFLP banding pattern with three primer combinations is presented in Fig. 1. Number and length of observed transcript-derived fragments (TDFs), varying from 50 bp to 500 bp, were dependent on PCs. In total, approximately 1100 TDFs were detected in the two barley genotypes under control and stress conditions. Of these, 70 fragments (6.3%) were differentially expressed in the control plants as compared to water stressed plants (3 h or 12 h dehydration). We selected 11 TDFs that displayed clear differences between sensitive and tolerant genotypes under 3 h or 12 h

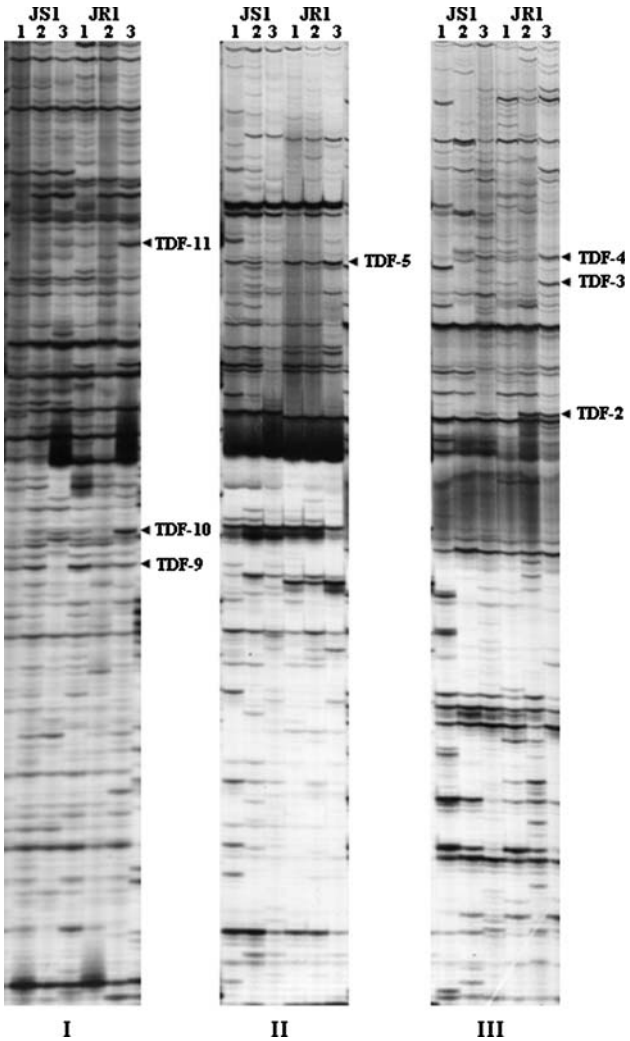

Fig. 1 cDNA-AFLP fingerprints of a sensitive (JS1) and tolerant (JR1) wild barley genotypes under control and dehydration stress. Lanes: 1. control plants; 2. 3 h dehydration; 3. 12 h dehydration. The primer combinations used here: I—*Eco*RI-C/*Mse*I-CG; II—*Eco*RI-CA/*Mse*I-CCC; III—*Eco*RI-CA/*Mse*I-CCG. Arrows indicate several differentially expressed bands, which were isolated and sequenced

dehydration stress. Differential amplification was confirmed by repeating the cDNA-AFLP PCR reaction with primer combinations that produced bands of interest.

cDNA-AFLP employs a highly stringent PCR regime. However, there remains the problem of overlapping and co-migration of bands in the gels, since each AFLP band may be composed of a number of fragments with identical size that differ in sequence (Meksem et al. 2001). To overcome this problem, we used a few steps of the procedure developed by Brugmans et al. (2003) for converting AFLP markers into single-locus markers. By using a generalized set of 16 degenerated *Mse*I primers and 16 degenerated *Eco*RI primers, we could determine the extra selective nucleotides adjacent to the *Mse*I and *Eco*RI primers for the 11 TDFs. For example, TDF-11 that was originally amplified with *Mse*I-CG/*Eco*RI-C primers was used as template for PCR amplification with *Mse*I-CG and a set of 16 degenerated *Eco*RI primers: (1) *Eco*RI primer + N + A or C or G or T; (2) *Eco*RI primer + NN + A or C or G or T primers; (3) *Eco*RI primer + NNN + A or C or G or T; (4) *Eco*RI primer + NNNN + A or C or G or T. The results of PCR with first four primers (+N) allowed the determination of the second selective nucleotide since the quantity of only one of the amplification products was high. The same happened with the amplification products of the second set of four primers (+NN) that allowed the determination of the third selective nucleotide. The third set of four primers (+NNN) allowed the determination of the fourth selective nucleotide, while the amplification with the fourth sets of primers (+NNNN) allowed the determination of the fifth selective nucleotide. According to these PCR results, we could determine four extra selective nucleotides adjacent to the *Eco*RI-C primers for the specific TDF. The same was done with the set of *Mse*I degenerated primers. Based on this analysis, new specific primers were designed for final PCR amplification of each TDF. The final PCR products were used for direct sequencing with no need in sub-cloning and sequencing of a few bands from each TDF. The TDFs gave good-quality sequences and the presence of the selective primers with additional extra four selective nucleotides in the 5′ or 3′ ends of all eleven TDFs confirmed the validity of our technique that was developed to overcome the problem of overlapping and co-migration of multiple bands in the gels.

The eleven TDFs were used to search the GenBank database and the *Triticeae* EST database (‘‘HarvEST’’). Hits were considered significant if the expected value (*E*-value) was less than 1.0E–5. The results of sequence comparisons are summarized in Table 2. From the eleven TDFs, six fragments showed significant homology with known-function genes. Five TDFs were homologous to EST sequences from *H. vulgare*, among them three corresponded to clones of EST libraries from disease and drought-stressed plants. TDF-2 and TDF-10 displayed very weak similarities to EST sequences. Of the six known-function genes, four are possibly involved in general cellular metabolism and organization, including pectin glucuronyltransferase (TDF-5), cystathionine β-lyase (TDF-11), 3-β-glucuronosyltransferase (TDF-13), and cullin3 (TDF-14). Another two drought responsive genes encoding for S-adenosylmethionine decarboxylase (TDF-12) and Rho-GTPase-activating protein (TDF-4), belong to the defense/stress and signal transduction category.

