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Hv-WRKY38: a new transcription factor involved in cold- and drought-response in barley

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Abstract

WRKY proteins constitute a large family of plant specific transcription factors implicated in many different processes. Here we describe *Hv-WRKY38,* a barley gene coding for a WRKY protein, whose expression is involved in cold and drought stress response. *Hv-WRKY38* was early and transiently expressed during exposure to low non-freezing temperature, in ABA-independent manner. Furthermore, it showed a continuous induction during dehydration and freezing treatments. A WRKY38:YFP fusion protein was found to localise into the nucleus upon introduction into epidermal onion cells. Bacterially expressed Hv-WRKY38 was able to bind *in vitro* to the W-box element (T)TGAC(C/T) also recognisable by other WRKY proteins. *Hv-WRKY38* genomic DNA was sequenced and mapped onto the centromeric region of the barley chromosome 6H. *Arabidopsis* and rice sequences homologous to *Hv-WRKY38* were also identified. Our results indicate that *Hv-WRKY38* transcription factor may play a regulatory role in abiotic stress response.

Abbreviations: ABA, Abscissic Acid; CAP, cleavaged amplified polymorphism; GMSA, gel mobility shift assay; GST, glutathione-S-transferase; YFP, yellow fluorescent protein

Introduction

Reprogramming of cellular function in response to external stimuli involves complex changes in gene expression. The transcription of a multitude of genes is subject to up- or down-regulation following defined temporal programmes (Fowler and Thomashow, 2002). The perception of an external stimulus immediately leads to the activation of early-responsive genes, mediated by pre-existing signalling components. In some case, the accumulation of the corresponding mRNAs is not only very rapid, but also transient in nature (Thomashow, 1999). The activation of these early-responsive genes, encoding signal proteins and transcription factors, is a prerequisite for the subsequent activation of secondary-responsive genes.

In plants, the regulation of the genes responsive to cold- and drought-stresses is a recent important insight. In *Arabidopsis* two main pathways leading to the expression of stress-induced genes in ABA-dependent or ABA-independent manner are known (Shinozaki *et al.*, 2003). Several MYC/MYB and bZIP transcription factors, with related *cis*-acting elements, are among the best known components of the signal transduction pathway in ABA-dependent genes expression (Bonetta *et al.*,

1998; Shinozaki and Yamaguchi-Shinozaki, 2000). On the other hand, the signal transduction pathway, promoting the ABA-independent gene expression in response to cold and dehydration, includes the DRE/CRT (dehydration-responsive element)/(C-repeat) *cis*-acting element and the AP2 transcription factors DREB1/CBF (DRE binding)/(CRT binding factor) and DREB2, induced by cold and dehydration, respectively (Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 2001).

Recently, the role of CBF-like genes was investigated in other plants. In barley, several CBF/DREB1 homologous have been characterised: *HvCBF3,* located on barley chromosome 5H, is transiently up-regulated by cold reaching its maximum level of expression after 2 h of treatment (Choi *et al*., 2002), while *HvCBF2* is constitutively expressed but its interaction with the core CRT/DRE motif is promoted by low temperature (Xue, 2003).

The WRKY gene family represents one of major groups of plant-specific transcriptional regulators. This class of transcription factors is defined by an amino acid sequence with DNA-binding activity known as WRKY domain, a 60 amino acid region highly conserved among family members. The WRKY domain includes a conserved WRKYGQK amino acidic sequence followed by a Cys_2His_2 or $Cys_2HisCys$ zinc-binding motif (Eulgem *et al*., 2000). These proteins were shown to bind specifically to W-box-Type [(T)TGCA(C/T)] DNA sequence elements both *in vitro* and *in vivo* (Rushton *et al*., 1995, 1996; de Pater *et al*., 1995; Eulgem *et al*., 1999; Yang *et al*., 1999; Du and Chen, 2000). WRKY genes have been found active during pathogen defence (Yu *et al*., 2001; Yoda *et al*., 2002), wounding response (Hara *et al*., 2000), as well as during senescence (Robatzek and Somssich, 2001), trichome development (Johnson *et al*., 2002) and germination (Zhang *et al.,* 2004). Seventy four WRKY genes (*At*WRKY1 to *At*WRKY74) have been identified in *Arabidopsis thaliana* through a genome wide search (Eulgem *et al*., 2000) and others WRKY factors have been isolated in different species including wild oat (ABF2) (Rushton *et al*., 1995), parsley (*Pc*WRKY1, 2, 3) (Rushton *et al*., 1996), sweet potato (SPF1) (Eulgem *et al*., 2000), tobacco (*t*WRKY1, 2, 3, 4) (Yang *et al*., 1999; Chen and Chen, 2000; Yoda *et al*., 2002) and rice (*Os*WRKY71) (Zhang *et al*., 2004). Latest knowledge has shown that WRKY factors are also involved in other stress responses. In barley a transcription factor belonging to the WRKY family, SUSIBA2 (a sugar responsive element binding factor), was found involved in the sugar signalling (Sun *et al*., 2003). In *Arabidopsis*, recent experiments made with microarray, have shown the presence of a number of WRKY transcription factors up-regulated by drought, cold or high-salinity stresses (Fowler and Thomashow, 2002; Seki *et al*., 2002).

In this study, we report on the cloning and mapping of *Hv-WRKY38* from barley. We present a detailed expression analysis showing the involvement of this gene in the abiotic stress response. Hv-WRKY38 protein was found to be localised into the cell nucleus and was shown to bind *in vitro* oligonucleotides containing W-box elements. These evidences indicate that *Hv-WRKY38* encode for a stress-related transcription factor.

Materials and methods

Plant materials and treatment conditions

The experiments were performed using winter barley genotypes (*Hordeum vulgare* cv. Nure) and barley albino mutant a_n (Burnham *et al*., 1971), characterised by a block in the early stage of chloroplast development, and therefore devoid of pigments and chlorophyll. Seeds were germinated in peat pots and grown at + 22 °C (9 h light 160 μmol photons m^{-2} s^{-1})/ + 16 °C (15 h dark) for 7 days to the first leaf stage and then subjected to cold-acclimation at +2, +6 or +10 °C for different lengths of time. Cold-acclimated plants were frozen at −3 or −8 °C and collected after 6, 12, 24, 36 and 48 h. Plants grown on 3 mm filter paper at 22 °C (9 h light/15 h dark) for 5 days were used for dehydration experiment and the progress of drought stress was followed measuring the variation in the Relative Water Content (RWC). Plants grown in peat pots were watered and sprayed with 1 mM ABA solution and their leaves were collected after 6 h.

Isolation of Hv-WRKY38

Hv-WRKY38 was initially isolated as a differential displayed fragment. Differentially expressed cDNA from control (22 °C) and cold-treated (6 h

at + 3 °C) mRNA samples isolated from leaves of *H. vulgare* cv. Nure were visualised on sequencing gels after the method developed by Liang and Pardee (1992). The messenger RNAs were treated according to RNAmap kit from GenHunter (Brookline, MA). Messengers reverse transcription PCR-amplification and cDNAs samples collection were carried out according to Baldi *et al.* (1999). The resulting differentially expressed cDNA fragments were cloned in the pCRII-cloning vector using the TA cloning kit (Invitrogen).

The *Hv-WRKY38* full length was isolated by screening a cDNA library (Uni-Zap XR vector, Stratagene) made with mRNA isolated from barley (cv. Nure) leaves exposed to +3 °C for 6 h. The library was plated and screened with the [α-^{32}P]dCTP-labelled (randomly primed reaction) differentially displayed fragment as probe. The isolated clones were verified by restriction analysis and sequenced. All DNA-blotting procedures were performed according to Sambrook *et al.* (1989). Sequencing was run in both directions using the ABI Prism^{TM}BigDye Terminator Cycle Sequencing kit on an ABI PRISM™ 310 Genetic Analyzer automated sequencing machine (PE Applied Biosystem). DNA sequences were compared to those in the non redundant databases by using the BLAST algorithms, available at the DNA analysis web sites maintained by the National Center for Biotechnology Information and The Institute for Genomic Research.

mRNA isolation and northern analysis

Frozen tissues were ground in liquid N_2 and resuspended in 50 mM Tris pH 9, 10 mM EDTA, 0.1 M NaCl, 2% (w/v) SDS. After three phenol-chloroform (1:1, v/v) extractions, the poly(A) RNAs were isolated by chromatography on oligo-dT-cellulose (Sambrook *et al.*, 1989). Messenger aliquots from each sample were separated onto a formaldehyde agarose gel and blotted onto a Hybond-N$^+$ membrane (Amersham Biosciences). Either the full-length cDNA or the differentially displayed fragment, corresponding to the 3′-UTR region of *Hv-WRKY38*, were used as probe with the same results. Nevertheless, due to the multigenic nature of the WRKY gene family, the 210 bp sequence (*dd10*) corresponding to the 3′ of the genes was routinely used in northern analysis to reduce the possibility of cross-hybridisation. cDNA fragment was labelled with [α-^{32}P]dCTP in a randomly primed reaction. The hybridization was performed at 65 °C in 6× SSC, 2× Denhardt's solution (Sambrook *et al.*, 1989), 0.1% SDS, and 100 μg ml^{-1} of denatured herring-sperm DNA. The filters were washed at 65°C three times with 2×, 1× and 0.5× SSC, 0.1% SDS for 20 min. To control the integrity and the amount of loaded poly(A)RNA, all filters were hybridized with an [α-^{32}P]-labeled probe corresponding to the barley gene coding for the ribosomal protein RPL12 (Baldi *et al.*, 2001).