Expression analysis of selected TDFs by RT-PCR

To verify the cDNA-AFLP results, an independent expression study was performed for seven TDFs by RT-PCR. New cDNA samples were prepared from a new set of JS1 and JR1 barley plants grown under the same conditions as used for the initial cDNA-AFLP analysis. Internal specific primer pairs were designed for fragments TDF-2, TDF-3, TDF-4, TDF-5, TDF-11, TDF-13, and TDF-14 based on their sequences and used for RT-PCR. The suitable primer pairs were designed for seven (relatively long) out of the sequenced 11 TDFs. Individual PCR conditions and primer pair sequences for each TDF are shown in Table 1.

RT-PCR analysis proved that the seven isolated cDNA-AFLP fragments are differentially expressed in sensitive and tolerant wild barley genotypes under water-stress condition (Fig. 2). All of the TDFs were up regulated in the tolerant genotype as compared to the sensitive genotype after 12 h of dehydration, and very good correspondence was found between cDNA-AFLP and RT-PCR expression patterns in JS1 and JR1 genotypes under control and dehydration stress (Fig. 2).

TDF-4 was selected as a promising candidate gene for drought tolerance due to high correspondence of its cDNA-AFLP and RT-PCR expression patterns and high homology to the gene encoding for GTPases known to be involved in many signal transduction pathways. Furthermore, RT-PCR using primers of TDF-4 confirmed the differential expression in another pair of sensitive (JS2) and tolerant (JR2) barley genotypes that was included in this expression assay.

Table 2 Homologies of TDF sequences isolated by cDNA-AFLP analysis

TDF name	Accession number**	Size (bp)	Expression pattern	Homology	Gene Bank Match	E value
TDF-2	EBl74194	135	Up-regulated in resistant genotype after 3 and 12h dehydration	*H. vulgare* seedling shoot EST library (dehydration stress)	BF630929	3.0
TDF-3	EB174195	202	Up-regulated in resistant genotype after 12h dehydration	*H. vulgare* leaf epidermis EST library (*Bipolaris sorokimana* infected)	CX627923	8.00E–38
TDF-4	EBl74196	223	Up-regulated in sensitive and resistant genotypes after 12h dehydration	Rho–GTPase-activating protein-like *(Oryza sativa)*	BAD87212	l.00E–32
TDF-5	EB174197	219	Up-regulated in resistant and down-regulated in sensitive genotype after 12h dehydration	Putative pectin-glucuronyltransferase *(O. sativa)*	XP466000	2.00E–50
TDF-7	EB174198	118	Up-regulated in sensitive genotype after 3 and 12h dehydration	*H. vulgare* spike EST library (*Fusarium graminearum* infected)	BI949919	4.00E–15
TDF-9	EBl74199	84	Down-regulated in sensitive genotype after 12h dehydration	*H. vulgare* ESTs from drought stressed seedlings	BM816475	3.00E–06
TDF-10	EBl74200	108	Up-regulated in sensitive and resistant genotypes after 12h dehydration	*H. vulgare* root EST library (non stressed)	AL502619	2.1
TDF-11	EBl74201	231	Up-regulated in resistant genotype after 12h dehydration	Cystathionine beta-lyase (CBL) *(A. thaliana)*	NP_191264	2.00E–27
TDF-12	EB174202	84	Up-regulated in resistant genotype after 12h dehydration	*Triticum aestivum* S-adenosylmethionine decarboxylase precursor	AF117660	l.00E–12*
TDF-13	EBl74203	334	Up-regulated in resistant and down-regulated in sensitive genotype after 12h dehydration	Putative 3-beta-glucuronosyitransferase (*pglcat6* gene) *(H. vulgare)*	HVU567371	9.00E–100*
TDF-14	EB174204	229	Up-regulated in resistant and down-regulated in sensitive genotype after 12h dehydration	Putative cullin 3 *(O. sativa)*	XP467770	2.00E–26

All are BLASTX scores except for those with *, which are BLASTN scores. ** Sequences were entered into the NCBI dbEST database

Expression analysis of the *Hsdr4* gene tested by quantitative RT-PCR (Q-PCR)

To quantitatively determine the expression pattern of the *Hsdr4* gene in tolerant and sensitive wild barley genotypes under dehydration stress, Q-PCR (quantitative expression analysis by real-time PCR) was used. Expression analysis of the *Hsdr4* gene under dehydration stress tested by Q-PCR analysis revealed no significant difference between sensitive and tolerant genotypes under control and after 3 h drought stress (Fig. 3). However, after 12 h dehydration, the tolerant genotypes (JR1 and JR2) showed 61% ($P = 0.011$) higher expression of the *Hsdr4* gene as compared to the sensitive ones (JS1 and JS2). These Q-PCR analysis of the *Hsdr4* gene validated the results obtained by both cDNA-AFLP and RT-PCR analysis and revealed higher expression level of the *Hsdr4* gene after 12 h dehydration stress in both tolerant genotypes (JR1 and JR2) as compared to the sensitive ones (JS1 and JS2).

Cloning and structure analysis of the *Hsdr4* gene

To obtain the complete nucleotide sequence of the gene corresponding to TDF-4, both 5′ - and 3′ - amplification of cDNA ends (RACE) was performed using TDF-4-specific primers and cDNA synthesized from dehydrated (12 h) wild barley genotype (JR1). The cloned 980 bp full-length cDNA, designated *Hsdr4* (*Hordeum spontaneum* drought-responsive), contains one open reading frame of 753 bp, encoding a protein of 250 amino acid residues. The 5′-UTR and 3′-UTR comprised of 34 bp and 193 bp, respectively. The database BLASTP search showed that the protein encoded by *Hsdr4* cDNA is 82% identical (E-value 3E–122, score 440 bits, 91% positivities) to the Rho-GTPase-activating protein-like of *Oryza sativa* (acc. no. BAD87212) and is 61% identical (E-value 1E–81, score 305 bits, 77% positivities) to an unknown protein of *Arabidopsis thaliana* (acc. no. NP_566369). Figure 4 illustrates the alignment of the deduced amino acid sequence of barley *Hsdr4* with the two most similar deduced proteins. Alignment revealed the SEC14 domain (Sec14p-like lipid-binding domain) (E-value 2E–11, score 63.5 bits, 100% aligned), that was first described in *Saccharomyces cerevisiae* phosphatidyl-inositol transfer protein (Sec14p), which plays an essential role in the formation of secretory vesicles and in protein transport from the Golgi complex (Bankaitis et al. 1990).

The whole *Hsdr4* gene was amplified from genomic DNA of wild barley using specific primers for 5′- and

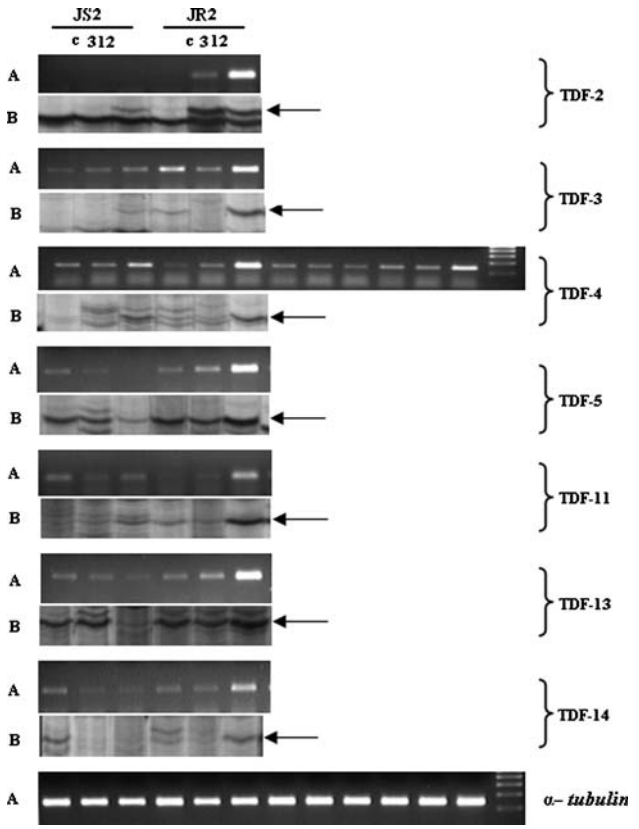

Fig. 2 Comparison of TDF expression patterns obtained by (A) RT-PCR and (B) cDNA-AFLP techniques. cDNA was obtained from seedlings of drought-sensitive (JS1) and drought tolerant (JR1) genotypes after dehydration treatments of 3 h and 12 h and control conditions (time 0). The expression of TDF-4 was compared by using a second pair of tolerant and sensitive genotypes JS2 and JR2. Barley *α-tubulin* gene was used as control for relative amount of RNA

3′-UTR. Comparison of the genomic DNA sequence with *Hsdr4* cDNA revealed that the gene consists of two introns and three exons. The splice junctions of both introns follow the GT/AG rule. The genomic sequence of the *Hsdr4* gene is available in the NCBI databases (acc. no. DQ464370). Southern blot of wild barley genomic DNA revealed that there is only one copy of this gene present in the wild barley genome (data not shown).

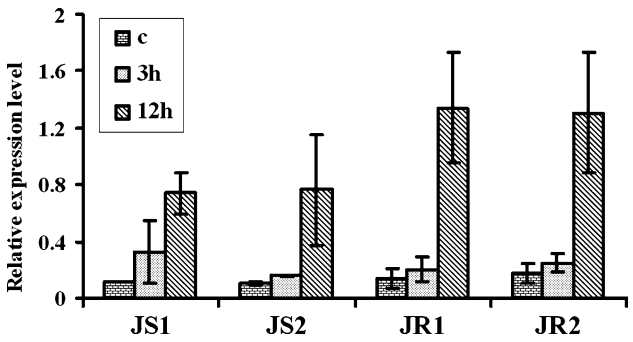

Fig. 3 *Hsdr4* gene expression upon dehydration stress detected by quantitative real time PCR (Q-PCR). Q-PCR was carried out with cDNA obtained from two sensitive (JS1 and JS2) and two tolerant (JR1 and JR2) wild barley genotypes under control (c) and after 3 h and 12 h of dehydration. Quantification is based on Ct values that were normalized using the Ct value corresponding to a barley *α-tubulin* gene. Each value is the mean ± SE (n = 5)

Isolation of *Hsdr4* promoter

The promoter region of the *Hsdr4* gene was isolated by screening the BAC library of cultivated barley (*H. vulgare L.* cv. Morex) (Yu et al. 2000). A positive BAC clone, 76G24, was used to subclone a 3.1-kb *Hin*dIII fragment carrying the *Hsdr4* gene into pBlueScript KS$^+$. This fragment was sequenced using *Hsdr4* and vector specific primers. Then, a new forward primer specific for 5′-end of *Hsdr4* of cultivated barley and reverse wild-specific primer of *Hsdr4* were used to PCR amplify the promoter region of the *Hsdr4* gene from wild barley, yielding a 1.7 kb fragment upstream of the translation start codon. Sequence analysis of the 5′-region of *Hsdr4* gene by comparison with the Eukaryotic Promoter Database (EPD) from NCBI showed no overall sequence homology to any other promoter. The putative transcription initiation site (TIS) was determined experimentally with FirstChoice RLM-RACE Kit (Ambion, USA) at 34 bp upstream of the first in-frame ATG codon. Using SignalScan software of PLACE database (Higo et al. 1999), the putative CCAAT motif was recognized at 52 bp upstream of the TIS. However, a putative TATA box was not revealed, suggesting that *Hsdr4* promoter belongs to the TATA-less class of promoters (Fig. 5).

Analysis of *Hsdr4* promoter

Putative cis-elements

For the identification of putative *cis*-acting elements in *Hsdr4* promoter the plant-oriented collection of transcription regulatory elements PLACE database was used. Fifteen putative regulatory *cis*-elements were found in the *Hsdr4* promoter (boxes A–N, Fig. 5). Their functions, sequences, and positions in *Hsdr4* promoter are summarized in Table 3. These elements from *Hsdr4* promoter could be separated into two groups according to their known functions. More than half of the identified putative signals belong to the group of typical stress-related *cis*-acting elements (boxes A–H, Fig. 5 and Table 3). Another group of putative binding sites for transcription factors, found in *Hsdr4* promoter, is composed of elements involved in hormone responses and developmental processes (boxes I-O, Fig. 5 and Table 3).