Genetic mapping and genomic sequence analysis

To find the map position of the *Hv-WRKY38* gene into the barley genome, the DNA of 136 F_1-derived doubled-haploid lines developed from the cross Nure × Tremois (Francia *et al.*, 2004) were amplified using the primer pair *wrky321-F* (5′-GGTTCTTGAGTCGGAGCTACAGCG-3′) and *wrky1503-R* (5′-TATGGAACGGAACATTTGAATGACTGC-3′). The resulting *wrky321-wrky1503* fragment was sequenced both in Nure and Tremois and a single nucleotide polymorphism (SNP) coincident with an *ApoI* restriction site was discovered. The segregation of the *ApoI* polymorphism was then scored in 136 double-haploid population and linkage analysis on the Nure × Tremois molecular map was performed using the software MAPMAKER 3.0 (Lander *et al.*, 1987).

The genomic structure of *Hv-WRKY38* was determined after genomic sequencing and comparison with the cDNA sequence. *Arabidopsis* homologous sequence was found after Blast search in genomic sequence databank available at www.arabidopsis.org. The rice homologous sequence was reconstructed by means of TC and BAC genomic sequence, retrieved in rice Gene Index at www.tigr.org. A genomic region of the rice BAC OJ1297_C09 characterised by incomplete sequence information was amplified with the primer pairs *Os600-F* (5′-TGCAGAGCCAGTTCAGCGACATGG-3′) and *Os1670-R* (5′-GAGATCTTGGGCTTGCACTCCTCG-3′) from the rice (*Oryza sativa* ssp. *Japonica*) cultivar Vialone nano and re-sequenced. Vector NTI software was used for contig construction and sequence alignment.

Plasmid constructs

The pBlueScript-*Hv-WRKY38* vector was digested with *BamHI* restriction enzyme. The *BamHI-BamHI Hv-WRKY38* fragment (from position 142 to 1163) containing the almost complete ORF was purified and introduced into the *BamHI* site of a pUC derived vector containing the *CaMV35S-NOS* cassette, resulting in the *pUC:CaMV35S:Hv-WRKY38:NOS* expression vector.

In vitro expression of recombinant Hv-WRKY38 protein was achieved by subcloning the *BamHI-BamHI Hv–WRKY38* fragment in frame with the gene coding for the glutathione-S-transferase into the pGEX-1N expression vector (Amersham Biosciences).

The construct *pGEM:CaMV35S:Hv-WRKY38:YFP* was developed to drive the *in planta* expression of the Hv-WRKY38 fused with the Yellow Fluorescent Protein. The expression cassette was assembled according to fusion PCR, a method allowing to melt complementary DNA fragments, present on separate plasmids, into a unique DNA fragment. Two PCR reactions were carried out. The *CaMV35S:Hv-WRKY38* cassette was amplified from the *pUC:CaMV35S:Hv-WRKY38:NOS* vector with two primers encompassing the promoter and a large part of *Hv-WRKY38* gene including the putative NLS. The PCR reaction was performed using the M13 primer and 5′-TCGCCCTTGCTCACCATGGTT TCCTGCTTGTGCTGCTGCTG-3′, a 42-mers primer designed from position 562 to 582 of the *Hv-WRKY38* sequence plus 21-mers overlapping in frame the 5′ region of the *YFP* gene. The *YFP:NOS* cassette was amplified from the *pUC:CaMV35S:YFP:NOS* vector using the 21-mers primer 5′-ACCATGGTGAGCAAGGGC GAG-3′, designed into the 5′ region of the *YFP* gene, and the M13 primer.

The PCR products were purified according to Wizard SV Gel Clean-Up System (Promega) and 30 ng of each purified fragment were pooled in the same reaction tube and amplified using forward and reverse M13 primers. All PCR reactions were carried out using *Pfu* Turbo DNA Polymerase (Stratagene) and standard protocol. The *CaMV35S:Hv-WRKY38:YFP:NOS* fragment yielded after fusion PCR was subcloned into *pGEM–T* Easy Vector (Promega).

All constructs were sequenced to verify the correct insertion of all fragments.

Production of recombinant Hv-WRKY38 protein

Overnight cultures in LB medium of *E. coli* strain DH5α, transformed with recombinant plasmids *pGEX:GST-Hv-WRKY38* were diluted 1:10 in fresh medium and grown for 1 h at 37 °C before addition of IPTG to 0.1 mM. After a further 3 h of growth, cells were pelleted and washed with 50 mM Tris–HCl pH 8. Since the fusion protein was located into inclusive bodies, the following protocol was used to achieve a partial purification of GST:Hv-WRKY38. The pellet was resuspended in 50 mM Tris–HCl pH 8, 20% Sucrose, 0.1 M EDTA, 3 mg/ml lysozyme, incubated for 30 min on ice and centrifuged at 13,000 × *g* for 10 min to harvest the spheroplasts. The pellet was resuspended in 20% Sucrose, 3 mM EDTA pH 7.3, and the spheroplasts were lysed by shaking with glass beads for 4 h at 4°C. The lysate was pelleted by centrifugation at 6000 *g* for 30 min at 4°C, than washed in 20% Sucrose, 3 mM EDTA pH 7.3 and resuspended in extraction buffer (20 mM Hepes pH 7.5, 1 mM EDTA, 50 mM NaCl, 5% v/v glycerol) as suggested by Rushton *et al.* (1995). Insoluble fraction was separated by centrifugation at 12 000 *g* for 10 min and the protein concentration of the supernatant was determined according to Bradford (1976).

Antibodies preparation

A first antibody was raised against the GST:Hv-WRKY38 fusion protein partially purified onto Tris–Tricina SDS-PAGE. After lisozyme digestion the pellet was dissolved in sample buffer and boiled at 100 °C for 4 min. The sample was loaded onto the gel and, after the electrophoretic separation, the band corresponding to the fusion protein was excised directly from gel and dialysed. An amount of 2 mg of purified proteins were injected into a rabbit to raise the corresponding polyclonal antibodies.

A second antibody was developed against immunogenic peptides of protein Hv-WRKY38. Protein secondary structure, antigenicity and accessibility of the protein domains was evaluated by means of public available softwares (at http://www.es.embnet.org/Services/MolBio/PredictProtein and http://bioinf.cs.ucl.ac.uk/psipred) and two

peptides were selected: WMGSQPSLSLDLHVG between amino acid positions 4 and 18, and CKRIREECKPVISKR between amino acid positions 174 and 181. The peptides were synthesised, then injected as a mix into a rabbit to raise the corresponding antibodies.

GMSA

GMSA was performed using Mobility Shift Optimization Kit from SIGMA (cat. SHIFT-1) and following the instructions manual. Six pmol of the forward and reverse oligonucleotides (described in Table 1) were end-labelled using polynucleotide kinase and γ-P^{32} labelled ATP, then purified from unincorporated label by G-25 Spin Chromatography (Amersham Biosciences). To obtain double stranded DNA, radiolabelled complementary oligonucleotides were annealed by cooling to 37 °C in 10 mM $MgCl_2$ and 50 mM NaCl after a denaturation step. 20 μg of protein extract were added to binding reaction mix prepared with 50 fmol of end-labelled probe, binding buffer (12 mM Hepes pH 8.0, 4 mM Tris–HCl, 60 mM KCl, 1 mM EDTA, 1 mM EGTA, 12% Glycerol), 2 μg poly(dI-dC), 20 μg BSA and 1.7 mM DTT. Igepal CA-360 0.07% was added in reaction mixes to stabilise the binding. 2.5 mM $MgCl_2$, 50 mM EDTA or 5 μM $ZnSO_4$ was used to assess the involvement of Zn and Mg in the protein–DNA interaction. To verify the involvement of protein–protein interaction, 1% sodium deoxycholate was added as protein dissociating agent (Després *et al*., 1995). Competition binding assays was run with cold target DNA or cold unrelated DNA in the reaction mix using at least 40-fold molar excess respect to the labelled oligonucleotide. For supershift analysis antiserum against GST:Hv-WRKY38 or Hv-WRKY38 peptides was added. The binding reactions were performed at room temperature for 20 min.

All samples were loaded without dye onto a 5% non-denaturing polyacryhilamide gels and run with 0.5× TBE (0.1 M Tris, 90 mM Boric Acid, 1 mM EDTA) buffer. Radioactively gels were exposed on storage phosphor screen, and revealed using the Typhoon instrument (Amersham Biosciences).