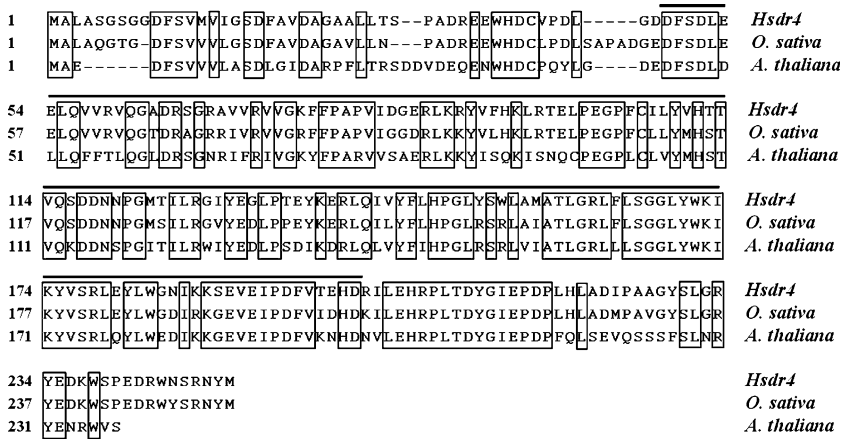

Fig. 4 Alignment of the full-length amino acid sequence of the *Hsdr4* with the two most similar proteins found in the databases, the rice Rho-GTPase-activating protein-like (acc. no. BAD87212) and unknown protein of *Arabidopsis* (acc. no. NP_566369). Enclosed boxes indicate identical amino acids. The SEC14 domain is indicated as a solid line. The alignment was generated by ClustalW algorithm using the MegAlign tool of the DS Gene program

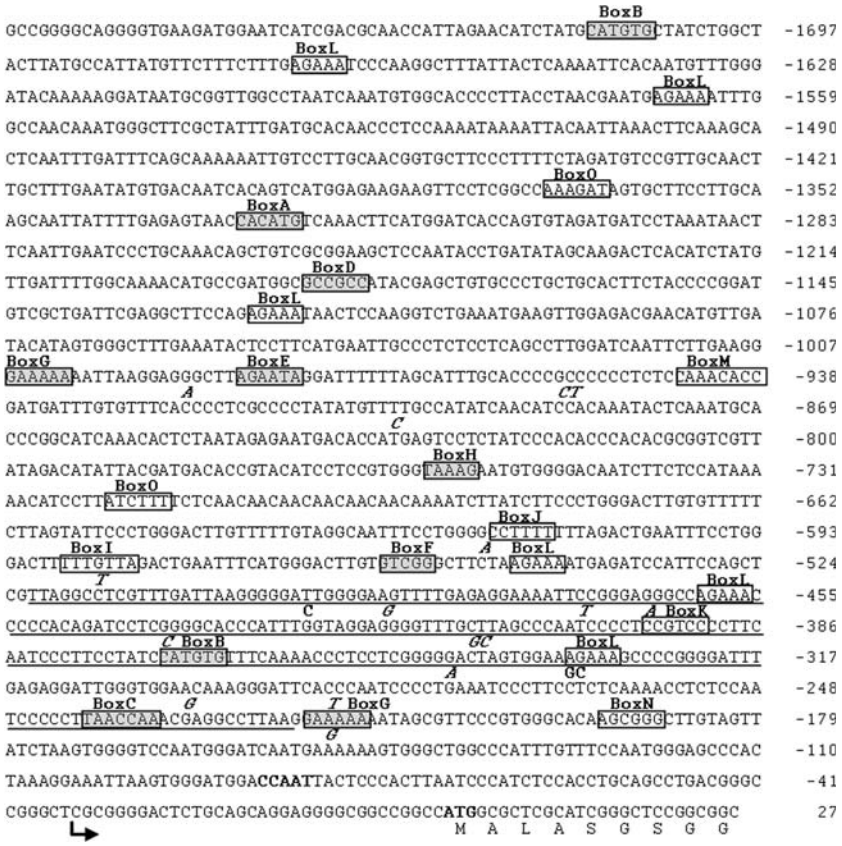

Fig. 5 Nucleotide sequence of the promoter region of the *Hsdr4* gene sequenced from tolerant barley genotype. Sequence of the 5′-flanking region and partial amino acid sequence of the first exon of the *Hsdr4* gene are shown together. The numbering of nucleotides relative to the translation start site (ATG) is shown on the right. The transcription initiation site is labeled with an arrow, and the putative CCAAT box is in bold letters. Putative promoter *cis*-elements are labeled with boxes (boxes A–O). The stress-related *cis*-elements are shown in shaded boxes. The miniature inverted-repeat transposable element (MITE), which potentially can form the hairpin structure, is underlined. The sequence differences in *Hsdr4* promoter of sensitive genotypes and cultivated barley are represented in bold and italic bold letters, respectively, under corresponding nucleotides in the *Hsdr4* promoter of tolerant genotypes

Table 3 Homology between *Hsdr4* promoter and several transcription factors (TF) binding sites