Nuclear localisation assay

Nuclear localisation of Hv-WRKY38:YFP was assessed with transient expression after particle

Table 1. List of the oligonucleotide sequences tested in GMSA experiments. The W-box motifs present in each sequences were highlighted: TTGACC (white letters in grey shaded box), TTGACT (black letters in light grey shaded box), TGACT (black letters in light grey shaded box), TGACC (white letters in grey shaded box) and TTGAC (white letters in black shaded box).

No.	Name	Sequence
1	Box2 *α-Amy2*	A**TTGACT**TGACCGTCATCGG
2	Box2 *Amy32b*	GCCCGGA**TTGACT**TGACCATCATCTG
3	BS65	ATCG**TTGACC**GAG**TTGACT**TT
4	BS65-MutA	ATCGTAGTACGAG**TTGACT**TT
5	BS65-MutB	ATCG**TTGACC**GAGTAGTATTT
6	BS65-5I	ATCG**TGACT**GAGTAGTAGCTA
7	BS65-5II	ATCG**TGACC**GAGTAGTAGCTA
8	BS65-5III	ATCG**TTGAC**GAGTAGTAGCTA
9	BS65-5I*5I	ATCG**TGACC**GAG**TGACC**GCTA
10	BS65-5II*5II	ATCG**TGACT**GAG**TGACT**GCTA
11	BS65-5III*5III	ATCG**TTGAC**GAG**TTGAC**GCTA
12	BS65-5I*5II	ATCG**TGACC**GAG**TGACT**GCTA
13	BS65-5I*5III	ATCG**TGACC**GAG**TTGAC**GCTA
14	BS65-5II*5III	ATCG**TGACT**GAG**TTGAC**GCTA
15	BS65-6A*5I	ATCG**TTGACC**GAG**TGACT**GCTA
16	BS65-6B*5I	ATCG**TTGACT**GAG**TGACT**GCTA
17	BS65-6B*5II	ATCG**TTGACT**GAG**TGACC**GCTA
18	BS65-6A*5II	ATCG**TTGACC**GAG**TGACC**GCTA
19	BS65-6B*5III	ATCG**TTGACT**GAG**TTGAC**GCTA
20	BS65-6A*5III	ATCG**TTGACC**GAG**TTGAC**GCTA
21	BS65-0b	ATCG**TTGACC****TTGACT**TTGAG
22	BS65-6b	ATCG**TTGACC**GAGGAG**TTGACT**TT

bombardment in onion epidermis cells. Inner epidermal peels (2 × 2 cm) of commercial white onion were directly placed on agar plates containing 4.3 g l^{-1} Murashige and Skoog (MS) salts, 3% (w/v) sucrose and 2% agar, pH 5.7 KOH. Gold microcarriers (1 μm diameter) were prepared essentially as described by Dal Bosco *et al*. (2003). An amount of 35 μl of resuspended microcarrier (60 mg/ml ethanol) were mixed with 25 μg of *CaMV35S:Hv-WRKY38:YFP:NOS* plasmid or *CaMV35S:YFP:NOS* in negative control experiment. The microcarriers were delivered to the freshly transferred explants, using the Biolistic Particle Delivery System PDS-1000/He (Bio-Rad, Hercules, CA; Lemaux *et al*., 1996) according to the manufacturer's instructions. A petri dish containing the plant tissue was placed 9 cm below the microcarrier launch assembly. The particles were fired using 1100-psi rupture discs (Bio-Rad) with a partial vacuum of 28 mm Hg. YFP was detected by reflected fluorescence system microscope, after recovering the transformed onion epidermis cells at + 22 °C for 18 h in the dark.

Results

Isolation and characterization of Hv-WRKY38

Hv-WRKY38 was originally isolated as a differential displayed fragment (cDNA *dd10*) in a Differential Display-RT-PCR experiment designed to compare the mRNA population extracted from leaves of barley plants exposed to + 3 °C for 6 h in presence of light with the corresponding mRNAs isolated from control plants grown at 22 °C, as described in Baldi *et al*. (1999). The differentially displayed *dd10* cDNA (211 bp) was subcloned and sequenced. Northern experiment showed that the steady state level of *dd10* corresponding mRNAs was transiently enhanced after cold treatment. Comparison of *dd10* nucleotide sequence with entries of GenBank and EMBL did not identify any significant homology in the databases. However the *dd10* sequence, submitted to the TIGR Barley Gene Index database, matched with TC121597, a 1842 bp sequence containing an ORF homologue to PIR/S61414 DNA-binding protein ABF2 in wild oat (Rushton *et al.,* 1995).

The cDNA corresponding to the full-length *dd10* clone was isolated by screening a cDNA library starting from mRNAs extracted from barley seedlings exposed to + 3 °C for 6 h. After phage purification and cDNA rescue, the sequence analysis of the positive clones allowed the identification of a cDNA corresponding to the full-length *dd10* sequence. The isolated clone showed a complete match with the differentially displayed fragment over all the 211 bp of *dd10*. This cDNA clone is 1567 bp long and contains a single long open reading frame representing a complete coding region of 353 aa. The molecular weight of the deduced protein is 38.6 kDa.

The comparison between *dd10* full-length sequence and TC121597 revealed that the TC121597 contains an insertion of 273 bp in the 3′ end region. Nevertheless, since the *dd10* cDNA exactly coincides with the 3′ terminal region of TC121597 and no introns are present in this region of the genomic clone (see below), we argued that a mistake in the EST overlapping is present in the TC121597 sequence.

Homologies searches run with the nucleotide and amino acid sequences of the full-length clone revealed a highly significant similarity to proteins belonging to WRKY family of transcription factors (Eulgem *et al*., 2000), particularly to ABF2 of *Avena fatua* (Rushton *et al.,* 1995). The isolated clone was therefore named *Hv-WRKY38* (38 kDa WRKY protein of *H. vulgare*). Recently, a barley WRKY gene identical to *Hv-WRKY38*, named *WRKY9-10*, was published in GenBank under the accession No. CAD60651. The amino acid sequence of *Hv-WRKY38* (Figure 1) is characterised by two motifs highly conserved within the WRKY family: a potential leucine-zipper-motif (LZ) (from position aa 63 to position aa 91) and a WRKY domain containing a single WRKYGQK motif (from position aa 200 to position aa 206) together with a zinc-finger-like motif ($C–X_{4-5}–C–X_{22-23}–H–X_1–H$) in the C-terminal region (from position aa 220 to position aa 252), which represent the peculiar features of all WRKY proteins.

Expression analysis

To investigate the involvement of *Hv-WRKY38* in plant response to abiotic stresses a detailed expression analysis was performed by means of

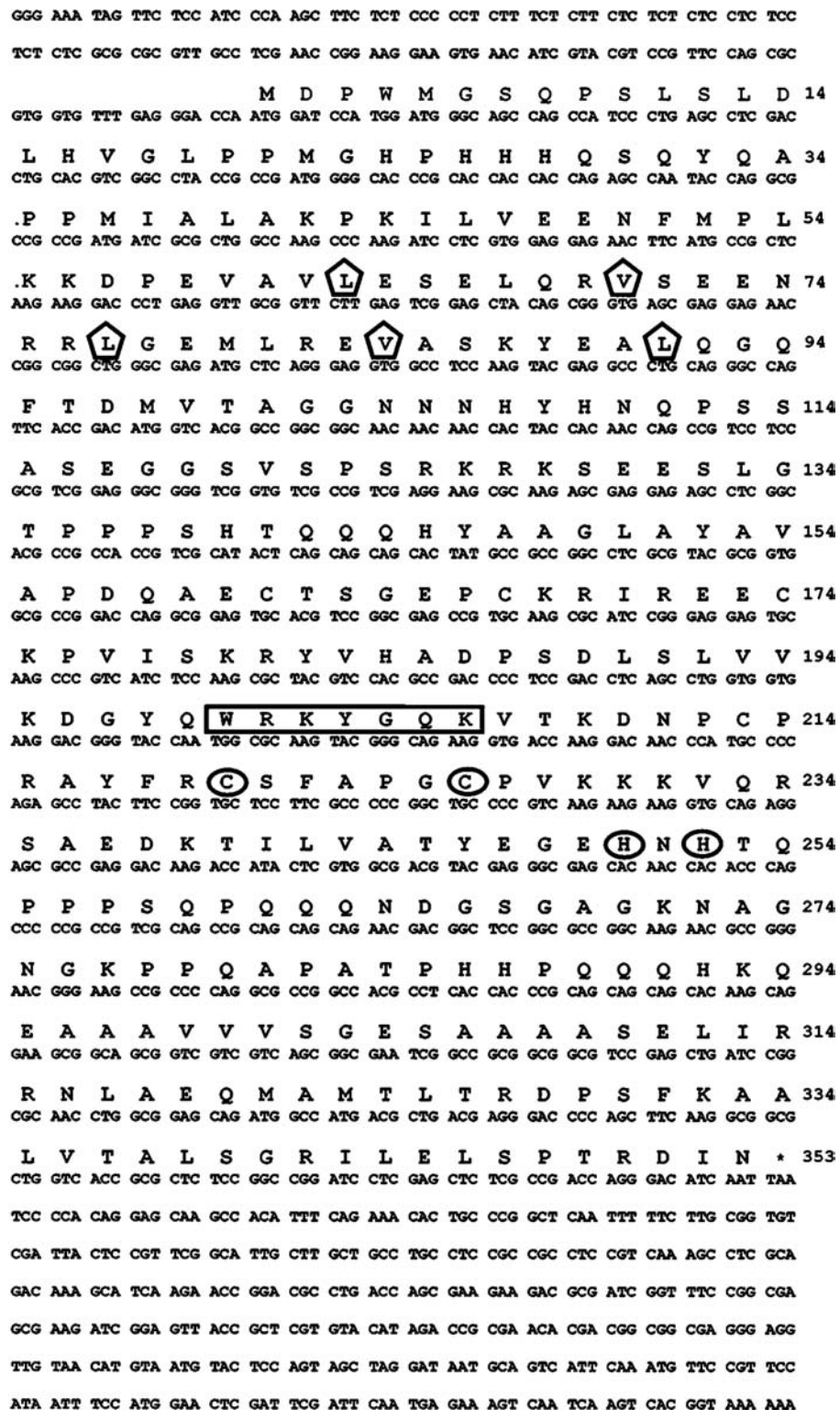
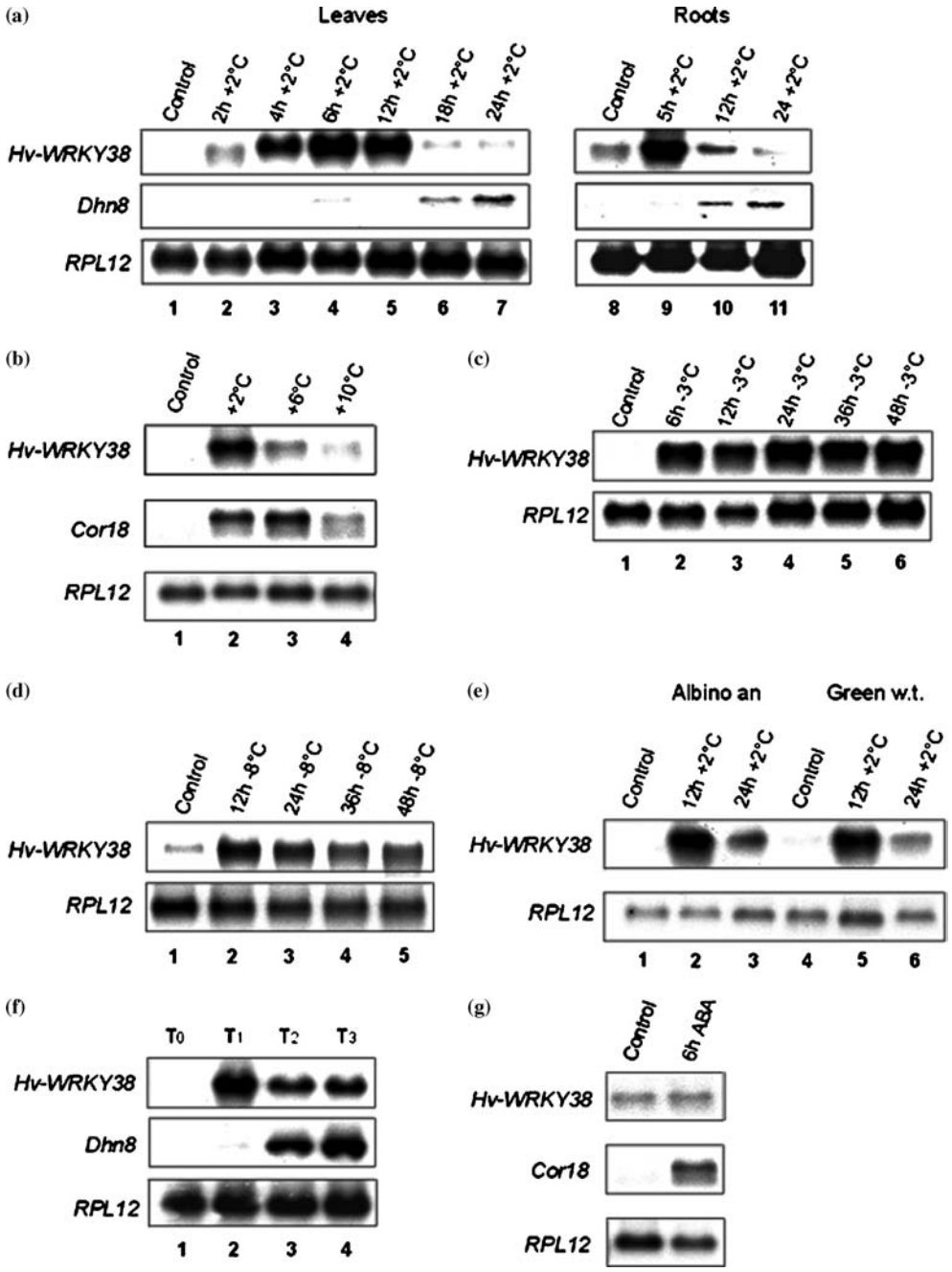


Figure 1. Nucleotide sequence and deduced amino acid sequence of *Hv-WRKY38*. The hydrophobic residues in the putative Leucine-zipper motif are inside of pentagons. The WRKYGQK sequence is indicated with a square. The two Cysteines and two Histidines of the putative zinc-finger motif are circled.



northern experiments. Since the cDNA was originally isolated from cold treated leaves, we first investigated the variation of the *Hv-WRKY38* steady state mRNA level in response to low temperature (Figure 2a). The amount of mRNA corresponding to *Hv-WRKY38* was transiently enhanced following exposure to low, no freezing, temperature (a typical hardening condition) both in leaves and roots. *Hv-WRKY38* mRNA showed a minimal level in control plants (higher in roots than in leaves) achieving its highest expression after 12 h in leaves and 5 h in roots. An enhancement of the mRNA level was detected in leaves already after 2 h of cold treatment suggesting an early and fast

induction. After 24 h of cold treatment the expression of *Hv-WRKY38* dropped to basal level. To compare the expression profile of *Hv-WRKY38*, the same filters were hybridised with a probe corresponding to *Dhn8,* a cold- and drought-stress induced dehydrin sequence (Choi *et al*., 1999). *Dhn8* related mRNA began to accumulate after the peak of *Hv-WRKY38* expression.

Hv-WRKY38 expression was found to be related to temperature drop. When young seedlings grown at +22 °C were moved down to +2, +6 and +10 °C for 8 h, *Hv-WRKY38* mRNA level increased, being gene expression higher when the plants were exposed to lower temperature (Figure 2b).

Since within 18–24 h of cold treatment the steady state mRNA level of *Hv-WRKY38* decayed close to the amount of untreated plants, it was possible to investigate the effect of a further temperature drop on *Hv-WRKY38* expression. Messenger RNA was isolated from leaves of plants acclimated at +2\1 °C for 7 days (control in Figure 2c) and from plants exposed to −3 °C after cold acclimation. Already after 6 h of freezing, the expression of *Hv-WRKY38* was strongly induced but, differently from +2 °C treatment, the mRNA level remained stable until 48 h of freezing. The same result was also obtained when 21 days acclimated plants were frozen to −8 °C for 48 h (Figure 2d). In both experiments the drop in the temperature induced a rapid and stable accumulation of *Hv-WRKY38* mRNA providing evidence that freezing stress still promotes a molecular response.

It has been reported that the expression of several barley cold regulated genes is controlled by a chloroplast derived molecular signal (Crosatti *et al*., 1999; Dal Bosco *et al*., 2003). To value the chloroplast role in the control of *Hv-WRKY38* expression, the cold induced mRNA accumulation of *Hv-WRKY38* was assessed in barley albino mutant a_n and in the corresponding wild type. Young seedlings of mutant and wild type plants exposed to 2 °C for 12 and 24 h showed the same level and the same kinetic of *Hv-WRKY38* induction suggesting that this gene is not chloroplast regulated (Figure 2e).

Since most of the chloroplast-independent stress-related genes are also involved in the dehydration response, drought and ABA induction of *Hv-WRKY38* was investigated. *Hv-WRKY38* was tightly induced by water deficit achieving its highest mRNA expression level already after 30 min of treatment (Figure 2F). Further dehydration slightly reduced *Hv-WRKY38* expression although the transcript was still detectable till the end of the experiment. Notably, when the same filter was hybridised with *Dhn8,* the drought expression of this dehydrin gene was delayed compared to *Hv-WRKY38*. Since ABA treatment had no effect on *Hv-WRKY38* expression (Figure 2G), this gene should be involved in cold- and drought-response in an ABA-independent way.