Box name	Factor or site name	Function	Location/Strand	Signal sequence	Reference
Box A	MYCATRD22	Recognition site for MYC and MYB transcription factors	–1330/(+)	CACATG	Abe et al. (1997, 2003)
Box B	MYCATERD1	Recognition box for MYC and NAC transcription factors	–371/(+) –1712/(+)	CATGTG	Simpson et al. (2003), Tran et al. (2004)
Box C	MYB1AT	MYB and MYC recognition site in dehydration-responsive gene *rd22* and many other genes in *Arabidopsis*	–240/(+)	WAACCAA(W=A/T)	Abe et al. (1997, 2003)
Box D	GCCCORE	Core of GCC-box found in many pathogen-responsive genes, function as ethylene- and jasmonate-responsive element	–1186/(+)	GCCGCC	Brown et al. (2003), Liu et al. (2006)
Box E	-10PEHVPSBD	Barley chloroplast *psb D* gene light-responsive promoter element	–985/(–)	TATTCT	Thum et al. (2001)
Box F	LTECOREATCOR15	Core motif of low temperature responsive element (LTRE)	–558/(–)	CCGAC	Baker et al. (1994) Jiang et al. (1996)
Box G	GT1GMSCAM4	''GT-1 motif'' plays a role in pathogen- and salt-induced *SCaM-4* gene expression in soybean	–220/(+) –1006/(+)	GAAAAA	Park et al. (2004)
Box H	TAAAGSTKST1	Target site for trans-acting StDofl protein controlling guard cell-specific gene expression	–761/(+)	TAAAG	Plesch et al. (2001)
Box I	MYBGAHV	Central element of gibberellin (GA) response complex (GARC) in high-pI alpha-amylase gene	–587/(–)	TAACAAA	Gubler et al. (1995)
Box J	PYRIMIDINEBOXOSRAMY1A	Pyrimidine box in rice alpha-amylase *(RamylA)* gene involved in sugar repression	–617/(+)	CCTTTT	Morita et al. (1998)
Box K	PALBOXAPC	One of *cis*-acting elements of phenylalanine ammonia-lyase genes in parsley	–396/(+)	CCGTCC	Logemann et al. (1995)
Box L	POLLEN1LELAT52	One of two co-dependent regulatory elements responsible for pollen specific activation of tomato *lat52* gene	–334/(+) –460/(+) –546/(+) –1122/(+) –1568/(+) –1670/(+)	AGAAA	Bate and Twell. (1998)
Box M	PROXBBNNAPA	''prox B'' (proximal portion of B-box) in promoter *napA* gene of *Brassica* is required for seed specific expression and ABA responsiveness	–945/(+)	CAAACACC	Ezcurra et al. (1999)
Box N	BS1EGCCR	BS1 binding site in *Eucalyptus gunnii* Cinnamoyl-CoA reductase (CCR) gene promoter	–193/(+)	AGCGGG	Lacombe et al. (2000)
Box O	NODCON1GM	One of two putative nodulin consensus sequences in the soybean leghemoglobin *Ibc3* and *N23* gene promoters	–1371/(+) –721/(–)	AAAGAT	Stougaard et al. (1990)

The results were obtained by searching the PLACE database (Higo et al. 1999). In the fourth column the position of the putative TF binding site in *Hsdr4* promoter relative to the translation start site and the *Hsdr4* strand (+ or –) in which the box was found, are indicated

Putative MITE structure

Structural analysis of 5′ region of *Hsdr4* gene revealed some additional interesting features. The portion (299 bp in length) of the proximal part of this promoter, located at –222 bp to –521 bp upstream of the start codon (Fig. 5), can potentially form a secondary structure because of several inverted repeats. A more detailed analysis revealed high resemblance of this sequence to the miniature inverted-repeat transposable

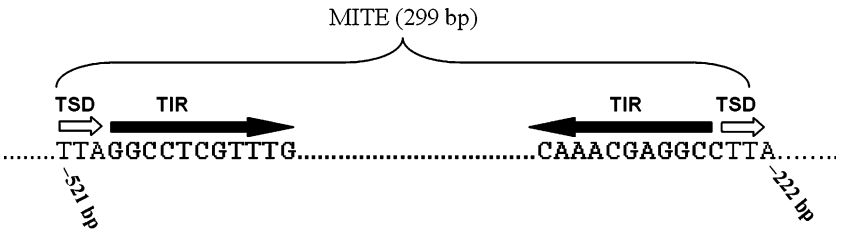

Fig. 6 Miniature inverted repeat transposable element (MITE) in the promoter region of the barley *Hsdr4* gene. MITE (299 bp in length) was identified –222 bp to –521 bp upstream of the start codon. The empty arrows refer to the target site duplication (TSD) sequences and filled arrows denote terminal inverted repeats (TIRs)

elements (MITEs). MITEs are non-autonomous DNA (class II) transposable elements characterized by short length (~60–700 bp), no coding capacity, usually short (10–30 bp) terminal inverted repeats (TIR), and target site (2–3 bp, rich in A and T) duplicated (TSD) at insertion, so that the MITE is flanked by a direct repeat (Fig. 6). MITEs have a potential to form a hairpin-like secondary structure and show preference to insert in introns or near the 5′ or 3′ ends of genes, but not in coding regions (Wessler et al. 1995; Feschotte et al. 2002).

The sequence of this 299 bp portion of *Hsdr4* promoter exhibited no homology to known repetitive elements in the Plant Repeat Database (http://www.tigr.org/tdb/e2 k1/plant.repeats/index.shtml). Nevertheless, its structure resembled the MITEs features. In our putative MITE, the insertion is flanked by 3 bp direct repeats (TTA), and the ends of the element are represented by 11 bp inverted terminal repeats (TIRs) (GGCCTCGTTTG) (Fig. 6). Its internal sequence displayed high similarity (E-value 5E–92, score 345 bits, 91% identities) to a part of the first intron of cyclic nucleotide-gated ion channel 4 (*nec1*) gene of barley (acc. no. AY972619). The detected MITE is especially capable of forming hairpin-like secondary structure (Fig. 7).

Chromosomal assignment of the *Hsdr4* gene

To map the *Hsdr4* gene on wild barley chromosomes one of the introns of this gene was amplified and sequenced from the two parental lines of the MA10-30 × WQ23-38 wild barley mapping population, which was evaluated for drought tolerance (Chen 2005). Sequence alignment revealed a few SNPs (single nucleotide polymorphism) between the parental lines. One of the SNPs corresponded to the *Taq1* restriction site that enabled us to map the cloned gene. Using the presence versus absence of restriction site and aligning our data with existing mapping data for this mapping

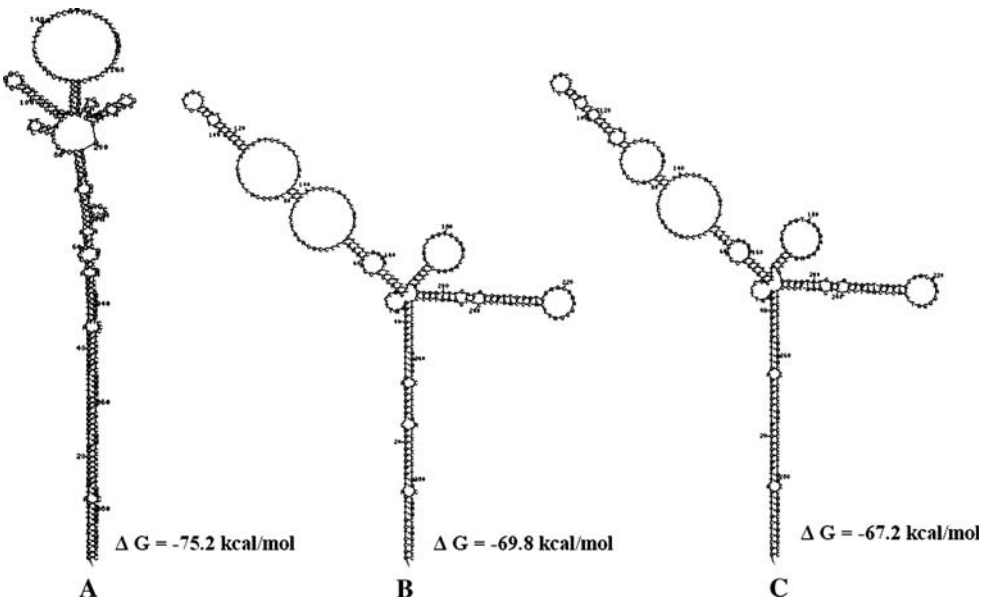

Fig. 7 Potential secondary structures formed by 299-bp putative MITE of promoter region *Hsdr4* gene from cultivated barley (A), and wild barley - sensitive (B) and tolerant (C) genotypes. The minimum-energy folding of elements and free energies (ΔG) are predicted by DNA MFOLD program (version 3.1) (Zuker 2003; http://www.bioinfo.rpi.edu/applications/mfold/old/dna/)

population, the *Hsdr4* gene was mapped to the long arm of chromosome 3H between markers *EBmac541* and *EBmag705* (Fig. 8).

Sequence comparison of *Hsdr4* between tolerant and sensitive barley genotypes

Since the expression pattern of *Hsdr4* gene under dehydration stress was different between tolerant and sensitive genotypes, we wondered whether it may derive from sequence differences in the promoter region. Moreover, keeping in mind our hypothesized importance of this gene in drought resistance, it was also interesting to look for possible differences between tolerant and sensitive genotypes in its transcribed part. *Hsdr4* gene and its promoter region of two tolerant and two sensitive genotypes were sequenced. Sequence alignment showed a high degree of sequence similarity between the four genotypes. The only differences found between tolerant and sensitive genotypes were in some SNPs in intron (data not shown) and in the promoter region (Fig. 5). Several SNPs were found in the putative MITE in comparisons between tolerant and sensitive wild barley genotypes and cultivated barley that may cause different folding patterns (Fig. 7).

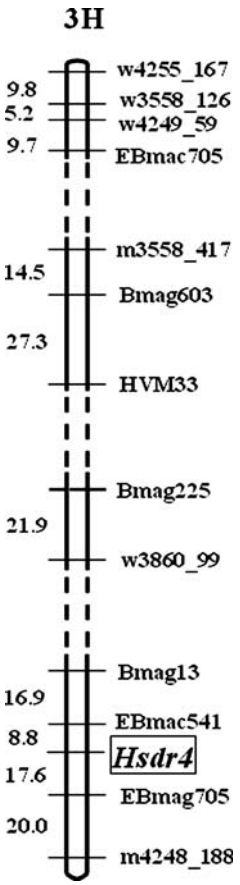

Fig. 8 Chromosome assignment of *Hsdr4* gene on barley chromosome 3H. The map location of *Hsdr4* gene is boxed. Units are in cM. Genetic mapping was conducted using MultiPoint program (http://www.multiqtl.com)

Discussion

Differential expression of wild barley under dehydration by cDNA-AFLP analysis

Plant adaptation to environmental stress is regulated through multiple physiological mechanisms at the cellular, tissue, and whole-plant levels (Hazen et al. 2003; Hazen et al. 2005; Ito et al. 2006; Yamaguchi-Shinozaki and Shinozaki 2005). Identification and detailed analysis of a large number of candidate genes involved in drought tolerance may enable to elucidate the molecular basis of drought resistance complexity.

The aim of our study was to investigate expression pattern of genes that are altered in response to dehydration stress and are potentially involved in the tolerance to dehydration in wild barley, by using the cDNA-AFLP fingerprinting method. cDNA-AFLP is one of the technologies that is successfully employed for characterization of gene expression under abiotic and biotic stresses in plants including water deficit (Dubos and Plomion 2003; Yang et al. 2003; Knight et al. 2006; Rodriguez et al. 2006). The cDNA-AFLP technique has the advantage of technical simplicity as compared with microarray, substracctive hybridization and less labor intensive as compared with other mRNA fingerprinting methods such as differential display (DDRT-PCR) (Liang and Pardee, 1992).

We used the cDNA-AFLP method to compare expression patterns of genotypes that under dehydration stress showed higher relative water content (RWC) and lower water loss rate (WLR), as compared to genotypes with lower RWC and lower WLR (Suprunova et al. 2004). The expression patterns of these genotypes that were regarded as tolerant and susceptible to water stress, were compared under control and dehydration stress. This kind of comparison enabled us to relate the function of the identified gene not only to those that are induced by dehydration stress but to those that are involved in mechanisms of tolerance or resistance to water stress. We have used this approach previously by comparing patterns of dehydrin gene expression in the same tolerant and sensitive wild barley genotypes (Suprunova et al. 2004). Recently, the same approach was described by Rampino et al. (2006) with resistant and tolerant wheat genotypes. This aspect makes the main difference between the

present study and most of others that use expression analysis by cDNA-AFLP to provide a genome-wise description of gene expression profiles. Moreover, we used for our study wild relative of cultivated barley, *Hordeum spontaneum*, which is a unique genetic resource harboring adaptive mechanisms of stress resistance from a unique (desert) ecogeographic region.