Figure 2. Analysis of *Hv-WRKY38* mRNA expression. (a) Expression of *Hv-WRKY38* at 2 °C: lane 1: RNA of leaves from plants grown for 7 days at 22 °C; lanes 2–7: RNA from leaves exposed to 2 °C for 2, 4, 6, 12, 18 and 24 h; lane 8: root RNA isolated from seedlings grown at 22 °C for 5 days; lane 9–11: RNA from roots exposed to 2 °C for 5, 12 and 24 h. The same filter was hybridised with *Dhn8* (Choi *et al*., 1999) to prove the effectiveness of low temperature treatment. (b) Accumulation of *Hv-WRKY38* leaves transcripts at different temperatures: lane 1: RNA of leaves from plants grown for 7 days at 22 °C; lanes 2–4: RNA from plants exposed for 8 h at 2, 6, and 10 °C. The same filter was hybridised with *Cor18* (Dal Bosco *et al*., 2003) to prove the effectiveness of low temperature treatment. (c) Expression of *Hv-WRKY38* transcripts in cold acclimated leaves during freezing at −3 °C: lane 1: RNA from seedlings grown for 7 days at 22 °C and then cold-acclimated for 7 days at 2 °C, lanes 2–6: RNA from plants exposed at −3 °C for 6, 12, 24, 36 and 48 h. (d) Expression of *Hv-WRKY38* transcripts in cold acclimated leaves during freezing at −8 °C: lane 1: RNA from seedlings grown for 7 days at 22 °C and then cold-acclimated for 21 days at 2 °C, lanes 2–5: RNA from plants exposed at −8 °C for 12, 24, 36 and 48 h. (e) Expression of *Hv-WRKY38* in the barley albino mutant a_n: lane 1: RNA from albino mutant leaves grown for 7 days at 22 °C; lanes 2 and 3: RNA from albino mutant leaves exposed to 2 °C for 12 and 24 h; lane 4: RNA from wild type leaves grown for 7 days at 22 °C; lanes 5 and 6: RNA from wild type leaves exposed to 2 °C for 12 and 24 h. (f) Expression of *Hv-WRKY38* in leaves during drought stress: lane 1: RNA from plants grown for 10 days at 24 °C; lanes 2–4: RNA from plants dehydrated for 30 min ($T_1 = 76\%$ RWC), 90 min ($T_2 = 74\%$ RWC) and 180 min ($T_3 = 72\%$ RWC). The same filter was hybridised with *Dhn8* to prove the effectiveness of drought treatment. (g) Expression of *Hv-WRKY38* in plants treated with ABA. The same filter was hybridised with *Cor18* (Dal Bosco *et al*., 2003) to prove the effectiveness of ABA treatment. Equal loading was assessed throught hybridisation with a cDNA coding for the ribosomal protein RPL12 (Baldi *et al*., 2001).

Genomic structure of Hv-WRKY38 *and identification of* Arabidopsis *and rice homologous sequences*

The Nure genomic sequence of *Hv-WRKY38* was amplified with the primer pair wrky98-F/wrky1503-R and sequenced. The comparison between the cDNA and the genomic sequence revealed a single 113 bp intron at position 317. The genomic sequence was deposited in the Genbank database under the accession No. AY541586.

The Nure and Tremois genomic sequences of *Hv-WRKY38* were amplified and the single PCR products obtained were subcloned in *pGEM-Teasy* vector. Sequencing of the two genomic sequences revealed several SNPs in the 3′ UTR of the gene. A transversion in the position 1246, from A (Tremois) to T (Nure), is associated with the presence in Tremois and the absence in Nure of the *ApoI* restriction site. This cleavaged amplified polymorphism (CAP) was used to map *Hv-WRKY38* onto the barley genome. The segregation data of the *ApoI* polymorphism were used for linkage analysis in the Nure × Tremois genetic map (Francia *et al*., 2004). *Hv-WRKY38* maps near to the sub-centromeric region of the short arm of chromosome 6H (*Triticeae* homologous group 6), between the SSR marker *Bmag0173* and *Bmag0009* (Figure 3).

In rice, two most similar TCs, TC150510 and TC148512, were identified, although we were unable to find a single TC covering the full *Hv-WRKY38* ORF. The rice TC148512 covers the WRKY domain and the COOH terminal region, while the rice TC150510 joins a region encompassing the NH_2 terminal and the LZ domain, with an additional region corresponding to COOH terminal suggesting a non correct assembling of the sequence. Both TCs are covered by the same BAC (OJ1297_C09) located on the chromosome 2 (www.tigr.org/tdb/e2k1/osa1), a region of the rice genome showing a general sintheny with the barley chromosome 6 (Devos and Gale, 1997) where *Hv-WRKY38* is located. We hypothesised that the rice genomic region on BAC OJ1297_C09 showing the highest homology to *Hv-WRKY38* might represent the rice homologous gene. Since each TC contains only a part of the putative rice *Hv-WRKY38* homologous, we examined the genomic sequence by means of TIGR annotation software to construct the full *Os-WRKY38* coding

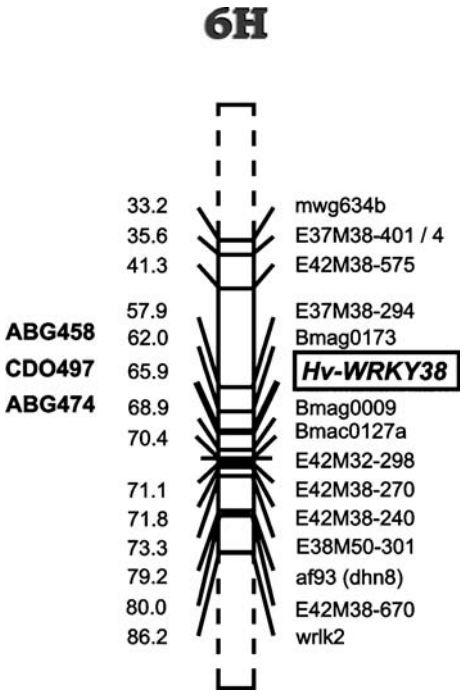


Figure 3. Chromosomal position of *Hv-WRKY38* in the Nure × Tremois barley map (Francia *et al*., 2004). Distances are in Kosambi cM, and inferred positions of markers mapped in other populations are shown on the left.

sequence. Two rice genes, *2495.m00163* and *2495.m00162*, were identified and a long intergenic sequence with a stop codon was present between them. The two genes are related to rice TC150510 and TC148512, respectively. The intergenic genomic region was not covered by any EST sequences, it did not show similarity with *Hv-WRKY38* or any other *WRKY* sequence and it contained a large number of undefined bases. In addition to the intergenic sequence three intronic regions were present, one in *2495.m00163*, two in *2495.m00162*. We hypothesised that the two TC identified and the two genes annotated could belong to a unique gene. To confirm this hypothesis we amplified the central region of the rice locus with the primer OsWRKYfor, localized on the 3′ terminal region of TC150510, and the primer OsWRKYrev, on the 5′ region of TC148512. The sequence of the unique 250 bp product was overlapped with the two rice genes, *2495.m00163* and *2495.m00162,* and a single ORF showing a high homology with Hv-WRKY38 was produced. We supposed that this protein might represent the product of the rice gene homologous to *Hv-WRKY38*. The built rice

sequence has been annotated in Genbank with accession No. AY541677. Our gene reconstruction was also confirmed by a recent work published while this manuscript was under revision, describing *OsWRKY71* gene (Zhang *et al.,* 2004).

When the *Hv-WRKY38* sequence was compared with the *Arabidopsis* databases (www.arabidopsis.org) a Blast search indicated AtWRKY40 as the most similar amino acid sequence (40%). AtWRKY40 protein (302 amino acid and molecular mass of 33.7 kDa) is coded by the locus *At1g80840* located on chromosome 1 and characterised by four exons and three introns. Maximum similarity was at the LZ and WRKY domains.

Figure 4 shows the alignment among Hv-WRKY38, ABF2, OsWRKY71 and AtWRKY40 proteins with the position of the introns respect to the amino acid sequence. The position of the first intron located at 315 bp corresponding to 59th amino acid is conserved in barley, rice and *Arabidopsis* sequences. Introns number two and three, absent in barley, are located in the WRKY domain in the same position in rice and *Arabidopsis*.

Nuclear localisation of Hv-WRKY38

The Hv-WRKY38 protein sequence, when submitted to PSORT (prediction program of protein localization sites, http://psort.nibb.ac.jp), revealed the presence of a monopartite nuclear localisation signal (NLS) RKRK, between position 125 and 128 of the amino acid sequence. To demonstrate the biological activity of the putative NLS, a *Hv-WRKY38* fragment containing the NLS motif was fused in frame to the *yfp* reporter gene and subcloned into an expression vector under the control of the *CaMV 35S* promoter. This construct was introduced into onion epidermal cells through particle bombardment and the subcellular localisation of the Hv-WRKY38:YFP fusion protein was focused by reflected fluorescence system microscope (Figure 5). Onion cells transformed with a plasmid expressing YFP alone, as a control, showed fluorescence throughout the cell. In contrast, fluorescence was detected mainly in the nucleus of cells transformed with the plasmid expressing the Hv-WRKY38:YFP fusion protein, suggesting that the NLS contained in the *Hv-WRKY38* gene should be active and able to drive the recombinant protein into the nucleus.

Hv-WRKY38 binds to conserved W-box motifs

The DNA binding activity of Hv-WRKY38 was investigated *in vitro* by a GMSA using a partially purified Hv-WRKY38 fused to GST. Since Hv-WRKY38 shows a high sequence similarity with ABF2 and OsWRKY71 (Figure 4), with an almost absolute identity in the putative binding domain and some differences in the adjacent regions, we tested two oligonucleotides corresponding to different Box2 motifs (Box2 α-*Amy2* and Box2 *Amy32b*) previously identified in the α-amylase gene promoters as the putative DNA target region of WRKY proteins. Box2 α-*Amy2*, from the wild oat promoter region of α-*Amy2*, was shown to bind to ABF2 (Rusthon *et al*., 1995), while Box2 *Amy32b* is part of the barley *Amy32b* promoter (also called O2S) and it was demonstrated to bind to OsWRKY71 (Zhang *et al*., 2004). We tested also the oligonucleotide BS65 reported to represent a binding site of some members of WRKY family proteins in *Arabidopsis* (ZAP1 – de Pater *et al*., 1996) and tobacco (WIZZ – Hara *et al*., 2000; TIZZ – Yoda *et al*., 2002). The list of all oligonucleotides used in GMSA experiment is reported in Table 1.

The GST:Hv-WRKY38 fusion protein efficiently bound to the BS65 and to the Box2 *Amy32b* oligonucleotides, as shown by the retarded probes (Figure 6A, lanes 1 and 3). To verify the specificity of complex formation, a parallel binding assay was performed with competitor DNA. Forty-fold molar excess of cold target DNA respect to the labelled oligonucleotide was sufficient to compete with the probe and to cause disappearing of the DNA-protein complex (Figure 6a, lanes 2 and 4). On the contrary, the GST:Hv-WRKY38 fusion protein produced a faint protein-DNA complex with Box2 α-Amy2 (Figure 6a, lanes 5 and 6).

Thorough characterisation experiments were performed on Box2 *Amy32b*, which had been bound with the highest efficiency by GST:Hv-WRKY38 fusion protein (Figure 6b). No probe retardation was detected without protein extract, with GST alone, or with protein extract from not induced recombinant culture (Figure 6B, lanes 1–3). The observed probe retardation was therefore assigned to the DNA binding activity of Hv-WRKY38. This result was further confirmed by antibody supershift

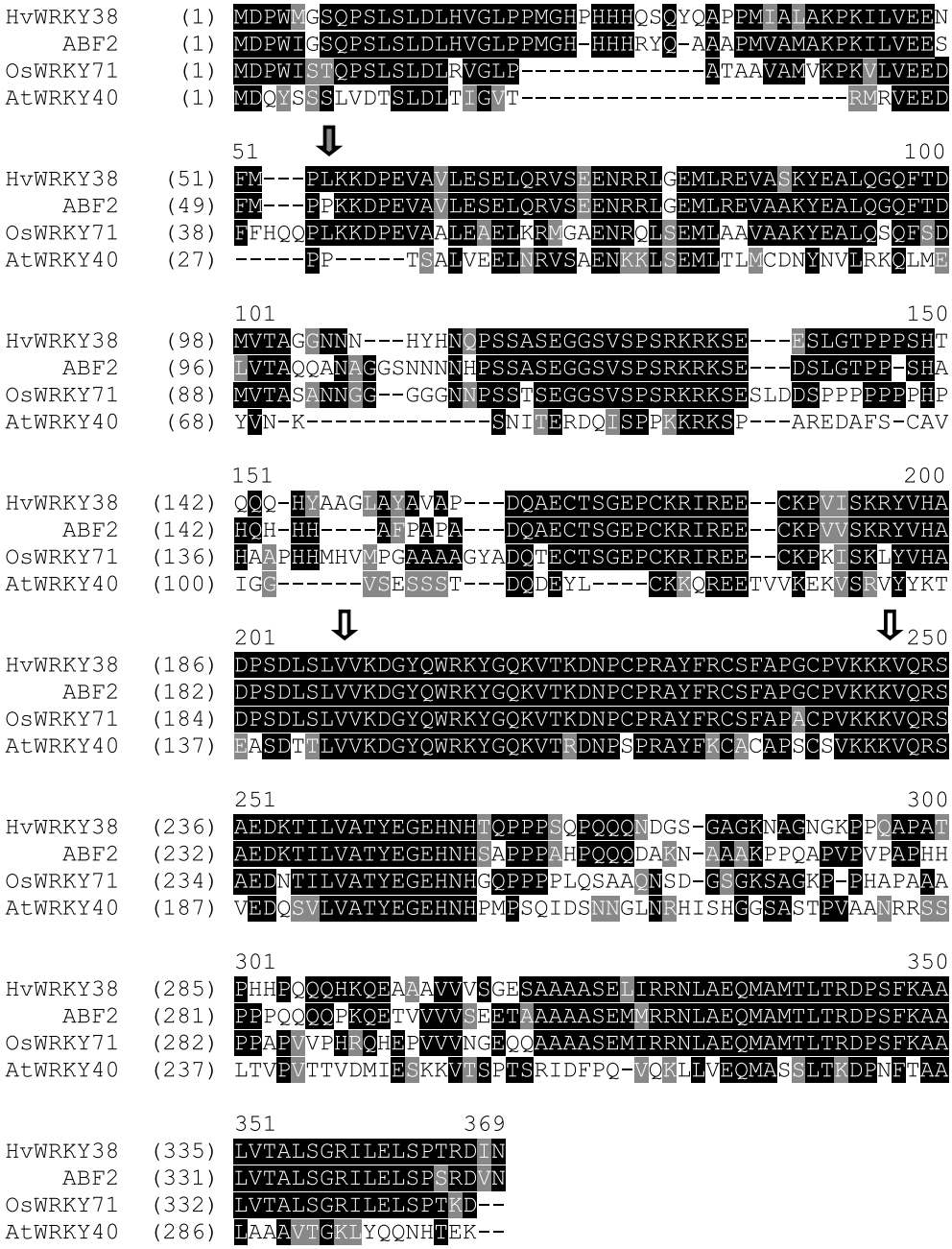


Figure 4. Alignment of Hv-WRKY38 (*Hordeum vulgare*), ABF2 (*Avena fatua*), OsWRKY71 (*Oryza sativa*) and AtWRKY40 (*Arabidopsis thaliana*), protein sequences. Identical residues are highlighted in black boxes, similar amino acids in grey boxes. The position of the intron common to all the species is indicated by grey arrowhead; the position of the introns common to rice and *Arabidopsis* is indicated by white arrowhead.

experiments performed with two antiserum raised against the whole fusion protein GST:Hv-WRKY38 and against two peptides of Hv-WRKY38 not involved in DNA binding domain. The Ig antiGST:Hv-WRKY38 prevented complex formation, instead, the Ig anti-Hv-WRKY38 peptides did not affect the formation of the protein-DNA complex (Figure 6b, lanes 4 and 5). As Hv-WRKY38 contains a Zinc-finger like motif putatively involved in the