For the efficiency of sequencing efforts, we carefully selected the candidate TDFs that were sequenced and further analyzed. For that, the method developed by Brugmans et al. (2003) was employed. By using this method we were able to isolate one amplicon per each TDF for direct sequencing, omitting other co-migrating fragments usually presented within the PCR products of the TDF excised from the gel. This was achieved by three steps of PCR amplifications: (a) re-amplification of each TDF with its corresponding selective primers, (b) second amplification by a set of degenerated primers in order to define additional four bases adjacent to the first one or two selective nucleotides; (c) third amplification with a pair of *Eco*RI primer + four additional nucleotides and *Mse*I primer + four additional nucleotides. Usually, DNA-AFLP fragments are cloned, confirmed by PCR or restriction analysis and then sequenced. Thus, Umezawa et al. (2002) used 64 PCs for selective amplification. They describe that from 130,000 fragments 140 bands were differentially expressed; their putative gene function according to BLAST comparison was described, but the expression of only four fragments was confirmed by RNA dot blot. Other studies describe the isolation of TDFs and re-amplification with non-selective primers (Bruggmann et al. 2005). These authors indeed used the maximal number of primer combinations (256); and out of the 363 TDFs isolated from the cDNA-AFLP gel, the induced expression of 92 TDFs was confirmed by gel blot; 1–8 clones per each TDF were sequenced because of mixed PCR products. Eventually, the expression of seven fragments was confirmed by quantitative RT-PCR. In the extensive work by Blanco et al. (2005) 128 PCs were used for amplification; however, these authors focused on analysis of 59 fragments out of 5680 TDFs. Eventually, the expression of 12 candidates was extensively described and analyzed. The resulting patterns of cDNA-AFLP are highly reproducible and sensitive and correlate well with Northern analysis, Q-PCR and microarrays (Donson et al. 2002; Avrova et al. 2003; Bruggmann et al. 2005). In our study, out of seven TDFs chosen for RT-PCR analysis, four displayed high coincidence of cDNA-AFLP and RT-PCR expression patterns. Q-PCR expression analysis of the *Hsdr4* gene validated the results obtained by cDNA-AFLP and RT- PCR analysis and revealed a higher expression level of the *Hsdr4* gene after 12 h drought stress in both tolerant genotypes (JR1 and JR2) as compared to the sensitive ones (JS1 and JS2).

Although cDNA-AFLP is widely used for expression analysis, there is still a disadvantage of low genuine ''yield'', when the objective is to isolate real relevant genes. By using the approach of (a) selecting only those TDFs that were up-regulated in tolerant plants under stress and (b) adopting the procedure (with some modifications) developed by Brugmans et al. (2003), we managed to achieve the goal with relatively low number of primer combinations.

Sequence analysis of the differentially expressed TDFs

Sequence analysis of the eleven TDFs revealed that they represent genes involved in basic metabolic activity as well as genes encoding for stress related proteins (Table 3). Pectin glucuronyltransferase (TDF-5) and 3-β-glucuronosyltransferase (TDF-13) belong to a large group of glycosyltransferases (GTs). One of the biological functions of GTs is to catalyze the biosynthesis of polysaccharides including cellulose, hemicelluloses, and pectins the main components of the cell wall (Zhong and Ye 2003). Cystathionine β-lyase (TDF-11) is an enzyme that plays a central role in the methionine (Met) biosynthesis pathway and, therefore, in plant growth and development (Maimann et al. 2000). Cullin3 (TDF-14) is a member of the family, which comprises the cullin-dependent ubiquitin ligases that control the rapid and selective degradation of important regulatory proteins involved in cell cycle progression and development (Thomann et al. 2005).

TDF-12 induced in the tolerant genotype under 12 h dehydration stress exhibited homology with *T. aestivum S*-adenosylmethionine decarboxylase protein (SAMDC). SAMDC is one of the rate-limiting enzymes in the biosynthesis of polyamines, which play a crucial role in morphogenesis, embryogenesis, floral and fruit development, and root formation (Walden et al. 1997). SAMDC was previously described as a stress-related protein involved in salt, drought, and cold tolerances of a variety of plants (Li and Chen 2000; Roy and Wu 2002; Hao et al. 2005).

The signal transduction and stress responding category also includes a gene encoding for Rho-GTPase-activating protein, homologous to TDF-4. Rho-GTPase-activating proteins (RhoGAPs) are among the components regulating the small GTPases of the Rho family that play an important role in molecular switches in many signal transduction pathways and

functions. The Rho-GTPase family has emerged as a key regulator of actin cytoskeleton in yeast and animal cells (Hall 1998). In plants, Rho-GTPases have been shown to control different cellular processes such as pollen and root hair tip growth and H_2O_2 production as a second messenger in plant signaling (Yang 2002). One of the most interesting findings about Rho-GTPases is the demonstration of its involvement in the control of stomatal closure and therefore plant water homeostasis. The *Arabidopsis* Rho-related GTPase protein, AtRac1, was identified as a central component in ABA-mediated stomatal closure process. It was shown that ABA treatment induced inactivation of AtRac GTPases, leading to stomatal closure through the disruption of the guard cell actin cytoskeleton (Lemichez et al. 2001).

Novel drought stress responsive gene, *Hsdr4* from wild barley

The isolated *Hsdr4* gene is composed of 3 exons and encodes 250 amino acid putative Rho-GTPase-activating protein that contains a conserved domain very similar to the SEC14 domain (Sec14p-like lipid-binding domain). Sec14p-related proteins are widespread and found in mammals, fungi, and plants. For example, *Arabidopsis* patellin1 (PATL1), characterized also by Golgi dynamics domains (GOLD), is involved in plant cell cytokinesis (Peterman et al. 2004). Another *Arabidopsis* phosphatidylinositol transfer protein, AtSfh1p, possesses the Sec14p-nodulin domain and acts as regulator of polarized membrane growth of root hairs (Vincent et al. 2005). Sec14p-nodulin domain is also shared by four members of PITP-like protein family (LjPLPs) from *Lotus japonicus.* These proteins may function in lipid-signaling pathways that regulate membrane biogenesis during nodulation (Kapranov et al. 2001). One of the members of the Sec14p family from soybean, Ssh1p, was characterized as a component of a stress response pathway that serves to protect the adult plant under osmotic stress (Monks et al. 2001).