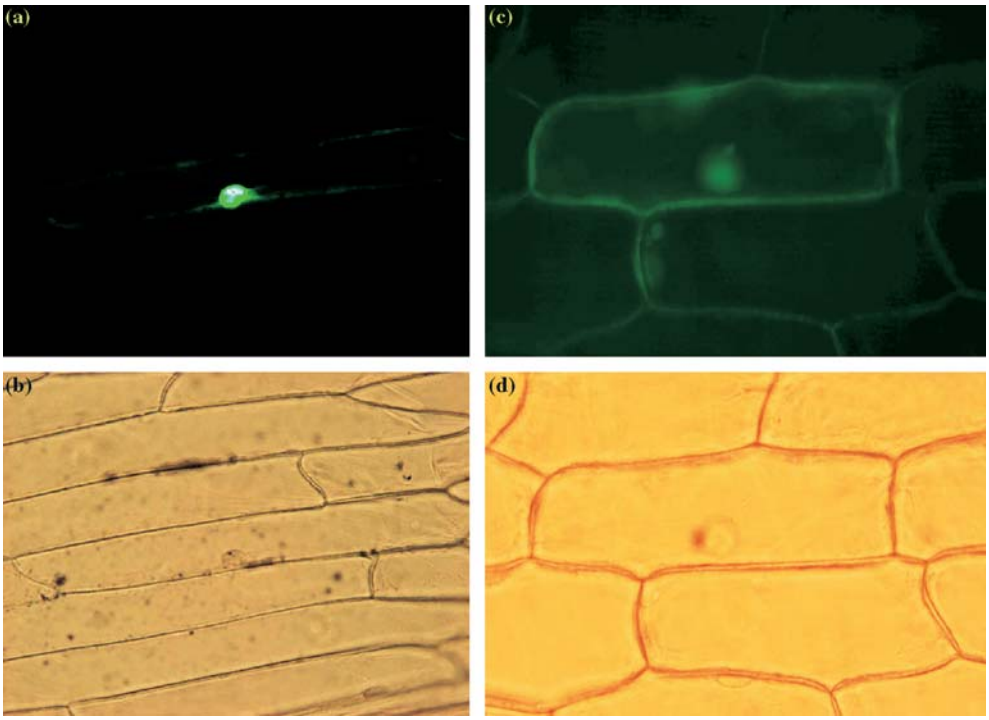


Figure 5. Nuclear localization of the Hv-WRKY38:YFP fusion protein by means of transient expression assays of YFP fluorescence in transformed onion cells. Onion epidermal cells were transformed by particle bombardment with a construct encoding the Hv-WRKY38:YFP fusion protein (a, b) or with a construct encoding YFP only (c, d). Yellow fluorescence excited by wavelength between 460 and 490 nm (a, c) and the corresponding images (b, d) under white illumination are shown at the same exposition time.

DNA binding activity, we assessed the involvement of Zn in protein-DNA interaction. Binding was stabilised by $ZnSO_4$ and to less extent by $MgCl_2$, while the chelant agent EDTA dramatically perturbed complex formation (Figure 6B, lanes 6–8). Since Hv-WRKY38 is also characterised by a putative Leucine zipper domain, the involvement of Hv-WRKY38 dimers in DNA-protein complex formation was hypothesised. When a binding reaction was treated with sodium deoxycholate, a detergent known to disrupt protein-protein and not DNA-protein interaction (Després *et al*., 1995), the DNA-protein complex disappeared (Figure 6B, lane 9) indicating that dimerization is essential for high-affinity binding to the target site as pointed out in literature for other WRKY proteins (Yang *et al*., 1999; Hara *et al*., 2000). Similar results have been obtained in a GMSA characterisation experiment performed on BS65 oligonucleotide (data not shown).

Since the oligonucleotides BS65 and Box2 *Amy32b* contain two W-box motifs, specific experiments were carried out to assess the involvement of each TGAC motif in the DNA-protein interaction and to test different types of W-box elements. In a first experiment five oligonucleotides carrying only a single W-box site (TTGACC; TTGACT; TGACC; TGACT and TTGAC – see oligonucleotides No. 4–8 in Table 1) were used in comparison with BS65 to assess the binding ability of the fusion protein. Oligonucleotides No. 4 and 5 (Table 1) are mutated version of BS65 where one of the six bases W-box element was disrupted, while the oligonucleotides No. 6–8 (Table 1) were chosen to test five bases W-box core sequences. No retardation was observed (data not shown), suggesting that two W-box motifs are required to promote the binding activity of Hv-WRKY38.

When oligonucleotides carrying two W-box motifs of five bases each (sequences Nos. 9–14 in Table 1) were tested, no DNA-protein complex was observed (data not shown). On the contrary, retardation probes appeared when oligonucleotides containing a six bases W-box motif in combination with a five bases W-box were used (sequence Nos. 15–20 in Table 1 and Figure 6c).

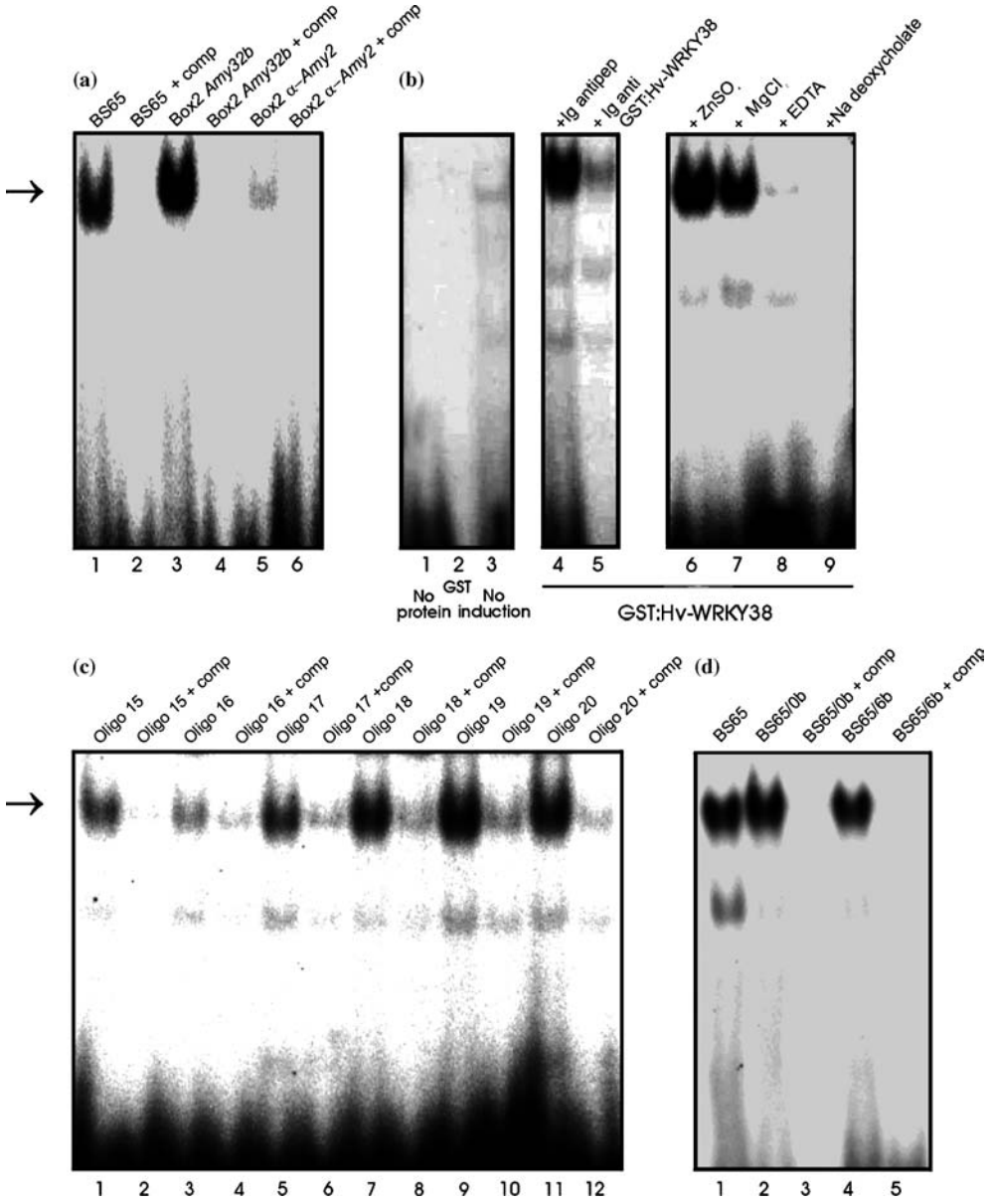


Figure 6. Gel Mobility Shift Assay of oligonucleotides containing W-box motifs with the recombinant GST:Hv-WRKY38 fusion protein. The sequences of all the oligonucleotides used in GSMA are reported in Table 1. (a) GMSA on BS65 oligonucleotide and on functionally defined W-box motifs. The recombinant GST:Hv-WRKY38 fusion protein was mixed with the following oligonucleotides: BS65, Box2 *Amy32b* and Box2 *α-Amy2* (reaction/lane 1, 3, 5, respectively). The reactions were controlled with corresponding cold competitors (reaction/lane 2, 4, 6). The arrow indicates the retarded probe. (b) Characterisation of DNA binding activity of Hv-WRKY38 to the oligonucleotide Box2 *Amy32b* by means of immunoglobulins, metal/chelant agents and a protein dissociating agent. Recombinant GST:Hv-WRKY38 fusion protein was used in all samples except in reaction/lane 1 (no protein added) and in reaction/lane 2 (GST protein). Protein extract from a not induced bacterial culture carrying the *GST:Hv-WRKY38* construct was used in reaction/lane 3. Gel shift assays were performed by adding in each reaction one of the following components: Ig raised against Hv-WRKY38 peptides (reaction/lane 4), Ig raised against GST:Hv-WRKY38 fusion protein (reaction/lane 5), 5 μM $ZnSO_4$ (reaction/lane 6), 2.5 mM $MgCl_2$ (reaction/lane 7), 50 mM EDTA (reaction/lane 8), 1% sodium deoxycholate (reaction/lane 9). (c) GMSA on oligonucleotides containing a six bases W-box motif in combination with a five bases W-box. The recombinant GST:Hv-WRKY38 fusion protein was mixed with the oligonucleotide Nos. 15–20 (reaction/lane 1, 3, 5, 7, 9, 11 respectively). The reactions were controlled with corresponding cold competitors (reaction/lane 2, 4, 6, 8, 10, 12 respectively). The arrow indicates the retarded probe. (d) Effects of distance between the two W-box motifs of the BS65 probe on the DNA binding activity of Hv-WRKY38. The recombinant GST:Hv-WRKY38 fusion protein was mixed with the oligonucleotides: BS65, BS65/0b, BS65/6b (reaction/lane 1, 2 and 4). The reactions were controlled with corresponding cold competitors (reaction/lane 3 and 5, respectively).

The signal intensity was, however, significantly different among the tested oligonucleotides, particularly the sequences containing the core motif TGACT showed a minimal DNA-protein complex formation (oligonucleotide Nos. 15 and 16 in Figure 6c).

The distance between two W-box sites was further investigated comparing the oligonucleotide BS65 (with two W-box spaced by 3 nucleotides) with the corresponding mutated forms spaced by 0 or 6 bases (sequence Nos. 21/BS65-0b and 22/BS65-6b). The results showed that nucleotides distance up to six bases did not affect binding activity (Figure 6d).

Discussion

In this work, we characterised a new gene, *Hv-WRKY38*, involved in cold and drought stress response in barley. *Hv-WRKY38* encodes a protein with the characteristic domain of the WRKY transcription factor family and a putative Leucine Zipper. The Hv-WRKY38 protein is localised in the nucleus and it is able to bind to W-box DNA motif as homodimers when assessed on GMSA. These findings suggest that *Hv-WRKY38* should represent a new transcription factor involved in stress response.

The expression of *Hv-WRKY38* at low temperature was detected transiently after a temperature decrease from 22 to 2 °C, an additional temperature drop promoted a further transcription of the gene providing evidences of the ability of the cell to induce gene expression also at subzero temperature. *Hv-WRKY38* was also found as one of the earliest, and not transient, response to dehydration. Since it is well known that cell dehydration is also a component of the cold stress due to ice formation in the apoplast (Yamada *et al*., 2002), the expression of *Hv-WRKY38* could therefore be associated with the presence of dehydration-related stimuli.

The *Hv-WRKY38* gene was mapped by linkage analysis onto barley chromosome 6HS. In barley, some cold-induced CBF-like transcription factor genes have been found to co-segregate with a QTL for frost resistance, representing therefore candidate genes underlying the frost tolerant locus (Francia *et al*., 2004). Nevertheless, *Hv-WRKY38* locus maps to an interesting position on the barley genome. The position of three RFLP markers (ABG458, CDO497, ABG474), mapped in other populations and associated to abiotic stress-resistance QTLs, has been inferred by comparative mapping using microsatellites Bmag0173 and Bmag0009 as bridges. It is noteworthy that ABG458 was found to be associated to salt- (Mano and Takeda, 1997) and cold- (Tuberosa *et al*., 1997) tolerance loci, as well as to grain protein concentration (See *et al*., 2002). Moreover, a QTL with main effects on grain carbon isotope discrimination, a measure associated with drought tolerance in terms of water-use efficiency and yield stability in drought-prone environments, has recently been observed spanning the Bmag0173–CDO497 interval (Teulat *et al*., 2002).

The WRKY proteins are a superfamily of transcription factors unique to plants and involved in various physiological programs, including senescence, trichome development and biosynthesis of secondary metabolites (Eulgem *et al*., 2000). Above all, current information suggests that WRKY factors play a key role in regulating the pathogen-induced defence program (Dong *et al*., 2003). Our work indicates an involvement of a WRKY transcription factor also in abiotic-stress response. This finding is also supported by few recent reports in the literature. In *Solanum dulcamara* a cold induced antifreeze protein characterized by a WRKY domain has been cloned (Huang and Duman, 2002). It showed a thermal hysteresis activity and a DNA specific binding activity to W-box. In the desert legume *Retama raetam* a WRKY transcription factor was found associated with dormancy, extreme drought tolerance and cold acclimation (Pnueli *et al.,* 2002). Expression analyses on the tobacco homologous sequence has additionally shown the involvement of this sequence in the response to a combination of drought and heat shock conditions (Rizhsky *et al.,* 2002). In *Arabidopsis*, array-based expression data also detected some WRKY transcription factors as early induced after drought, salinity and cold stress (Seki *et al*. 2002; Fowler and Thomashow, 2002). Although the complexity of the WRKY gene family (72 member in *Arabidopsis*) (Dong *et al.,* 2003) makes it difficult to identify the sequence homologous to *Hv-WRKY38*, comparison of the amino acid sequences and genomic structures in barley, rice and *Arabidopsis* suggested that *AtWRKY40* might represent the homologous

Arabidopsis sequence. A single rice sequence, *OsWRKY71,* showed a clear homology at amino acid level (75% identity plus similarity) with *Hv-WRKY38*, nevertheless barley and rice sequences were characterised by different genomic structures (one and three introns, respectively). The same rice genomic structure was then found in the *Arabidopsis AtWRKY40* locus. These findings might suggest that the loss of the second and third intron in barley is a recent evolutionary event following the rice divergence. Besides the similarities in the gene structure and sequences, *AtWRKY40* was also found induced in response to cold (Seki *et al*., 2002; Chinnusamy *et al*., 2003), drought and salinity (Seki *et al*., 2002), an expression profile similar to that of *Hv-WRKY38*.

In *Arabidopsis*, the major transcription system regulating ABA independent gene expression in response to dehydration and cold stress includes the DREB/CBF family and *ice1,* a key regulator of the cold induced transcriptome. Based on array data, *ice1* defective mutant failed to induce *AtWRKY40* in response to low temperature suggesting that this gene belongs to ICE signal transduction pathway (Chinnusamy *et al*., 2003). In confirmation of this hypothesis, three MYC recognition sites (the putative target motifs of ICE1) have been found in the upstream region of *AtWRKY40* homologous. Given that the same promoter region does not contain any CRT/DRE *cis* elements, *AtWRKY40* could represent a transcription regulator belonging to a pathway alternative and parallel to CBF signal transduction cascade. The extensive conservation of the CBF system between *Arabidopsis* and barley (Choi *et al*., 2002; Francia *et al*., 2004) may suggest a similar relationship also in cereals.

The WRKY sequences most similar to *Hv-WRKY38* are *ABF2* from wild oat (Rushton *et al.,* 1995), a gene involved in regulation of α-*Amy2*, and *OsWRKY71* from rice (Zhang *et al*., 2004), a gene coding for a repressor of the gibberellin signaling pathway in aleurone cells. The corresponding proteins, ABF2 and OsWRKY71, were shown to bind to similar oligonucleotides (Box2 α-*Amy2* and Box2 *Amy32b*, respectively) characterised by two W-box motifs. The GST:Hv-WRKY38 fusion protein showed a stronger binding activity with Box2 *Amy32b*, but only traces of DNA-protein complex were detected with the Box2 of the α-*Amy2* promoter. Since the W-box motifs in Box2 α-*Amy2* and Box2 *Amy32b* are identical, the results suggest that the *cis*-elements surrounding regions could be determinant for the specificity of DNA binding. Although the alignment between Hv-WRKY38 and ABF2 reveals an absolute identity within the WRKY domain, the differences present in the surrounding regions could explain the different DNA binding specificity (Eulgem *et al*., 2000).

Transcription factors may act as either positive or negative regulators of gene expression depending on promoter context. Published data suggest that the WRKY transcription factors can display both activities. *AtWRKY6* has been shown to promote the expression of sequences involved in senescence and pathogen defence, as well as to act as a repressor of its own promoter activity (Robatzek and Somssich, 2002). Maleck *et al*. (2000) proposed that *PR-1* (phatogenesis-related) regulon genes may be co-repressed by WRKY transcription factors in absence of pathogen attack, while the expression of PR-1 during systemic acquired resistance could be the results of a WRKY-mediated de-repression (Rowland and Jones, 2001). Recently, Zhang *et al*. (2004) demonstrated that OsWRKY71 blocks the activation of the *Amy32b* through an interaction with the GA-inducible transcription activator OsGAMYB. OsWRKY71 and OsGAMYB bind to different sites (Box2 also known as O2S and the GA responsive element (GARE), respectively) located close to each other on the promoter of the α amylase gene *Amy32b*. The O2S motif was found to promote GA-induction *in vitro* when associated with GARE, as well as ABA induction when associated with the ABA Responsive Element (ABRE) (Rogers and Rogers, 1992), suggesting that the role of the O2S binding protein changes according to the promoter context. These results may suggest that Hv-WRKY38 could interact with other transcription factors to modulate the cold and drought dependent expression of stress-regulated genes containing a cluster of functional W-box in their promoters. Further studies with over-expressing and knocked out plants are in progress to establish the role of Hv-WRKY38 in the regulation of gene expression during stress response.

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