All hitherto characterized plant proteins with the Sec-14-like domain belong to the phosphatidylinositol transfer protein (PITP) family. To the best of our knowledge, the Sec-14-like domain was not previously reported in any Rho GTPase-activating proteins (RhoGAPs) in plants. The GAP catalytic domain and the Cdc42/Rac-interactive binding (CRIB) domain are characteristic of this family (Borg et al. 1999; Wu et al. 2000). Nevertheless, several mammalian RhoGAP-like proteins, including neurofibromin NF1 and members of the multifunctional Dbl family were reported recently to possess Sec14p lipid binding domain (Aravind et al. 1999). It was proposed that these proteins with Sec14p-like domain play a role in lipid regulation of the Rho-mediated signaling pathway. Here, we report for the first time on a new putative Rho GTPase-activating protein from wild barley that possesses the Sec14p-lipid binding domain. Studying of the function of the protein encoded by this transcript will be the next major step towards determining its role in stress response, including water stress.

Identification and mapping of relevant QTLs and/or co-localization of QTLs with candidate genes are useful approaches for dissecting the genetic basis of complex traits such as drought tolerance. *Hsdr4* gene was mapped on the long arm of the barley chromosome 3H between markers *EBmac541* and *EBmag705*. None of the QTLs associated with drought tolerance were revealed in this chromosome region in our mapping population of wild barley (Chen 2005). However, according to QTL mapping of another barley population, Tadmor × Er/Apm, this region contains a QTL for osmotic potential (OP) and a QTL that affects the relative water content (RWC) (Diab et al. 2004). Moreover, the *Hsdr4* rice orthologue, encoding for Rho-GTPase-activating protein-like (BAC clone AP003259), is located in rice chromosome 1 at position 167.2–169.5 cM. In this region two QTLs, associated with total root dry weight (TRDW) and penetrated root thickness (PRT) related to drought resistance, were identified in rice (Nguyen et al. 2004). Our finding on the higher expression level of *Hsdr4* gene under dehydration stress in drought tolerant as compared with drought sensitive genotypes and its co-localization with QTLs associated with drought tolerance allow us to suggest that *Hsdr4* could be a viable candidate gene for the determinant of water-stress tolerance. To investigate the gene further to validate its possible role in tolerance to dehydration stress, transgenic work will be conducted.

Sequence analysis of the *Hsdr4* promoter region

Analysis of the 1.7-kb sequence corresponding to the *Hsdr4* promoter resulted in the identification of several potential binding sites for transcription factors. More than half of the identified motifs are typical stress-related *cis*-acting elements that have been identified in the promoter regions of a number of other drought- and ABA-induced genes. For example, MYC and MYB recognition sites (box A and C, Fig. 5 and Table 3) in *Arabidopsis rd22* promoter function as *cis*-acting elements in drought-inducible expression of *rd22* gene (Abe et al. 1997, 2003). Another MYC-like

motif (box B) was found in drought-, salt-, and dark-induced *Arabidopsis erd1* gene. GCC-motif (box D) seems to be associated with jasmonate-responsive gene expression (Brown et al. 2003; Liu et al. 2006). Box E (Fig. 4) is a light-responsive element that is sufficient to mediate circadian cycling of the barley chloroplast *psbD* gene promoter activated by blue, white, or UV-light (Thum et al. 2001). Low temperature responsive element, LTRE (box F), seems to be involved in the expression of such cold-regulated genes as *cor15a* from *A. thaliana* (Baker et al. 1994), *bn115* from winter *Brassica napus* (Jiang et al. 1996), and *wcs120* from wheat (Ouellet et al. 1998). GT-1 *cis*-element (box G) interacts with a GT-1-like transcription factor that plays a role in pathogen- and salt-induced *SCaM-4* gene expression in both soybean and *Arabidopsis* (Park et al. 2004). Finally, box H (Fig. 5) showed similarity with TAAAG motif found in the promoter of the potato *KST1* gene encoding for a K^+ influx channel of guard cells. TAAAG elements are target sites for trans-acting Dof (zinc finger) transcription factors controlling guard cell-specific gene expression (Plesch et al. 2001). Our results of *Hsdr4* promoter analysis are consistent with the expression data of this gene. Strong indications of *Hsdr4* induction by dehydration stress and preliminary results on salt induction (not shown) allow us to speculate that the revealed MYC, MYB, LTRE, and GT-1 elements may be related to stress responsiveness. Whether different responsive elements of the *Hsdr4* promoter are *in vitro* targets for corresponding transcription factors remains to be elucidated in future studies.

The MITE insertion, which was identified in the promoter region of the *Hsdr4* gene, seems to be a new transposable element in barley. MITEs are high-copied DNA transposons associated with the non-coding part of genes and, therefore, are useful in plant genome analysis as molecular markers and potential tools in plant systematic and phylogenetic studies (Feng 2003). The close proximity of MITEs to the coding regions suggests their role in transcription, splicing, and translational regulation of the genes. For example, MITE insertion in intron 2 of one of the *nec1* gene alleles in barley caused alternative splicing, frame shift, and production of a defective protein (Rostoks et al. 2006). MITEs have been suggested to supply *cis*-acting elements affecting, presumably, gene expression (Bureau and Wessler 1994; Pozueta-Romero et al. 1996; Yang et al. 2001). Several features known to be critical for the characteristic of MITEs, are also characteristic of the MITE-like insertion detected in our study: (i) the proximity to the start codon of *Hsdr4*; (ii) presence of a few putative *cis*-element in the internal sequence; and (iii) the predicted ability to form sequence-depending alternative hairpin structures with different free energy (Fig. 7). These features may provide the different regulatory mechanism since such structures are known to be critical for transcriptional regulation (Wadkins et al. 2000).

In summary, we demonstrated that the study of wild relatives of cultivated plants from diverse ecogeographic regions combined with functional genomic tools such as the cDNA-AFLP technique has great potential for identification of novel candidate genes related to stress tolerance. We described here the sequence, structure and expression, and genetic mapping of a new candidate gene (*Hsdr4*) for water-stress tolerance in wild barley. *Hsdr4*, induced by dehydration stress in tolerant young plants of wild barley, encodes a putative Rho-GTPase-activating protein with a Sec14p-like lipid-binding domain, and has several potential stress-related *cis*-elements in the promoter region. Due to its expression patterns and structure, *Hsdr4* is a promising candidate gene for further studies on the molecular genetic basis of water-stress tolerance in plants.

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